The role of human papillomavirus in the development of tonsillar squamous cell carcinomas

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General introduction

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Role of human papillomavirus in the development of head and neck squamous cell carcinomas

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Introduction

Squamous cell carcinoma is the most prominent malignancy of the head and neck region. It develops from the mucosal lining of the upper aerodigestive tract, with the most frequently affected sites being the oral cavity, the oropharynx and the larynx (Figure 1). Head and neck squamous cell carcinomas (HNSCC) comprise about 5% of all newly diagnosed cancer cases in the Northern and Western European countries and the United States (US), and is the fifth most common cancer type worldwide[1]. The median age at presentation is 60 years and two-thirds of patients are men[2]. Between 1985 and 1990 the world-wide incidence of cancer of the oral cavity and pharynx increased from 26.8 to 33.8 cases per 100 000[2,3].

In The Netherlands, the incidence of HNSCC is 15 cases per 100.000 citizens. In about 30% of all new cases of HNSCC it concerns a laryngeal carcinoma. Between 1998 and 2003 there was a significant reduction in the incidence of these carcinomas in men (9.5 versus 6.6 per 100.000 men, respectively), whereas the incidence in women remained stable (1.3 per 100.000 women)[4]. In 1989 the incidence of carcinomas of the oral cavity measured 4.1 per 100.000 for men and 2.7 per 100.000 for women. Since then an annual increase of 3.5% is detected in the total number of patients with these carcinomas. With an incidence of about 1 per 100.000, carcinomas of the hypopharynx are relatively rare. The incidence of these cancers remained stable between 1989 and 2001[5]. The incidence of oropharyngeal carcinomas was 2.4 cases per 100.000 for men and 1.2 cases per 100.000 for women in 1998. Between 1989 and 1998 the total fraction of patients with oropharyngeal carcinomas increased with about 5% annually. The increased incidence was mainly seen in the patient group of 45 to 59 years of age[6].

A recent study of the US National Cancer Institute[7] reviewing 45769 cancer cases between 1973 and 2004 also reported an increase in incidence of carcinomas arising from the oropharynx, particularly those of the base of the tongue, the lingual tonsil, the tonsil, and Waldeyer ring, whereas carcinomas arising from other subsites, such as other parts of the tongue, gum, mouth, and palate showed a reduction in incidence. This is in agreement with an earlier report of Frisch et al.[8], describing a fourfold increase in the incidence of tonsillar squamous cell carcinoma (TSCC), which accounts for three quarters of all tonsillar malignancies[9], among white women in Connecticut during 1945-1994, whereas in men there were annual increases of 2.7% in Afro-Americans and 1.9% in whites. No such increases could be observed at

non-tonsillar sites. These data suggest that TSCC may differ etiologically from other oral and pharyngeal cancers.

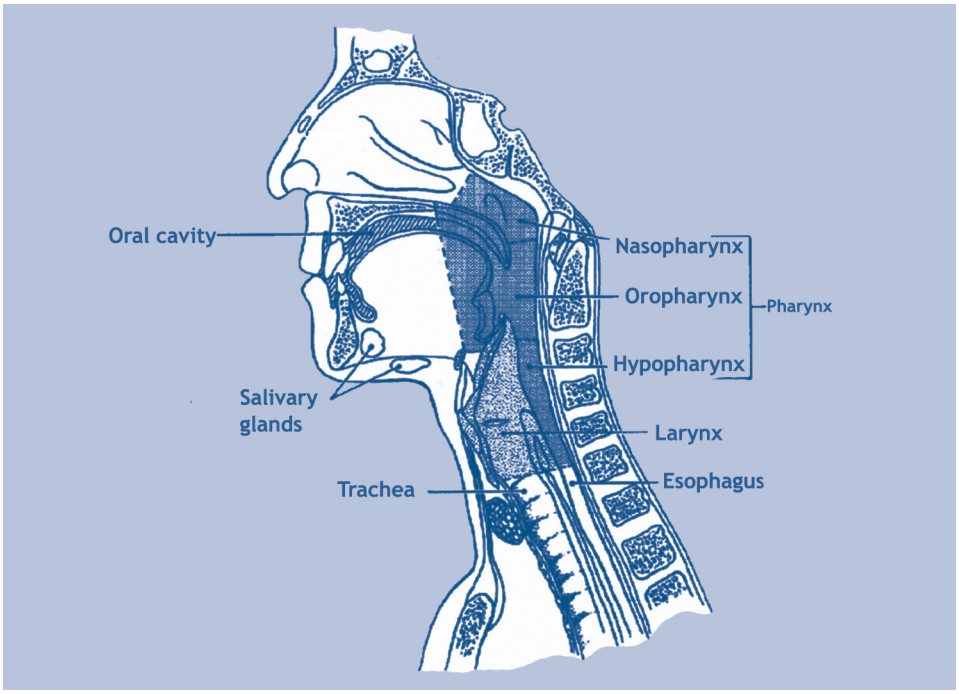

FIGURE 1. LATERAL VIEW OF THE MAIN SITES IN THE HEAD AND NECK REGION.

Anatomy of the pharynx

The pharynx is the common passageway for air and food extending from the nasal and oral cavities to the esophagus and larynx. The pharynx is divided into the nasopharynx, oropharynx and hypopharynx (Figure 1). The oropharynx is located between the soft palate and the upper edge of the epiglottis. It connects to the nasopharynx cranially, to the hypopharynx caudally, and to the oral cavity anteriorly. The tonsils form the boundary between the oropharynx and the oral cavity. The tonsils are embedded between an anterior and posterior fold extending from the soft palate to the tongue (Figure 2). The tonsils consist of lymphatic nodules lying in a subepithelial position, separated from underlying tissues by a connective tissue capsule. The lymphoid tissue of the tonsil is penetrated by deep crypts lined by

stratified squamous non-keratinized epithelium that is continuous with the same type of epithelium overlying the tonsil. The epithelium of the tonsillar crypts is usually infiltrated by lymphocytes. The tonsils function as a first outpost of the body's immune defense system against bacteria, viruses and fungi entering the body through the mouth.

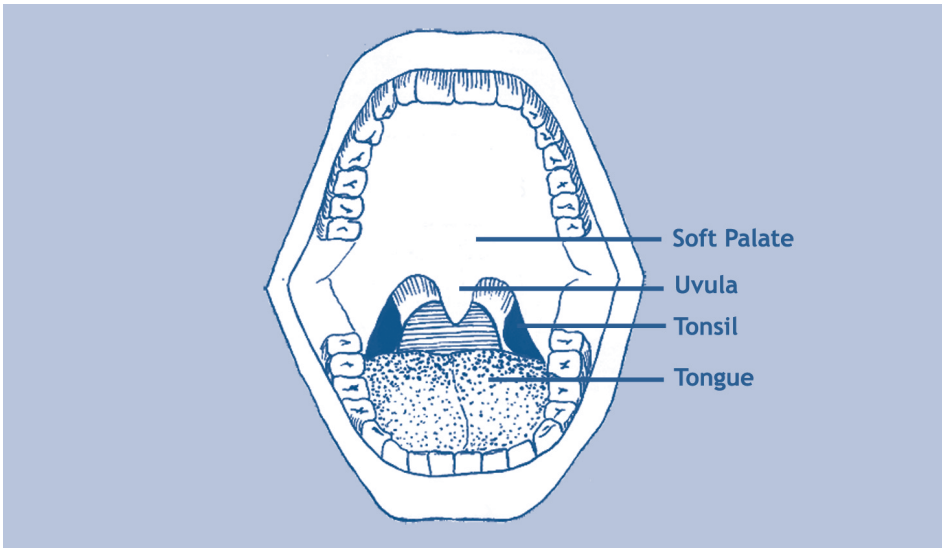

FIGURE 2. FRONTAL VIEW OF THE ORAL CAVITY.

Etiology of HNSCC

Tobacco smoking, extensive alcohol drinking and betel quid chewing are well-known risk factors in the etiology of HNSCC[10,11]. The age at which an individual starts smoking appears to be inversely associated with the relative risk of developing HNSCC[10,12]. Tobacco smoking and alcohol intake are independent risk factors, but a synergistic effect is observed when they are combined[10]. Other known risk factors include environmental exposure to wool dust, wood dust and mineral fibers, low intake of vegetables and fruit and infection with Epstein-Barr virus, which is strongly linked to the development of nasopharyngeal carcinoma[10,13]. Kreimer et al.[14] suggested that a low body mass index might increase the risk of oral cancer, and that vegetables and fruits may modulate the carcinogenic effects of tobacco and alcohol. However,

an increasing number of patients develop oral cancer in the absence of exposure to the above-mentioned risk factors and independent of any obvious predisposing genetic defect, suggesting the presence of additional risk factors[15,16].

In 1933, Shope and Hurst[17] observed that infection with the cottontail rabbit papillomavirus led to the subsequent development of keratinous lesions, some of which progressed to invasive epithelial neoplasms. This observation led to the discovery of the first DNA virus that causes tumors in mammals. Substantial evidence now supports the role of human papillomavirus (HPV) in the development of (pre)malignant lesions of the vulva, penis, anus and uterine cervix[18]. In addition, epidemiological and molecular data suggest that HPV may also promote head and neck carcinogenesis[18-23]. A carcinogenic role for HPV is also suggested by the finding that patients with cervical cancer develop an excess of cancers in the head and neck region, especially of the oropharynx, in comparison with females with other cancers[24]. Schwartz et al.[25] reported that HPV infection of the oral cavity is linked to an elevated cancer risk, independent of tobacco smoking and alcohol abuse, and that HPV may therefore be a risk factor for both uterine cervical cancer and HNSCC[26]. Indeed, individuals with detectable serum levels of antibodies against HPV-16 demonstrate an approximately two-fold higher risk for developing HNSCC and an approximately ten-fold increased risk for developing tonsillar carcinomas, although only 37% of cases with HPV-positive HNSCC seem to have these antibodies in their serum[27]. Recently, a case control study of D'Souza et al.[28] showed that a high lifetime number of vaginal-sex partners (26 or more) was associated with oropharyngeal cancer, as was a high lifetime number of oral-sex partners (6 or more). The degree of association increased with the number of partners. These findings have led to the hypothesis that some HNSCC, and particularly TSCC, are sexually transmitted diseases[25,27].

Head and neck carcinogenesis

The development of cancer involves the accumulation of genetic and epigenetic alterations. These alterations include gain of function of growth stimulating genes, the oncogenes and loss of function of growth-inhibiting genes, the tumor suppressor genes. This process of activation of oncogenes and/or inactivation of tumor suppressor genes results in a cell population with an uncontrolled and increased

growth potential as well as a decreased cell death fraction, which can evolve into a malignant tumor.

Head and neck cancer progresses through a series of well-defined clinically and histologically recognizable stages, reflecting the multi-step process of carcinogenesis (Figure 3). First, a hyperplastic lesion may develop in which the epithelial cells increase in number but individually exhibit a relatively normal differentiation behavior and normal morphology. Hyperplasia may then proceed to dysplastic lesions of the squamous cell epithelium that are characterized by cellular atypia and loss of normal maturation. When the lesion occupies the whole thickness of the epithelium, but stromal invasion has not taken place, it is classified as carcinoma in situ. Finally, the term invasive carcinoma is used when carcinoma cells invade the stomal compartment. In this stage the blood and lymph system may be reached by the invasive cells, which can result in further spreading of the cells through the body and the formation of regional or distant metastases. Multiple genetic changes have been identified that underlie the multistep carcinogenesis of the head and neck mucosa. In Figure 3 the most important genetic changes, as described in literature, have been implemented in a progression model of head and neck carcinogenesis[29,30].

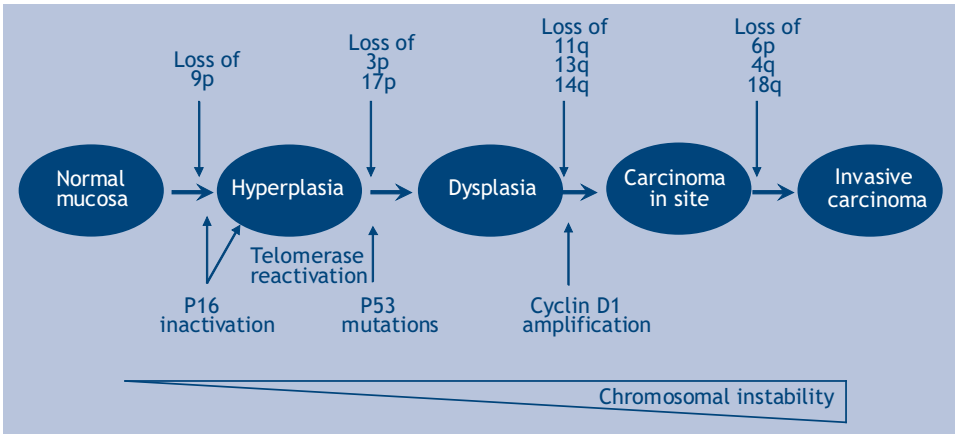

FIGURE 3. GENETIC PROGRESSION MODEL OF THE CARCINOGENIC PROCESS LEADING TO HNSCC. Important genetic alterations and candidate genes that are associated with the histopathological progression of HNSCC are indicated[26,27].

Important events in the early tumorigenesis of HNSCC are losses of chromosomes

9p21 and 3p, which are accompanied by inactivation of tumor suppressor genes on the remaining chromosomes, such as $p16^{INK4A}$ and FHIT. Furthermore, reactivation of telomerase and overexpression of EGFR are frequently identified as well as mutations in the p53 tumor suppressor gene in up to 50% of HNSCC. In the course of the tumorigenic process the number of molecular aberrations further increases, leading to chromosome instability, including DNA deletions and amplifications, such as those at 11q13 and 3q26[11,29-32].

Patients with HNSCC often suffer from multiple head and neck lesions, both pre-malignant and malignant. This phenomenon was originally described by Slaughter et al. in 1953[33]. These authors formulated the concept of field cancerization, meaning that a prolonged carcinogenic exposure of the entire mucosal lining of the upper aerodigestive tract results in multiple lesions that have acquired independent genetic changes. Later, an alternative theory has been proposed by Bedi et al.[34] advocating intramucosal spread and expansion of (pre)malignant cells harboring identical genetic alterations as an explanation for the occurrence of multiple squamous cell lesions. The observation of unknown primary tumors harboring genetic alterations that are identical to those identified later in head and neck mucosa biopsies is also in accordance with the latter theory[35].

Human papillomaviruses

HPVs are small epitheliotrophic DNA viruses of about 45-55 nm in diameter with circular double-stranded DNA genomes of approximately 8 kb[18]. Based on their DNA and capsid structure they originally have been grouped together with the polyomaviruses to the family of Papovaviridae. Differences in genome size, organization and nucleic and amino acid sequences, however, have led to the separation of both virus groups in two different families, Papillomaviridae and Polyomaviridae[36]. The viral genome is organized into 3 segments: the long control region LCR (about 10% of the genome), the early (E) E1, E2 and E4-7 genes (about 50% of the genome) and the late (L) L1 and L2 genes (about 40% of the genome) (Figure 4).The 8 HPV genes are designated as E or L according to their expression in early or late stage of the assembly process of the virus, respectively. As a result E1, E2, E5, E6 and E7 are expressed early in differentiation, E4 is expressed throughout, and L1 and L2 are expressed during the final stages of differentiation of the epithelium[37]. A new HPV

subtype is defined if the E6, E7 and L1 regions display < 90% sequence homology with any other known HPV type[38]. Nearly 120 HPV genotypes have been identified so far, which are subdivided into benign subtypes on the one hand, and malignant or oncogenic subtypes on the other hand[18,19]. The benign subtypes, including HPV-6 and -11, induce hyperproliferations of epithelia such as mucosal warts and papillomatosis. Recurrent respiratory papillomatosis of the larynx caused by HPV is the most common benign neoplastic disease of the larynx and is very difficult to treat[39]. Other subtypes, particularly HPV-16 and -18, are strongly associated with malignancy.

HPVs are thought to infect proliferating cells in microlesions of the skin or mucosa (in the basal layer) or at the transformation zones between different types of epithelium, e.g. at the squamocolumnar junction of the uterine cervix[40]. Except for some limited expression of E5, E6 and E7, viral gene expression is largely suppressed in these cells. As a result of cell division HPV spreads laterally or migrates into the suprabasal differentiating cell layers. In these cells late viral gene expression is initiated, resulting in replication of the circular genome and production of structural proteins. In the upper cell layers, viral particles are assembled and released[18].

So far, most HPV studies have concentrated on its role in the etiology of uterine cervical cancer[18]. In particular the high-risk oncogenic HPV types 16 and 18 induce preneoplastic lesions with an increased risk of progression to cancer. The transition from dysplasia to invasive cancer appears to be associated with integration of the viral DNA into the cellular host genome[41]. Recent studies suggest that mostly singular integration events occur by means of non-homologous sequence-specific recombination at a single locus or a few chromosomal loci (most probably fragile sites) in individual cell clones[41-44]. Molecular studies have shown that HPV integration results in disruption of the E1/E2 open-reading frames of the HPV genome, leading to increased expression of the E6 and E7 proteins in particular (Figure 4)[18,19].

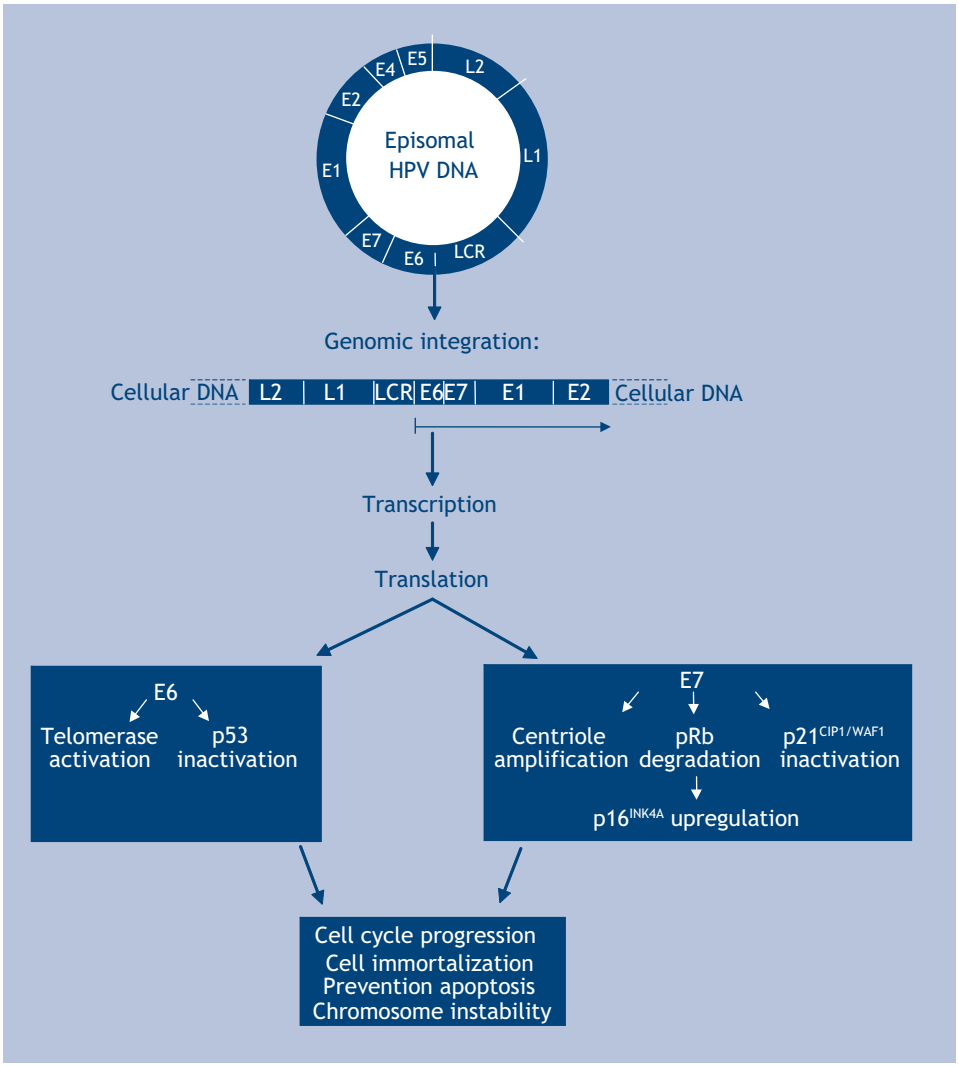

FIGURE 4. STRUCTURE OF CIRCULAR (EPISOMAL) AND INTEGRATED HPV DNA. In premalignant lesions the 8 kb-large HPV genome, harbouring six E and two L genes, is usually present in many copies as circular DNA in the nucleus of infected cells. During cancer development the viral DNA becomes integrated into the host cell genomic DNA, thereby frequently losing (part of) the E2, E4, E5 and L2 genes. In most cases the integrated viral DNA contains the intact E6 and E7 genes of which the expression is upregulated, resulting in cell cycle progression, cell immortalization, prevention of apoptosis and chromosome instability.

HPV E5, E6 and E7 proteins

The E5 protein is expressed in productive infections and may fulfill a function in the early expansion of an infected cell clone by activation of growth factor receptors or other protein kinases[35]. In uterine cervical carcinoma cells, however, the E5 open reading frame is frequently deleted, indicating the absence of an essential role of this gene in maintaining the malignant phenotype of cells.

Many data are available relating to the function of the E6 and E7 oncoproteins[18,19]. They are consistently expressed in the majority of HPV-carrying malignant tumors. Although E6 and E7 proteins may independently promote carcinogenesis of human mucosal cells, their cooperative interaction leads to substantially enhanced cell immortalization[45]. Different interactions of the oncogenic E6 and E7 proteins with host-cell proteins have been reported. These data provide important clues towards understanding the oncogenic properties of the high-risk HPV types.

The HPV E6 protein can interact with the cellular wild-type p53 tumor suppressor protein, inducing p53 degradation via the ubiquitin-mediated pathway (Figure 4). Normally, p53 prevents cells with damaged DNA from progressing through the cell cycle by arresting them at the transition from the G1 to the S phase, thus allowing time for DNA repair. In the case of excessive DNA damage, the cell will undergo p53- and BAX-mediated apoptosis. Therefore, inactivation of p53 by E6 leads to deregulation of the cell cycle and promotes accumulation of mutations as well as chromosome instability. In addition, the E6 oncoprotein activates host-cell telomerase, a ribonucleotide protein complex that synthesizes telomere repeat sequences critical for cell immortalization during cell growth[46]. Furthermore, E6 binds to numerous other cellular proteins that can be divided into four broad classes: transcriptional co-activators, proteins involved in cell polarity and motility, tumor suppressors and inducers of apoptosis, and DNA replication and repair factors[35].

The most important function of the E7 protein is its interaction with the cellular retinoblastoma tumor suppressor protein (pRb), resulting in pRb ubiquitination and degradation by proteasomes[18,47]. As a consequence, the cyclin-dependent kinase 4/6 inhibitor $p16^{INK4A}$ is upregulated and the transcription factor E2F is released, which activates both cell cycle progression by stimulation of the S-phase genes cyclin A and E, and viral DNA synthesis (Figure 4)[18]. The importance of the binding of E7 to pRb is supported by the observation that there is a correlation between the ability of the E7 proteins to bind cell growth regulatory proteins and the oncogenicity of

the HPV type. For example, the E7 protein from the oncogenic HPV types 16 and 18 binds pRb more strongly than does E7 from the benign HPV types[48]. Furthermore, E7 appears to block the function of cyclin-dependent kinase inhibitor $p21^{CIP1/WAF1}$ and to induce centriole amplification. These actions of the E7 protein further contribute to tumorigenesis by stimulating cell cycle progression and the development of genetic instability and aneuploidy (Figure 4)[18,35,49,50].

HPV and head and neck cancer

Epidemiological and molecular data have clearly indicated that oncogenic HPVs may promote head and neck tumorigenesis in the absence of more traditional risk factors such as tobacco smoking and alcohol consumption[19-23]. However, discrepancy exists with respect to the percentage of HNSCC harboring HPV, as the reported frequencies in head and neck lesions vary over a wide range (0-100%). These differences may be due to the detection method used (Southern blot hybridization, polymerase chain reaction (PCR) or in situ hybridisation), the anatomic location of tumors, the type of HPV detected, and/or the number of tissue samples analyzed in the various studies. A 1998 review[19] of studies on HPV in head and neck lesions reported that: the frequency of HPV (both low and high risk types) in benign and precancerous lesions ranged from 19% to 36%; the overall prevalence of HPV in HNSCC ranged from 18% (in situ hybridisation studies) to 35% (PCR studies); the majority of HPV-positive tumors contained the high-risk HPV types 16 (40%) and 18 (12%); and (iv) HPV was most often detected in tumors of the oral cavity (59%), followed by those of the pharynx (43%) and larynx (33%).

As a result of more recent studies[20-23,51-53] it has become clear that 15-25% of HNSCC harbor high-risk HPV types, which in about 90% of cases appears to be HPV-16, and that oropharyngeal carcinomas, particularly TSCC, are significantly more often HPV-positive (about 50% of cases) than cancers in other locations. An important issue to consider here is the reliability of the PCR data, particularly in terms of providing evidence for a biological association between HPV and the tumor cells. If patients are to benefit in the near future from HPV-targeted therapeutic strategies (e.g. vaccines), then a clonal association between the virus and (a large proportion of) the tumor cells is a prerequisite[54]. Several reports, however, have shown that up to 50% of cases that tested positive for high-risk HPV types using PCR

turned out to be negative in control experiments using Southern blotting and reverse transcriptase PCR to determine E6/E7 mRNA expression levels[21,22,54,55]. This may be due to contamination or to the detection of ''biologically irrelevant'' HPV copies in the DNA extracts using PCR. Therefore, control experiments are essential in order to help improve estimates of the proportion of HNSCC for which there is a biologically relevant association with HPV[56]. Alternative approaches for doing so include the detection of high-risk HPV DNA by multiplex PCR or PCR analysis of microdissected tumor cells, demonstrating viral load data between 1 and 150 HPV DNA copies per ß-globin copy[51], or use of highly sensitive fluorescence in situ hybridization (FISH) protocols[23,57]. The FISH procedure enables HPV DNA detection up to the level of a single copy per cell nucleus, and to discriminate between replicative (episomal) and integrated virus on the basis of the nuclear staining pattern. In this way HPV association with the tumor can be directly analyzed under the microscope. Because of these advantages of the FISH procedure over the other detection methods we decided to use the FISH procedure in our studies.

Detailed epidemiological data concerning the normal course of high-risk HPV infection in the oropharyngeal region are unavailable at present. Therefore, and because premalignant lesions of the oropharynx are scarcely observed in the clinic, studies on the prevalence of HPV-16 and -18 in normal mucosa of healthy tonsils, as well as carcinoma containing tonsils, using cytological tissue samples, biopsies or resection material, should shed further light on their role in promoting oropharyngeal carcinogenesis[58-61].

Clinical course

With respect to the clinical course and prognosis of HPV-related HNSCC, varying observations have been reported. Most authors describe a survival advantage of HPV-positive HNSCC patients when compared to HPV-negative cases[21,52,62,63], while others show the opposite result[64] or could not show a difference in survival outcome[65,66]. In addition, it has been suggested that the clinical course of the disease might be gender-specific, since males with a HPV-positive tumor had a better prognosis than males with a HPV-negative tumor, whereas this correlation could not be detected in women[52]. It has also been postulated that HPV would be more prevalent in younger

patients[65]. Some studies describe that HPV-positive tumors have a better response to radiation and chemo- therapy compared to HPV-negative tumors[67].

It is evident from the foregoing that more detailed prognostic studies are needed before HPV status can affect clinical decision making.

Outline of this thesis

As described above, it is obvious that, besides the well known risk factors such as tobacco smoking and alcohol intake, HPV also plays an important role in the development of HNSCC. Despite the evident association of HPV with HNSCC in a subgroup of patients, the exact mechanisms by which the virus causes tumor initiation and progression are not fully understood.
In this thesis the hypothesis is put forward that HPV-positive and HPV-negative tonsillar squamous cell carcinomas are two different entities.

The following questions were raised:

1) Can we detect integrated DNA of oncogenic HPV in premalignant lesions, HNSCC and their metastases?
2) Since overexpression of p53 is frequently observed in HNSCC, the question had to be answered whether this is related to the presence of HPV and/or the result of p53 mutations.
3) What is the impact of HPV, tobacco smoking and other risk factors on the clinical course of TSCC?
4) Can $p16^{INK4A}$ overexpression reliably be used as a surrogate marker for HPV infection in head & neck cancer and predict the presence of HPV in normal tonsil tissue?
5) Which key cell cycle proteins in the retinoblastoma (pRb) en p53 pathways are involved in HPV-related carcinogenesis of TSCC, and are these proteins related to a favorable prognosis?

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Chapter 2

A subset of head and neck squamous cell carcinomas exhibits integration of hpv 16/18 DNA and overexpression of $p16^{INK4A}$ and p53 in the absence of mutations in p53 exons 5-8

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Abstract

Besides well-known risk factors such as tobacco use and alcohol consumption, oncogenic human papillomavirus (HPV) infection also has recently been suggested to promote head and neck tumorigenesis. HPV is known to cause cancer by inactivation of cell cycle regulators p53 and pRb via expression of viral oncoproteins E6 and E7. This indicates that p53 mutations are not a prerequisite in HPV-induced tumor development. However, discrepancy exists with respect to the frequency of head and neck squamous cell carcinomas (HNSCC) harboring DNA of oncogenic HPV and the fraction of these tumors showing p53 mutations. In our study, we examined the frequency of HNSCC demonstrating HPV 16/18 integration as identified by fluorescence *in situ* hybridization (FISH) and investigated their p53 (mutation) status by immunohistochemistry and single-strand conformation polymorphism (SSCP) analysis of exons 5–8. Paraffin-embedded, archival biopsy material from 27 premalignant mucosal lesions and 47 cases of HNSCC were analyzed. Ten of the 47 (21%) HNSCC unequivocally exhibited HPV 16 integration, including 8 of 12 (67%) tonsillar carcinomas. This is supported by the immunohistochemical detection of $p16^{INK4A}$ overexpression in all 10 HPV-positive tumors. Although FISH is considered to be less sensitive than PCR-based methods for HPV detection, our data clearly demonstrate clonal association of HPV with these tumors, as illustrated by the presence of integrated HPV 16 in both the primary tumor and their metastases in 2 patients. In contrast, HPV 16/18 DNA could not be detected in the premalignant lesions. In 30 of 47 (64%) HNSCC accumulation of p53 was observed, including 8 of the 10 HPV-positive carcinomas. However, in none of the latter cases could mutations in exons 5–8 be identified, except for a polymorphism in codon 213 of exon 6 in one patient. Evaluation of clinical data revealed a significant inverse relation between tobacco use with or without alcohol consumption, and HPV positivity of the tumors.

Chapter 2

Introduction

Head and neck squamous cell carcinomas (HNSCC) account for 6.5% of annual cancer cases worldwide. During the last decades, the incidence of HNSCC has increased in Western Europe. For example, the incidence of cancer of the mouth and pharynx increased from 26.8 to 33.8 per 100,000 from 1985 to 1990[1,2]. Recent data suggest that this increase in incidence is especially high in patients younger than 40 years of age[3,4]. Median age at presentation of HNSCC is 60 years, and 66.7% of the patients are men.

Tobacco smoking, alcohol drinking and betel quid chewing are well-known risk factors in the etiology of HNSCC, responsible for 90% of the cases[5,6]. The age at smoking initiation appears to be inversely associated with a higher relative risk of developing a carcinoma[5,7]. Tobacco and alcohol use are independent risk factors, but when combined a synergistic effect is observed[5]. In addition, epidemiologic and molecular data suggest that human oncogenic papillomaviruses (HPVs), known to cause cervical and other anogenital cancers, may also promote head and neck carcinogenesis[8-10]. Discrepancy exists with respect to the percentage of HNSCC harboring HPV, as the reported frequencies in head and neck lesions vary over a wide range (2–76%)[8,11]. These differences may be due to the detection method used (Southern blot hybridization, polymerase chain reaction (PCR) or *in situ* hybridization), the anatomic location of tumors, the type of HPV detected and/or the number of tissue samples analyzed in the various studies[8,11].

Most studies have so far concentrated on the role of HPV in the etiology of uterine cervical cancer[12]. Particularly the high-risk oncogenic HPVs, such as HPV type 16 and 18, induce preneoplastic lesions with an increased risk of progression to cancer. The transition from dysplasia to invasive cancer appears to be associated with integration of the viral DNA into the host genome, most probably at fragile sites in chromosomes[13-15]. Molecular studies have shown that HPV integration results in upregulation of the viral oncoproteins E6 and E7[8,12]. The E6 protein contains zinc-binding motifs and can complex with the host cell p53, thereby inducing p53 degradation through the ubiquitin-mediated pathway and thus preventing a cell cycle block and induction of apoptosis in DNA-damaged cells. The E7 protein forms complexes with hypophosphorylated forms of the retinoblastoma tumor suppressor protein (pRb), resulting in a decrease of the cellular pRb level and a release of E2F, a transcription factor involved in cell cycle progression[16,17]. Since HPV inactivates

both p53 and pRb, it is expected that inactivating mutations in these genes do not play an important role in HPV-infected HNSCC. This is not only the case in cervical cancers[10,12] but also in half of the HNSCC[6]. Some studies on HNSCC, however, have reported the concomitant presence of HPV DNA and p53 overexpression and/or mutation[9,18-21]. This gives rise to the question as to whether HPV is causally related to the development of a subgroup of HNSCC.

Because HNSCC are thought to arise via a multistep process with histologically distinct precursor phenotypes harboring specific genetic alterations[6,22], we sought to clarify the relationship between the presence of HPV 16/18 and p53 alterations in premalignant lesions of the head and neck mucosa, HNSCC and their metastases. For this purpose, we have applied fluorescence *in situ* hybridisation (FISH) combined with tyramide signal amplification to formaldehyde-fixed, paraffin-embedded tissue sections because this sensitive approach enables not only the direct visualization of up to 1 copy of HPV DNA in cells[23,24] but also allows for the distinction between the integrated or replicative (episomal) state of HPV 16/18 on basis of a punctate or a diffuse hybridization signal, respectively[25,26]. p53 accumulation in cell nuclei was detected by immunohistochemistry, and the HPV-positive tumors were also examined by single-strand conformation polymorphism (SSCP) analyses of p53 exons 5–8 to investigate if p53 accumulation was related to a mutation in the gene. HPV-positive and negative oropharyngeal carcinomas, in addition, were stained for pRb and $p16^{INK4A}$ to assess the expression levels of these proteins. Clinical data and alcohol and tobacco intake were related to the presence of HPV in the tumor.

Material and methods

Tumor material and patient data

Formaldehyde-fixed, paraffin-embedded archival biopsy and resection material from 74 patients was selected from the archives of the Department of Pathology, University Hospital Maastricht, The Netherlands. These included 27 patients with premalignant mucosal lesions and 47 patients (13 female, 34 male; mean age at diagnosis, 57 [range, 27–84] years) with primary HNSCC and 9 metastases corresponding to these primary carcinomas. The distribution by anatomic site was as follows: premalignant lesions: 21 were located in the larynx and 6 in the oral cavity (7 hyperplasia, 10 mild dysplasia, 7 moderate dysplasia and 3 severe dysplasia); HNSCC: 7 were located

in the larynx, 19 in the oral cavity (10 tongue, 4 palate, 3 floor of the mouth, 2 alveolar process), 16 in the oropharynx (12 tonsil, 4 base of the tongue) and 5 in the hypopharynx. Demographic data, including age at diagnosis, gender, alcohol and tobacco exposures, were obtained from medical records. Tumor site, grade and pTNM classification were determined from review of pathologic, radiologic and surgical reports[27].

A series of 4 µm-thick sections were cut from the specimens for (i) hematoxylin-eosin staining and detailed histopathologic classification (F.J.B.)[27], including determination of the absence or presence of basaloid features[9]; (ii) FISH to detect HPV 16/18; (iii) immunohistochemistry to visualize p53, pRb and $p16^{INK4A}$ expression; (iv) extraction of genomic DNA to be analyzed by SSCP for p53 gene mutations.

Detection of HPV 16/18 DNA by FISH

FISH was performed on 4-µm tissue sections as described previously[22,28]. Formaldehyde-fixed, paraffin-embedded sections were deparaffinized, pretreated with 85% formic acid/0.3% H_2O_2 for 20 min at room temperature and subsequently dehydrated with 70% ethanol containing 0.01 M HCl (acid dehydration), 90% ethanol and 100% ethanol for 3 min each prior to air drying. The slides were incubated in 1 M NaSCN for 10 min at 80°C, followed by acid dehydration and digestion with 4 mg/ml pepsin (800-1,200 U/mg protein from porcine stomach mucosa; Sigma, St. Louis, MO) in 0.02 M HCl. The slides were rinsed 3 times in 0.01 M HCl and acid dehydrated. After air drying, sections were postfixed in 1% formaldehyde in PBS for 15 min at room temperature and dehydrated in an ascending ethanol series. The digoxigeninlabeled HPV 16, 18 and HPV 16/18 mixture probes (Kreatech, Amsterdam, The Netherlands) were applied under a coverslip according to the manufacturer's instructions. Probe and target DNA were denatured simultaneously for 5 min at 80°C prior to hybridization overnight at 37°C in a humid chamber. After hybridization the preparations were washed stringently in 50% formamide, 2×SSC at 42°C (2 times 5 min). The digoxigenin-labeled probes were detected conventionally by application of mouse anti-digoxin (Sigma), rhodamin-conjugated rabbit anti-mouse IgG and rhodamin-conjugated swine anti-rabbit IgG or by using tyramide signal amplification (TSA) as previously described[29,30]. In short, the digoxigenin-labeled probe was detected with peroxidaseconjugated sheep anti-digoxigenin Fab fragments (Roche, Basel, Switzerland; 1:100 diluted in 4×SSC containing 5% nonfat dry milk), followed by a TSA reaction using rhodamin-labeled tyramide. Fifty microliters of rhodamin-

labeled tyramide (1:500 diluted from a 1 mg/ml stock solution in ethanol) in PBS containing 0.1 M imidazole, pH 7.6, and 0.001% H_2O_2 were applied under a coverslip for 10 min at 37°C. Finally, the slides were washed in PBS containing 0.05% Tween-20 (Janssen Chimica, Beerse, Belgium), dehydrated in an ascending ethanol series and mounted in Vectashield (Vector Laboratories, Burlingame, CA) containing 4',6-diamidino-2-phenyl indole (DAPI; Sigma: 0.2 µg/ml). Microscope images were recorded with the Metasystems Image Pro System (black and white CCD camera; Sandhausen, Germany) mounted on top of a Leica DM-RE fluorescence microscope equipped with DAPI and rhodamin filters.

Controls and evaluation of FISH results

Controls included hybridizations on 70% ethanol suspensions and formaldehyde-fixed, paraffin-embedded sections of known HPV 16- and 18-positive human cervical carcinoma cell lines (CaSki [ATCC; CRL1550; 500 integrated HPV 16 copies], HeLa [ATCC; CCL2; 20–50 integrated HPV 18 copies] and SiHa [ATCC; HTB35; 1–2 integrated HPV 16 copies]) to guarantee probe specificity, sensitivity and interpretation accuracy[23,29], as well as hybridizations on tissue sections of cervical lesions with proven integration or episomal presence (replication) of HPV genomic DNA. Negative controls consisted of HPV PCR- and FISH-negative cell lines and tissue sections and hybridizations omitting the viral probe. Evaluation of nuclear hybridization signals was performed by 3 investigators (H.C.H, E.J.M.S. and A.H.) according to the criteria described by Cooper et al.[25], i.e., punctuate and/or diffuse signals throughout the nucleus indicating integrated and episomal HPV DNA, respectively. In addition, the number of HPV integration spots per nucleus was scored in the tissue.

Immunohistochemical staining of p53, pRb and $p16^{INK4A}$

Immunohistochemical protein staining on 4 µm-thick formaldehyde- fixed, paraffin-embedded tissue sections was performed as described earlier[27]. Briefly, sections were deparaffinized and subsequently pretreated with 0.3% H_2O_2 in methanol to quench endogenous peroxidase activity. Antigen retrieval was performed by microwave heating in 0.01 M citrate buffer (pH 6.0). The monoclonal antibodies DO-7 (Dako A/S, Glostrup, Denmark), NCl-Rb (Novocastra, Newcastle upon Tyne, UK) and E6H4 (Dako A/S) were used to detect p53, pRb and $p16^{INK4A}$ proteins, respectively. After incubation with a biotinylated secondary antibody, immunohistochemical detection was performed by an avidin-biotinylated peroxidase complex (ABC)

procedure (Vectastain-Elite-ABC kit; Vector). Peroxidase activity was detected using diaminobenzidine/ H_2O_2. Sections were counterstained with hematoxylin and mounted in Entellan (Merck, Darmstadt, Germany). In each analysis, negative and positive controls were included. Analysis was performed by 3 independent observers (H.C.H., E.J.M.S. and A.H.) and consensus was acquired.

Although the DO-7 antibody binds to both normal and mutant p53 protein, in general normal levels of wild-type p53 protein are too low to detect by immunohistochemistry. The number of cells exhibiting a strong, positive nuclear p53 staining was scored as: (i) negative when <30% of the cells exhibited a nuclear staining; (ii) positive when ≥30% of the cells exhibited a nuclear staining[31]. pRb nuclear staining in normal squamous epithelium is abundant and includes parabasal and the lower suprabasal layers (high intensity was semiquantitatively scored as 2+ or 3+). Staining in ≥ 30% of the tumor cell nuclei was scored accordingly or as weaker staining (± or +) or absence of staining (-)[32]. Normal expression levels of p16INK4A in squamous epithelium are under the detection limit of immunohistochemistry. Strong nuclear and cytoplasmic $p16^{INK4A}$ staining in ≥ 30% of tumor cells was considered positive and in < 30% of cells negative[33].

SSCP and mutation analysis of p53 exons 5-8

SSCP analysis of exons 5-8 of the p53 gene was performed in 9 of the 10 tumors that were positive for HPV 16/18 DNA by FISH. Five to ten 10 µm-thick sections were stained with hematoxylin and eosin to select parts of the tissues composed of >70% tumor cells. These areas were microdissected by scraping the tissue from the histologic glass slides. Genomic DNA was extracted according to the tissue protocol of the QIAamp DNA mini kit using proteinase K (Qiagen, Westburg, Leusden, The Netherlands). Exons 5-8 of the p53 gene were investigated by PCR SSCP analysis as previously described[34]. In short, each exon was amplified in 2 overlapping fragments and tumor DNA was always compared with normal DNA from the same patient. PCR was performed with 30 ng isolated DNA in a final reaction volume of 15 µl containing: 1.5 mM MgCl2, 0.02 mM dATP, 0.2 mM dGTP, dTTP and dCTP each, 0.8 µCi α-^{32}PdATP (Amersham, Buckinghamshire, UK), 20 pmol of each primer and 0.2 unit Taq polymerase (Promega, Madison, WI). PCR was performed for 35 cycles (denaturing at 95°C for 30 sec, annealing at 55°C for 45 sec and extension at 72°C for 1 min) in a Biometra thermocycler (Biometra, Göttingen, Germany). A final extension was carried out at 72°C for 10 min. PCR products were diluted with loading buffer (95%

formamide, 10 mM EDTA (pH 8.0), 0.025% bromophenol blue and 0.025% xylene cyanol), denatured at 95°C for 4 min and snap-cooled on ice. The samples were run overnight at 7W on a nondenaturing 6% polyacrylamide gel containing 10% glycerol in 1×TBE running buffer. After electrophoresis, gels were fixed in 10% acetic acid, dried on blotting paper on a vacuum gel dryer and exposed to X-ray film overnight at -70°C, using intensifying screens. Films were evaluated by visual inspection. The aberrant band from the PCR-SSCP analysis of the single HPV-positive tongue tumor (tumor 1 in Table 1) was excised from an additionally prepared Sybr Green-stained SSCP polyacrylamide gel, reamplified, purified using a QIAquick gel extraction kit (Qiagen) and sequenced by cycle sequencing as described previously[35].

Statistical analysis

Factors associated with HPV status were selected on cross-tabulations, which were analyzed by the use of the Fisher exact test (2-tailed) and/or X^2 test. A significance level of $p \leq 0.05$ was chosen.

Results

Presence of HPV 16/18 DNA in cell lines

To examine the sensitivity and reliability of our protocols, the Siha, Hela and Caski cell lines were subjected to FISH using the HPV 16- and 18-specific probes, followed by conventional or TSA detection procedures. Figure 1a-d shows that our FISH procedures on 70% ethanol fixed and pepsin-pretreated cell suspensions enable the detection of all 5 HPV 18 integration sites in Hela cells (integrated at 8q24) as well as the 1-2 HPV 16 integration sites in Siha cells (integrated at 13q21) by both detection methods. This could also be achieved by hybridization to paraffin sections of these cell lines (data not shown). As the TSA procedure allows evaluation at lower magnification due to its higher sensitivity, we have used this system to further analyze the series of head and neck lesions.

Presence of HPV 16/18 DNA in premalignant lesions and HNSCC

In total, 27 premalignant mucosal lesions, 47 primary carcinomas and 9 corresponding metastases were examined for the presence of HPV 16 and 18 DNA by FISH. In none of the tested premalignant lesions could HPV 16/18 be identified by our FISH procedure.

Ten of the 47 (21%) HNSCC exhibited HPV 16- specific FISH signals (Table 1) and only in case no. 8 a simultaneous infection with HPV 18 was observed. Interestingly, these 10 HNSCC comprised only 1 nonoropharyngeal tumor (tongue carcinoma) and 9 of the 16 oropharyngeal carcinomas (56%), i.e., 8 tonsillar and 1 base of the tongue tumors, indicating a highly significant ($p < 0.001$) correlation between HPV positivity and localization in the oropharynx. The 10 HPV-positive tumors all harbored punctate FISH patterns indicating HPV integration (Figure 1e,f and Table 1), while 7 of these tumors showed evidence for a concomitant replication of the virus in specific areas of the tumor (Figure 1g). Seven of the 10 HPV-positive tumors showed 1 HPV integration site per nucleus (Figure 1e), 2 tumors harbored areas with more than 1 integration site per nucleus (Figure 1f) and 1 tumor showed both a large and a smaller hybridization spot in the nuclei, suggesting different copy numbers of virus integrated at 2 sites in the cellular genome (case number 2, Table 1). Only in 2 of the 10 HPV-positive tumors did all tumor cells contain the viral DNA (tumor numbers 1 and 3, Table 1), whereas in the other 8 cases the HPV DNA-containing cells were either present as clusters or scattered throughout the tumor. From 2 patients, of which lymph node metastases were available for analysis, both the metastasis as well as the primary tumor displayed identical punctate nuclear HPV 16 signals, indicating stable integration of the virus prior to and during tumor spread (tumor numbers 3 and 4, Table 1 and Figure 1h–k).

Correlation between HPV 16 integration and absence of p53 mutations in exons 5-8

Fifteen of the 27 premalignant lesions (55%) showed accumulation of p53, predominantly observed in the basal cell layers of the epithelium (data not shown). In 30 of the 47 HNSCC (64%) p53 overexpression was detected, including 8 of the 10 HPV-positive carcinomas (Tables 1 and 2, and Figure 2a,b). To verify whether p53 accumulation in the HPV-positive HNSSC correlates with the presence of a gene mutation, 9 of the 10 tumors were also examined by SSCP analysis of p53 exons 5–8 (Figure 1l). Only the analysis of tumor DNA from patient number 1 (Table 1) resulted in a gel shift from a polymorphism in codon 213 of exon 6 of the p53 gene, also detected in the control DNA of the patient (Figure 1l–n). Thus, in our study no p53 mutations could be identified in HPVpositive HNSCC, despite the fact that these tumors often accumulate wild-type p53.

TABLE 1. CLINICOPATHOLOGIC DATA, HPV 16/18 AND P16^{INK4A}, PRB AND P53 STATUS IN 16 OROPHARYNGEAL CARCINOMAS AND 1 TONGUE CARCINOMA

Patient no.	Age at diagnosis	Gender	Smoking behaviour[1]	Alcoho consumption[2]	Tumorsite	Tumor classification[3]			Nuclear HPV FISH pattern[4]			Immuno-histochemistry[5]		
						T	N	M	Punctuate	Spot no.	Diffuse	p53	p16^{INK4A}	pRB
HPV positive														
1	46	Male	25	3U	Tongue	3	0	0	HPV16	1and >1	HPV16	Pos	Pos	3+
2	54	Female	0	0	Base of tongue	4	3	0	HPV16	2	HPV16	Pos	Pos	n.a.
3	48	Male	0	0	Tonsil LN	2	1	0	HPV16	1	HPV16	Neg	Pos	±
4	64	Female	0	0	Tonsil LN	2	2b	0	HPV16	1 and >1	HPV16	Neg/pos	Pos	2+
5	71	Male	0[6]	0	Tonsil	2	2b	0	HPV16	1	No	Pos	Pos	+
6	54	Female	0	0	Tonsil R L	1	0	0	HPV16[7]	1	HPV168	Pos	Pos	+
7	56	Female	0	5U	Tonsil	2	2b	0	HPV16	1	HPV16	Pos	Pos	2+
8	54	Male	0	4U	Tonsil	2	2a	0	HPV16+18	1	HPV18	Pos	Pos	±
9	69	Male	20	0	Tonsil	3	1	0	HPV16	1	No	Neg	Pos	±
10	54	Male	33	7U	Tonsil	4	2b	0	HPV16	1	No	Pos	Pos	±
HPV negative														
11	84	Male	20	0	Base of tongue	3	2b	0				Pos	Neg	Neg
12	58	Female	15	15U	Base of tongue	1	2b	0				Pos	Neg	
13	49	Male	0	0	Base of tongue	3	2c	0				Pos	Neg	Neg
14	47	Male	10	15U	Tonsil	2	2b	0				Pos	Neg	2+
15	49	Male	48	15U	Tonsil	4	3	0				Po	sNeg	3+
16	51	Male	8	3U	Tonsil	4	2b	0				Neg	Neg	2+
17	61	Male	25	4U	Tonsil	2	0	0				Neg	Neg	+

[1]The number of cigarettes smoked per day.-[2]The units of alcohol beverages consumed per day (1 unit ~12 g alcohol).-[3]Classification according to the pTNM classification system[27].-[4]FISH signals were scored according to Cooper et al.[25]: punctate signals, HPV integration sites; spot no., number of integration spots/nucleus; diffuse signals, HPV replication (episomal appearance).-[5]Protein staining in tumor cells was scored as follows: p16^{INK4A}: positive, ≥30% cells (nuclei and cytoplasm) stained; negative, <30% nuclei stained. pRb: 2+/3+, strong nuclear staining in ≥30% of cells, ±/+, weak nuclear staining; neg, no nuclear staining; n.a., not available. p53: positive, ≥30% nuclei stained; negative, <30% of nuclei stained.-[6]Former smoker: stopped smoking more than 10 years ago.-[7]Predominantly in the right tonsil.-[8]Predominantly in the left tonsil.

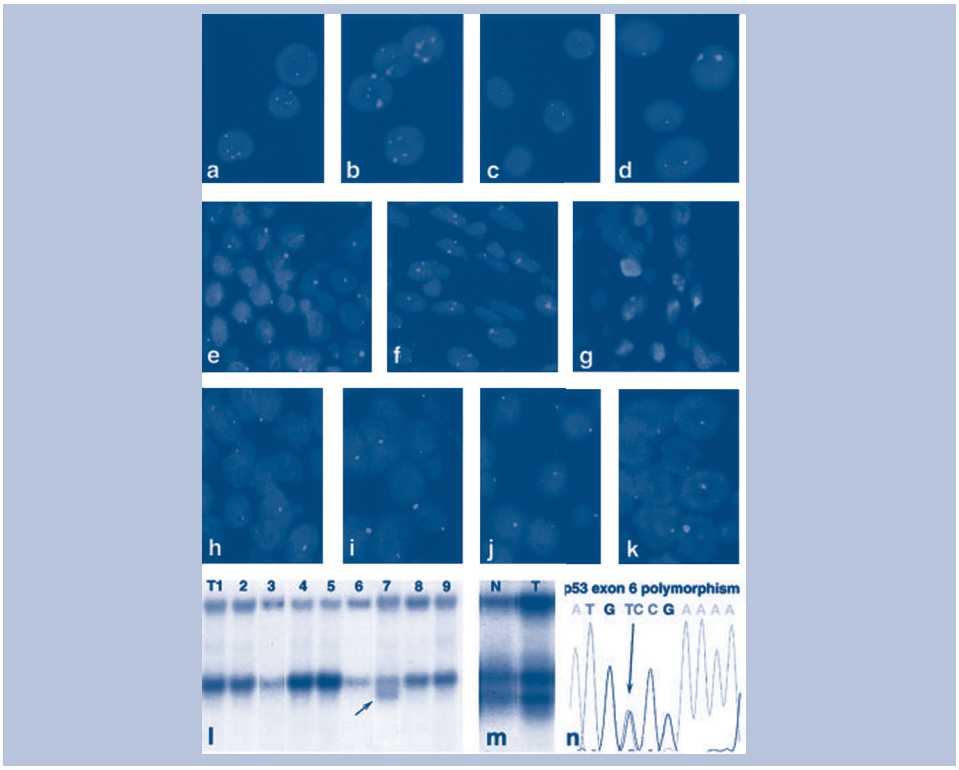

FIGURE 1. (a-k) RESULTS OF HPV 16/18 FISH ANALYSIS AND (l-n) SSCP ANALYSIS OF CELL LINES (a-d), TISSUE SECTIONS (e-k) AND EXTRACTED GENOMIC DNA (l-n) FROM SQUAMOUS CELL CARCINOMAS OF THE CERVIX (a- d) AND HEAD AND NECK REGION (e-n). (a,b) Hela cell nuclei (dark) showing the expected 5 HPV 18 integration sites (punctate signals, light) as visualized by conventional (a) and TSA (b) probe detection. (c,d) SiHa cell nuclei (dark) showing the expected 1-2 HPV 16 integration sites (punctate signals, light) as visualized by conventional (c) and TSA (d) detection. (e-g) Example of HPV 16-positive tongue carcinoma (patient no. 1, Table 1) showing different tumor areas harboring nuclei (dark) with 1 integration site (e), 2 integration sites (f) and episomal appearance of the virus (g) (light punctate (e,f) and diffuse (g) signals, respectively). (h- k) Primary tonsillar carcinoma and corresponding metastasis of patient nos. 3 (h,i) and 4 (j,k) demonstrating clonal association of HPV 16 during tumor spread as 1 integration site (light) per nucleus (dark). (l-n) p53 exon 6 II PCR-SSCP results of 9 of the 10 HPV-positive HNSCC patients. (l) Lanes 1-9 correspond to patient nos. 9, 5, 4, 6, 8, 3, 1, 7 and 2 in Table 1, respectively. The arrow indicates an aberrantly migrating fragment in tumor 1. (m) Comparison of tumor and normal DNA from patient 1 showing the aberrant fragment in both tissue samples. (n) Identification of an exon 6 codon 213 polymorphism (arrow) after sequencing the exon 6 II PCR products of tumor 1. (see page 152 for color figure)

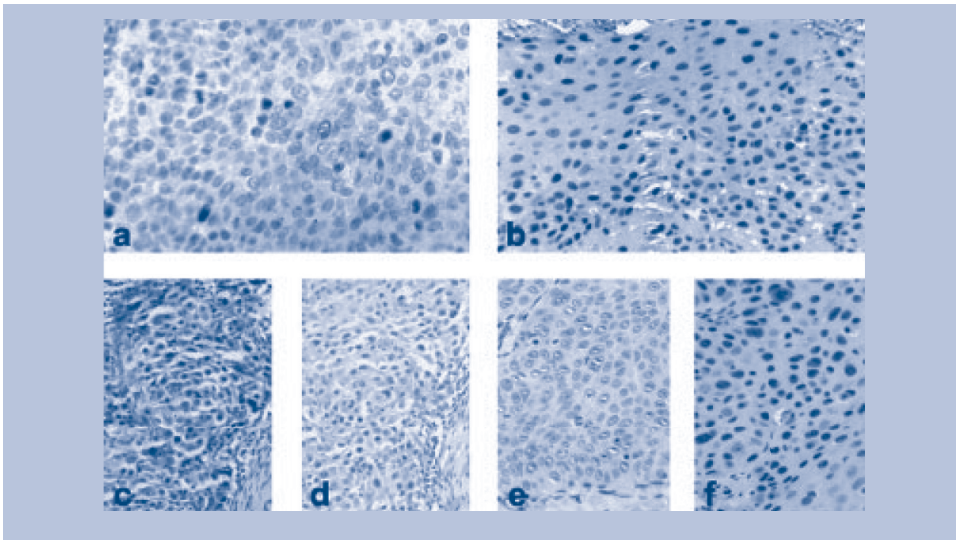

FIGURE 2. IMMUNOHISTOCHEMICAL STAINING FOR P53 (a,b), $P16^{INK4A}$ (c,e) AND PRB (d,f). (a,b) HPV-positive HNSCC no. 4 (a) and 1 (b) (Table 1) showing distinct nuclear staining for p53 protein in <30% and 100% of tumor cells. (c,d) HPV-positive tonsillar carcinoma no. 8 (Table 1) showing strong nuclear and cytoplasmic staining for $p16^{INK4A}$ (c) but only weak nuclear staining for pRb (d) in the tumor cells. (e,f) HPV-negative tonsillar carcinoma no. 15 showing no $p16^{INK4A}$ staining (e) but strong nuclear pRb staining in the tumor cells (f). (see page 153 for color figure)

Correlation between HPV status and $p16^{INK4A}$ overexpression

To assess the level of pRb and $p16^{INK4A}$ expression in HNSCC associated with HPV, immunohistochemical staining was performed on tissue sections of the 17 tumors listed in Table 1. Representative examples are presented in Figure 2c–f. A remarkable correlation was found between $p16^{INK4A}$ accumulation and the presence of HPV because all 10 HPV-positive HNSCC exhibited strong nuclear and cytoplasmic staining in the tumor areas harboring cells with integrated HPV (Figure 2c), while the HPV-negative cancers were also negative for $p16^{INK4A}$ (Figure 2e). Staining of pRb could be performed in 16/17 tumors and intense nuclear staining was observed in 3/9 HPV-positive and 3/7 HPV-negative tumors (in total 37%; Figure 2f). Accordingly, in 63% of carcinomas pRb expression was reduced or negative, including 6 of 9 HPV-positive cases (Table 1 and Figure 2d). These data are in agreement with the results of Wiest et al.[32] and demonstrate that besides a wild-type p53 status HPV-positive HNSCC are characterized by $p16^{INK4A}$ overexpression, as well as reduced pRb levels in most cases.

TABLE 2. HPV 16 AND P53 STATUS ACCORDING TO PRIMARY TUMOR SITE.

	p53 +[1]		p53 -	
	HPV +	HPV -	HPV +	HPV -
Oral cavity (n = 19)	1	10		8
Oropharynx (n = 16)	7	5	2	2
Hypopharynx (n = 5)		4		1
Larynx (n = 7)		3		4
Total (n = 47)	8	22	2	15

[1]p53 accumulation in ≥ 30% of tumor cell nuclei.

Correlation between HPV status, clinicopathologic parameters and risk factors

Clinical data, including gender, age at diagnosis and pTNM-classification of all oropharyngeal tumors and 1 tongue carcinoma as well as alcohol and tobacco consumption data are listed in Table 1. The mean age of patients with HPV-positive tumors was identical to that of patients with HPV-negative tumors, i.e., 57 years. In addition, neither pTNM-classification nor tumor grade (data not shown) was correlated with HPV 16/18 status. Four HPV-positive tumors showed basaloid characteristics (i.e., case numbers. 3, 7, 8 and 9 in Table 1). Four of the 9 HPV-positive patients with an oropharyngeal carcinoma (44%) were alcohol and/or tobacco consumers. This is in contrast to the HPV-negative patients, where 6 of the 7 (86%) patients consumed tobacco and 5 of the 7 (71%) consumed both tobacco and alcohol. Statistical analysis showed a significant correlation between HPV presence and the absence of tobacco exposure, i.e., 7 of 9 patients with HPV-positive tumors were nonsmokers compared to 1 of 7 patients who presented with an HPV-negative tumor (p = 0.0406). This also holds true when comparing HPV presence with consumption of both cigarettes and alcohol (1 of 9 patients with HPV-harboring tumors vs. 5 of 7 with HPV-negative cancers; p = 0.0350). In contrast, there was no clear relation between HPV status and alcohol consumption alone or in combination with tobacco intake.

Discussion

Similar to the situation in the uterine cervix, where oncogenic HPV has been firmly established as an etiologic risk factor for the development of squamous cell carcinoma and adenocarcinoma[12], several molecular and epidemiologic studies suggest that HPV also plays a role in the carcinogenesis of the head and neck mucosa in a subset of patients[8-10,32,36]. In conjunction with these suggestions, our data provide strong evidence that HPV 16 integration into the tumor cell genome is linked to a defined subset of HNSCC, particularly oropharyngeal carcinomas. These cancers, which exhibited $p16^{INK4A}$ overexpression but no p53 mutations in exons 5–8, may comprise a distinct pathologic entity, predominantly occurring in patients without a history of extravagant tobacco and/or alcohol consumption.

Although the prevalence of HPV in lesions of the head and neck mucosa has already been suggested by the end of the 1980s[37], the use of different detection methods and HPV-specific probes, as well as varying numbers of tissue samples of different locations, has caused confusion with respect to the frequency of HPV-positive lesions, ranging from 2–76%[8,11]. Especially the transition in the 1990s from in situ hybridization techniques to the highly sensitive PCR procedures to detect HPV DNA and its transcripts has actually decreased the discriminative power of HPV detection. For example, almost every lesion of the uterine cervix tested by these sensitive protocols will be classified HPV-positive[38]. Such an outcome will only be meaningful when evidence for the biologic association of HPV with the tumor cells is provided. This can, for example, be achieved by determining the viral copy number, E6/E7 expression levels or replication and/or integration state, the latter being linked to malignant progression in uterine cervical lesions[10,15,25,32,33,36]. This additional information can be generated by molecular approaches such as Southern blotting, (quantitative) (RT)PCR analysis of (microdissected) tumor cells and/or FISH. In our study, we have applied a highly sensitive FISH procedure enabling both HPV DNA detection up to the level of a single copy per cell nucleus, as well as discrimination between replicative (episomal) and integrated virus on the basis of the nuclear staining pattern. Our data show that 56% of oropharyngeal carcinomas, in particular tonsillar tumors (67%), exhibit HPV 16/18 genomic DNA integration in comparison to 3% of nonoropharyngeal carcinomas (p = 0.001) (in total 21% of HNSCC). HPV integration was predominantly seen as 1 punctate FISH signal per tumor nucleus. The strong association of HPV 16 with oropharyngeal cancer is in agreement

with recent data in the literature generated by PCR-based techniques[8-10,32,36,39] and further underlines the sensitivity of our FISH protocol. Nevertheless, a slight underestimation of HPV-infected HNSCC cannot be ruled out because we only focused on HPV types 16 and 18. In 2 patients where tissue from a lymph node metastasis was available, HPV integration could be identified in both the primary tumor and the metastasis, suggesting a clonal association of HPV with tumor cells. Besides HPV DNA integration, 7 of 10 HPV-positive HNSCC also harboured scattered (groups of) tumor cells with diffuse FISH signals, indicating replicative, extrachromosomal HPV. This finding is in agreement with in situ HPV staining results obtained in squamous cell carcinomas of the uterine cervix[25].

It has been proposed that transition of oncogenic HPV DNA from the replicative to the integrated state may reflect a progression towards the malignant phenotype in uterine cervical lesions[15,25]. If this hypothesis also holds for head and neck mucosal lesions, one would expect predominantly diffuse HPV staining patterns in preinvasive mucosal lesions. However, in our study we were unable to detect HPV 16/18 DNA in all these premalignant cases. This may be explained by the fact that in all cases it concerned preinvasive, nonoropharyngeal lesions (larynx, oral cavity). Although HPV has been reported to be present in these lesions, this mostly concerned HPV types 6 and 11, viruses that are considered of low oncogenic potential. Because premalignant lesions of the tonsils are scarcely observed in the clinic, studies on the prevalence of HPV 16/18 in normal mucosa of tonsils of healthy persons as well as tonsils with carcinomas, e.g., by using cytologic cell scrapes[40], should shed further light on its role in promoting tonsillar carcinogenesis. Alternatively, one could search for HPV-positive cells in premalignant lesions present in resection margins of operated tonsillar carcinomas. As it has been suggested that HPV-positive tonsillar carcinomas are a sexually transmitted disease[9,39], further epidemiologic studies should determine HPV prevalence in tonsils of the healthy population in relation to sexual behavior[36].

Our findings indicate that within the oropharynx in particular the palatine tonsils are at risk for HPV infection. Why particularly the tonsils are susceptible to HPV infection is unclear to date. Explanations to be considered comprise the fact that (i) tonsils, like the uterine cervix, are easily accessible and appear to undergo metaplastic processes[41]; (ii) tonsils contain deep invaginations of the mucosal surface (crypts), creating an extensive monolayered epithelial surface that may facilitate viral access to basal cells and intensive antigenic stimulation[36,42]; (iii) the presence

of cytokines produced by lymphoid tissue may affect HPV transcription and cellular transformation[41,43].

We noticed that all 9 HPV-positive HNSCC analyzed by PCR-SSCP for exons 5–8 of the p53 gene were devoid of mutations. This finding is in accordance with previous studies reporting an inverse relationship between p53 mutations and the presence of HPV[9,10,32], and underlines the etiologic role of HPV in a subset of HNSCC. HPV integration may thus stimulate tumor progression by targeting the nonmutated p53 for ubiquitination and degradation via E6 oncoprotein. This inactivation of p53 is achieved in half of the HNSCC by the mutation of the protein[6]. The literature reports large discrepancies in the number of HPV-PCR-positive HNSCC exhibiting simultaneous p53 exon 5–8 mutations, ranging from 13–46%[9,10,18,19,21,32,44]. In 2 of these studies[10,32], it was observed that when also taking E6 expression into account as an additional indicator of a causal relationship between HPV and HNSCC, maximally 8% of the tumors harbored p53 mutations. In addition, part of these tumors exhibited downregulation of pRb and accumulation of $p16^{INK4A}$ further indicating that in these carcinomas HPV is indeed etiologically involved. This implies that the high sensitivity of HPV detection by PCR also identifies "biologically irrelevant" cases due to the presence of only a few viral genomes that may not be clonally associated with the tumor or the use of crude extracts of frozen or paraffin sections with HPV contamination. Therefore, multiple PCR approaches are recommended to unequivocally prove the causal involvement of HPV in HNSCC. It is tempting to speculate that our sensitive FISH approach for assessing the involvement of HPV in HNSCC might be the optimal substitute for those multiple PCR assays. In particular, this approach can link HPV integration into the cellular genome directly to the histologic context. Although we are aware of the fact that we may underestimate the real number of tumors associated with HPV because of the use of only the HPV 16/18-specific probe mixture in our study, the clear correlation between HPV 16 integration as identified by FISH and $p16^{INK4A}$ overexpression (with simultaneous pRb downregulation in 6 of 9 cases) in our study underlines this speculation.

Despite the lack of p53 mutations, 8 of 10 FISH-positive HNSCC unexpectedly presented with nuclear accumulation of p53 in >30% of the tumor cells. This apparent discrepancy between p53 overexpression and absence of p53 exon 5–8 mutations has been observed previously[18,19,31]. The molecular mechanisms that may underlie this stabilization and/or overexpression of p53 protein are yet to be identified. Possible explanations comprise (i) the presence of mutations occurring outside exons 5–8[45];

(ii) upregulation of the wild-type p53 protein by genotoxic insults, e.g., ultraviolet radiation or hypoxia[46]; (iii) nonmutational p53 stabilization by mdm2[47] or viral proteins such as large T-cell antigen of SV40; and/or (iv) the lack of functional E6 expression[18]. We are currently in the process of studying some of these hypotheses in our series of HNSCC. Preliminary results so far did not demonstrate clear-cut evidence for transcriptional activity of p53 as concluded from the fact that the expression levels of the p53 transcriptional targets p21 and mdm2 do not show a consistent correlation with p53 overexpression in the HPV-associated tumors.

Our data show a significant correlation between HPV integration on the one hand and strongly reduced or absent exposure to the known risk factors of HNSCC, i.e., tobacco and alcohol consumption, on the other. These observations provide further evidence for the suggestion that HPV-positive HNSCC represent a separate tumor entity[9,36]. In these studies, a correlation with less alcohol intake was, however, more significant than with smoking, whereas in our study HPV integration was more strongly associated with smoking alone or in combination with alcohol intake. The reason for these differences may be due to the more accurate determination of HPV integration by FISH than by PCR, or on the other hand the relative low number of HPV-positive HNSCC in our study in comparison with others[9]. An improved prognosis, as has been reported for patients with HPV-positive HNSCC[9,48], could not be confirmed in our study because of insufficient follow-up. Although our study shows no correlation of HPV status with tumor grading, 4 oropharyngeal carcinomas exhibited a basaloid morphology, which previously has been reported to be associated with HPV positivity[9]. Speculations that HPV-positive tumors might be associated with younger age of patients[49], tumor size and gender could neither be substantiated in the current study, nor by earlier investigations[9,36].

From our study we conclude (i) that a subset of HNSCC, particularly tonsillar carcinomas, harbors HPV 16 shown by a sensitive FISH approach to be integrated in the tumor genome; (ii) that this subset of tumors is characterized by $p16^{INK4A}$ accumulation, as well as reduced pRb and overexpressed wild-type p53 in most of these cases; and (iii) that these tumors develop in patients showing no extravagant tobacco and/or alcohol consumption.

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Chapter 3

Marked differences in survival rate between smokers and non-smokers with HPV 16-associated tonsillar carcinomas

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Abstract

Oncogenic human papillomavirus (HPV) is a causative agent in a subgroup of head and neck carcinomas, particularly tonsillar squamous cell carcinomas (TSCC). This study was undertaken because controversial data exist on the physical status of HPV-DNA and the use of $p16^{INK4A}$ overexpression as surrogate HPV marker, and to examine the impact of HPV and tobacco consumption on the clinical course of TSCC. Tissue sections of 81 TSCC were analyzed by HPV 16-specific fluorescence in situ hybridization (FISH) and $p16^{INK4A}$-specific immunohistochemistry. Results were correlated with clinical and demographic data. HPV 16 integration was detected by FISH as punctate signals in 33 out of 81 (41%) TSCC, 32 of which showed $p16^{INK4A}$ accumulation. Only 5 out of 48 HPV-negative tumors showed $p16^{INK4A}$ immunostaining ($P < 0.0001$). The presence of HPV furthermore correlates significantly with low tobacco ($P = 0.002$) and alcohol intake ($P = 0.011$), poor differentiation grade ($P = 0.019$), small tumor size ($P = 0.024$), presence of a local metastases ($P = 0.001$) and a decreased (loco)regional recurrence rate ($P=0.039$). Statistical analysis revealed that smoking significantly increases the risk of cancer death from TSCC and that non-smoking patients with HPV-containing TSCC show a remarkably better disease-specific survival rate. HPV 16 is integrated in 41% of TSCC and strongly correlates with $p16^{INK4A}$ overexpression, implicating the latter to be a reliable HPV biomarker. Patients with HPV-positive tumors show a favorable prognosis as compared to those with HPV-negative tumors, but tobacco use is the strongest prognostic indicator. These findings indicate that oncogenic processes in the tonsils of non-smokers differ from those occurring in smokers, the former being related to HPV 16 infection.

Introduction

Head and neck squamous cell carcinomas (HNSCC) account for 4% of all malignancies in the Western world, for up to 50% of all malignancies in Southeast Asian countries and for 6.5% of all annual cancer cases worldwide[1]. HNSCC is associated with severe disease- and treatment-related morbidity and because treatment has not improved greatly in recent years, the 5-year survival rate remains approximately 50%. HNSCC develop in various anatomical defined regions, including the oral cavity, larynx and pharynx. These organ-specific tumors each show specific clinical presentations and outcome, and are treated by different strategies[2,3]. The median age at presentation is 60 years and approximately two third of patients are male[1].

Well-known risk factors in the etiology of HNSCC are cigarette smoking combined with alcohol consumption in Western countries, or with betel quid chewing in Asia. A history of tobacco use is present in 90% of patients who develop oral cavity cancers[2,3]. Despite these evident associations, the exact mechanisms by which these factors cause tumor initiation and progression are not fully understood. Furthermore, the fact that most tobacco and alcohol users do not develop HNSCC and that in recent years more often individuals without a history of these traditional risk factors have been witnessed[4], underlines the complexity of HNSCC pathogenesis and a role for additional factors in the disease process.

Increasing evidence suggests that human oncogenic papillomaviruses (HPVs), known to cause uterine cervical and other anogenital cancers, may also be of importance in the pathogenesis of HNSCC[5]. The strongest association has been found for oropharyngeal carcinomas, especially tonsillar carcinomas[6-11]. Sero-positive patients for HPV 16 or with a history of HPV-related anogenital cancer also show increased risk rates of developing oropharyngeal cancer[12,13]. The prevalence of HPV-exhibiting HNSCC, however, varies broadly amongst several studies (2-76%) due to differences in the population, combination of histological subsites, type and number of specimens analyzed, and detection methods used[7,14]. Thus, besides determining the presence of HPV DNA it has been suggested to better define the biological association of oncogenic HPV with these tumors, e.g. by means of assessing the viral copy number per cell, the viral oncoprotein E6/E7 expression levels, perturbation of pRb-dependent cell cycle control, or the physical status of the virus (episomal or integrated)[15]. In this way, several reports have shown that HPV 16 is predominantly identified in oropharyngeal carcinomas, with a frequency of approximately 50-70%[9,16-18].

Integration of high-risk HPV DNA, such as HPV 16 DNA, into the human cellular genome is considered an important step in malignant transformation[19]. From studies on lesions of the uterine cervix it has become clear that viral integration marks the transition from a dysplastic lesion to (micro)invasive cancer[20,21]. After integration and disruption of (part of) the viral early gene E2, an upregulation of the oncoproteins E6 and E7 is detected[7,19]. On the one hand the E6 protein interacts specifically with the host-cell tumor suppressor protein p53 and induces its degradation. The subsequent inability to inhibit cell growth and induce apoptosis results in genetic instability. P53 mutations are therefore not a prerequisite in HPV-related tumorigenesis, and are therefore stand seldomly identified[8,9,18]. On the other hand, the E7 protein inactivates the retinoblastoma tumor suppressor protein pRb, resulting in release of the transcription factor E2F and upregulation of $p14^{ARF}$ and $p16^{INK4A}$ [22,23]. In oropharynx and especially tonsillar carcinomas, however, the literature is controversial with respect to viral integration. Results range from virus being present only in an episomal form to 100% viral integration, with or without concurrent episomal HPV[18,24,25]. Also immunohisto-chemical detection of $p16^{INK4A}$ overexpression, which has been postulated as a fast, easy and less expensive alternative for the detection of HPV infection in tonsillar cancer, may remain negative in cases where the gene is inactivated[17,26-28].

With respect to the clinical course and prognosis of HPV-related tonsillar squamous cell carcinomas (TSCC), varying observations have been reported. Some authors described a survival advantage of HPV-positive TSCC patients when compared to HPV-negative cases[8,29-31], while others showed the opposite result[32] or could not show a difference in survival outcome[33,34]. In addition, it has been suggested that the clinical course of the disease might be gender-specific, since males with a HPV-positive tumor had a better prognosis than males with a HPV-negative tumor, whereas this correlation could not be detected in women[29]. It has also been postulated that HPV would be more prevalent in younger patients[33,35].

In the present study we have analyzed a series of 81 TSCC for HPV 16 using fluorescence in situ hybridization (FISH). This technique allows the visualization of 1 copy of HPV DNA in tumor cells, and at the same time the determination of the physical status (integrated versus episomal) of the virus on basis of the nuclear staining pattern. HPV 16 detection in the tumor was also correlated with $p16^{INK4A}$ overexpression, in order to investigate whether or not this method is reliable to distinguish between HPV-positive and HPV-negative TSCC. Furthermore, clinico-

pathological characteristics, smoking, alcohol intake and disease outcome were correlated with HPV status.

Materials and methods

Tumor material and patient data

The study population consisted of 81 patients with a TSCC diagnosed between 1992 and 2001. Formaldehyde-fixed, paraffin-embedded archival biopsy and resection materials from these patients were selected from the archives of the Department of Pathology, University Hospital Maastricht, The Netherlands. Information on patient gender, age, smoking and alcohol consumption, treatment modality, date and cause of death, as well as tumor site, differentiation grade and TNM classification were collected from review of clinical, pathological, radiological and surgical reports. Patients were classified as daily tobacco smokers (≥ 1 cigarette, pipe, and/or cigar per day) or non-smokers (never smoker (n = 10) or former smoker (n = 2), which are those who had stopped smoking more than 10 years before the diagnosis of TSCC). Patients were also classified as drinkers (consumption of > 2 whiskey equivalents per day (1 whiskey equivalent ~10 g alcohol)) or non-drinkers (0-2 whiskey equivalents per day). All patients were treated by surgery, radiotherapy, chemotherapy or a combination irrespective of their HPV status. The study protocol was approved by the institutional ethical committee, and all of the patients gave informed consent.

When available the tissue sections were taken from the resection specimen. Otherwise biopsy material was used for examination. A series of 4 µm-thick sections was cut from the specimens for detailed histopathological reclassification on basis of hematoxylin-eosin staining (F.J.B.), including the scoring of tumor grade (i.e., well, moderately, or poorly differentiated) according to the criteria of the World Health Organization[36]. Furthermore, we applied FISH to identify HPV 16 infestation and immunohistochemistry to visualize $p16^{INK4A}$ expression.

Detection of HPV 16 DNA by FISH

FISH for the detection of HPV 16 was performed on 4-µm thick tissue sections as described previously[18,21]. Formaldehyde-fixed, paraffin-embedded sections were deparaffinized, pre-treated with 85% formic acid/0.3% H_2O_2 for 20 minutes at room temperature, and subsequently dehydrated in an ethanol series starting with 70%

ethanol containing 0.01 M HCl (acidic dehydration). After air drying, the preparations were treated with 1 M NaSCN for 10 minutes at 80°C, followed by acidic dehydration and digestion with 4 mg/ml pepsin (800-1,200 U/mg protein from porcine stomach mucosa; Sigma Chemical Co., St. Louis, MO, USA) in 0.02 M HCl. The slides were rinsed three times in 0.01 M HCl, acidically dehydrated, air dried, post-fixed in 1% formaldehyde in PBS for 15 minutes at room temperature and dehydrated in an ethanol series starting with 70% ethanol in distilled water. The digoxigenin-labeled HPV 16 probe (Panpath, Amsterdam, The Netherlands) was applied under a coverslip at a concentration of 1 ng/µl in 60% formamide, 2× SSC, 10% dextran sulphate, and a 50x excess of carrier DNA (salmon sperm DNA). Probe and target DNA were denatured simultaneously for 5 minutes at 80°C prior to hybridization overnight at 37°C in a humid chamber. After hybridization the preparations were washed stringently in 50% formamide, 2×SSC, pH 7.0 at 42°C (2 times 5 min). The digoxigenin-labeled probe was detected with peroxidase-conjugated sheep anti-digoxigenin Fab fragments (Roche, Basel, Switzerland; 1:100 diluted in 4xSSC containing 5% non-fat dry milk), followed by a tyramide signal amplification (TSA) reaction using rhodamine-labeled tyramide[18,21,37]. 50 µl rhodamine-labeled tyramide (1:500 diluted from a 1 mg/ml stock solution in ethanol) in PBS containing 0.1 M imidazole, pH 7.6, and 0.001% H_2O_2 was applied under a coverslip for 10 minutes at 37°C. Finally, the slides were washed in PBS containing 0.05% Tween-20 (Janssen Chimica, Beerse, Belgium) and PBS, dehydrated in an ascending ethanol series and mounted in Vectashield (Vector Laboratories, Burlingame, USA) containing 0.2 µg/ml 4',6-diamidino-2-phenyl indole (DAPI; Sigma). Slides were evaluated under the microscope and images were recorded with the Metasystems Image Pro System (black and white CCD camera; Sandhausen, Germany) mounted on top of a Leica DM-RE fluorescence microscope equipped with DAPI and rhodamine filters.

Controls and evaluation of FISH results

Controls included hybridizations on 1) 70% ethanol-fixed cell suspensions and formaldehyde-fixed sections of paraffin-embedded cell pellets of human uterine cervical carcinoma cell lines with known HPV 16 copy number, i.e. CaSki (ATCC; CRL1550; 500 integrated HPV 16 copies), SiHa (ATCC; HTB35; 1-2 integrated HPV 16 copies) and HeLa (ATCC; CCL2; 20-50 integrated HPV 18 and no HPV 16 copies); and 2) formaldehyde-fixed, paraffin-embedded tissue sections of human uterine cervical lesions with proven integration or episomal presence (replication) of HPV 16 genomic

DNA to guarantee probe specificity, sensitivity and interpretation accuracy[18,21]. Negative controls consisted of HPV PCR- and FISH-negative cell lines (the bladder transitional cell carcinoma line T24 and the endocrine pancreatic tumor line BON-1) and tissue sections.

Evaluation of FISH signals was performed by three investigators (H.C.H, A.H., E.J.M.S.) according to the criteria first described by Cooper et al.[38], i.e. punctate and/or diffuse nuclear signals indicate the presence of integrated and/or episomal HPV DNA, respectively[18,21]. These criteria are based on the correlation of these FISH signal patterns with restriction digestion and Southern blot hybridization results to detect integrated or replicative (episomal) HPV[38]. In a recent preliminary study HPV integration as detected by punctate FISH signals also strongly correlated with the presence of fusion transcripts as detected by the amplification of papilloma virus oncogene transcripts (APOT) assay[20] in 10 TSCC and 10 uterine cervical SCC (unpublished observations).

Immunohistochemical staining of $p16^{INK4A}$

Immunohistochemical staining of $p16^{INK4A}$ on 4 µm-thick formaldehyde-fixed, paraffin-embedded tissue sections was performed as described earlier[18]. Sections were deparaffinized and subsequently pretreated with 2% H_2O_2 in methanol for 30 minutes to quench endogenous peroxidase activity. Antigen retrieval was performed by microwave heating in 0.01M citrate buffer (pH 6.0). The monoclonal antibody E6H4 (Dako, Glostrup, Denmark) was used to detect the $p16^{INK4A}$ protein. After incubation with biotinylated horse anti-mouse antibody, immunohistochemical detection was performed by the avidin-biotinylated peroxidase complex (ABC) procedure (both Vectastain-Elite-ABC kit; Vector). Peroxidase activity was detected using diaminobenzidine/H_2O_2. Sections were counterstained with hematoxylin and mounted in Entellan (Merck, Darmstadt, Germany). In each analysis, negative and positive controls were included. Analysis was performed by three independent observers (H.C.H., M.S. and E.J.M.S.) and consensus was acquired. Levels of $p16^{INK4A}$ expression in normal squamous epithelium are under the detection limit of immunohistochemistry. Strong nuclear and cytoplasmic $p16^{INK4A}$ staining in ≥ 25% of tumor cells was considered as positive[18,23,27].

Detection of high risk HPV DNA by PCR

Genomic DNA was extracted from 5-10 10 µm-thick tissue sections of a subset of 25 TSCC according to the tissue protocol of the QIAamp DNA mini kit using proteinase K (Qiagen, Westburg, Leusden, The Netherlands)[18]. ß-Globin gene PCR was performed with all DNA samples to demonstrate that they contained sufficient DNA of adequate quality and were free of substances inhibitory to PCR (268 bp PCO4/GH20 PCR product)[17]. HPV 16-specific PCR was performed according to Baay et al.[39]. In 5 tumors that were negative for HPV 16 but positive for $p16^{INK4A}$, both a general primer GP5+/6+ PCR (150 bp product) and a nested PCR with degenerate primers A5/A10 (527 bp product) and A6/A8 (268 bp product) for HPV were performed[40]. PCR products (5µl) were separated on 2% agarose gels and visualized by ethidium bromide staining. For HPV typing biotinylated A6/A8-PCR products were hybridized with 37 type-specific digoxigenin-labeled oligonucleotide probes in an enzyme-immunoassay (EIA) as previously described[41].

Statistical analysis

Factors associated with HPV status, including presence of $p16^{INK4A}$ overexpression, gender, age at time of diagnosis, smoking and alcohol use, TNM status and grade of the tumors, were analyzed by cross-tabulations using the 2-tailed Fisher exact test. A significance level of $P \leq 0.05$ was chosen for all analyses.

Disease-specific and overall survival curves were calculated using the Kaplan-Meier method[42]. Survival was calculated from the date of diagnosis until death or until the last date the patient was known to be alive. Patients that died of other causes than tonsillar carcinoma were considered censored observations in the disease-specific survival analyses. Disease-free survival was calculated from the date of diagnosis until the date of recurrence (local, regional or distant, whichever occurred first). Patients without recurrence were censored at the date of the last follow-up or the date of death. The statistical significance of differences between survival times was determined by the log rank test in univariate analyses[43] using a significance level of $p \leq 0.05$.

Multivariate analyses were performed using the Cox proportional hazards model. Variables included were HPV, smoking, alcohol consumption, and T-classification. Variables remained in the model if their P values were below 0.10. All calculations were performed by use of the SPSS Base System version 11.5.

Results

Clinico-pathologic characteristics of the study population

Table 1 provides demographic and clinical features of the 81 patients included in this study. Seventy-three percent of the patients were male. Patient ages ranged from 39-87 years with a mean of 58.9 years.

Data concerning smoking and alcohol intake could be obtained from 80 patients. Sixty-eight of 80 (84%) patients were smokers and 49 of 80 (61%) consumed more than 2 units of alcohol per day. Twenty-three (28%) of the 80 patients consumed only tobacco, 4 (5%) only alcohol and 45 (56%) used both tobacco and alcohol, while there were only 8 (10%) patients without intoxication of alcohol or tobacco.

At time of diagnosis 40 of the 81 (49%) patients presented with a tumor < 4 cm in diameter. Fifty-nine (73%) patients had lymph node metastasis at time of diagnosis, and in 22 (27%) ultrasonography and MRI could not detect a lymph node metastasis. The grade of the squamous cell carcinomas was poor or moderate in 29 (36%) patients, while the tumors were well differentiated in 49 (60%) patients.

Treatment modalities consisted of surgery, radiotherapy, chemotherapy or combinations of these (Table 1). Thirty patients were never disease free, 17 (21%) patients developed a recurrent disease (12 at the primary site (local) and 5 in the neck (regional)) and 34 (42%) patients remained disease free after primary treatment. In addition, 6 patients developed a second primary tumor.

HPV 16-containing tonsillar carcinomas and correlation with p16 ^{INK4A} overexpression

Thirty-three (41%) out of the 81 TSCC contained punctate, nuclear HPV 16 FISH signals in > 25% of tumor cells, indicating viral integration into the cellular genome (Figure 1A). In the other tumors no specific HPV 16 FISH signals were detected. Twenty-six of the HPV-positive tumors showed 1 integration site per nucleus, while the remaining tumors harbored either 2 integration sites per nucleus (n = 1), or two tumor areas with, respectively, 1 and 2 integration sites per nucleus (n = 1), or tumor areas with, respectively, 1 integration site per nucleus and > 1 nuclear signals varying significantly in size and intensity (termed granular FISH pattern; n = 5). In addition, 11 of the 33 HPV 16-positive tumors showed evidence for a concomitant replication of the virus in specific areas of the tumor as visualized by diffusely stained nuclei (Figure 1B). Interestingly, 32 out of the 33 (97%) HPV-positive TSCC also exhibited

TABLE 1. P16^{INK4A} AND CLINICOPATHOLOGICAL DATA OF THE STUDY POPULATION GROUPED BY HPV 16 INTEGRATION STATUS.

Characteristic	Total TSCC n = 81 (%)	HPV-positive n = 33 (%)	HPV-negative n = 48 (%)	Fisher exact P
P16^{INK4A} overexpression[1]				
Yes	37 (46)	32 (97)	5 (10)	< 0.0001
No	44 (54)	1 (3)	43 (90)	
Gender				
Male	59 (73)	24 (73)	35 (73)	NS
Female	22 (27)	9 (27)	13 (27)	
Mean age at diagnosis (y)	58.9	59.8	58.2	NS
Intoxication				
Smoking	23 (28)	11 (33)	12 (25)	
Alcohol	4 (5)	3 (9)	1 (2)	
Smoking + alcohol	45 (56)	11 (33)	34 (71)	
None	8 (10)	7 (21)	1 (2)	
Unknown	1 (1)	1(3)	0	
Smoking[2]				
Yes	68 (84)	22 (70)	46 (96)	0.002
No	12 (15)	10 (30)	2 (4)	
Unknown	1 (1)	1	0	
Alcohol intake[3]				
Yes	49 (61)	14 (42)	35 (73)	0.011
No	31 (38)	18 (58)	13 (27)	
Unknown	1 (1)	1	0	
Complaints at diagnosis				
Local	56 (69)	15 (46)	41 (85)	0.001
Metastasis	20 (25)	14 (42)	6 (13)	
Unknown	5 (6)	4 (12)	1 (2)	
Lymph node metastasis[4]				
Positive	59 (73)	26 (79)	33 (69)	NS
Negative	22 (27)	7 (21)	15 (31)	
T-classification				
≥ 4 cm (T3-4)	40 (49)	11 (33)	29 (60)	0.024
< 4 cm (T1-2)	41 (51)	22 (67)	19 (40)	
Tumor grade[5]				
Poor/moderate	29 (36)	17 (52)	12(25)	0.019
Well	49 (60)	15 (45)	34(71)	
Unknown	3 (4)	1 (3)	2(4)	
Primary therapy				
Surgery	8 (10)	5 (15)	3 (6)	
Surgery + RT	36 (44)	17 (52)	19 (40)	
RT	23 (28)	8 (24)	15 (32)	
CT + RT	4 (5)	0	4 (8)	
CT	3 (4)	2 (6)	1 (2)	
Surgery+CT+RT	5 (6)	1 (3)	4 (8)	
None	2 (3)	0	2 (4)	
Recurrent disease				
Yes	17 (21)	4 (12)	13 (27)	0.039
No	34 (42)	19 (58)	15 (31)	
Never disease free	30 (37)	10 (30)	20 (42)	
Second primary tumor				
Yes	6 (7)	0	6 (13)	NS
No	75 (93)	33 (100)	42 (87)	

HPV= human papillomavirus; NS= not significant; RT= radiation therapy; CT= chemotherapy. [1]Strong nuclear and cytoplasmic p16^{INK4A} staining in ≥25% of tumor cells was considered positive and in <25% of cells negative. [2]Patients were classified as daily tobacco smokers (≥ 1 cigarette, pipe, and/or cigar per day) or non-smokers (never smoker (n = 10) or former smoker (n = 2), which are those who had stopped smoking more than 10 years before the diagnosis of TSCC). [3]Patients were classified as drinkers (consumption of > 2 whiskey equivalents per day (1 whiskey equivalent ~10 g alcohol)) or non-drinkers (0-2 whiskey equivalents per day). [4]As determined by ultrasonography and MRI scanning. [5]Tumor grade was scored as well-, moderately-, or poorly differentiated according to the criteria of the World Health Organization.[36]

strong nuclear and cytoplasmic staining for $p16^{INK4A}$ in the tumor areas harboring cells with nuclear HPV signals (Figure 1C-D). Forty-three out of the 48 (90%) HPV-negative TSCC did not show any expression of $p16^{INK4A}$. Thus, a highly significant correlation was found between accumulation of p16 ^{INK4A} and the presence of HPV 16 ($p<0.0001$; Table 1).

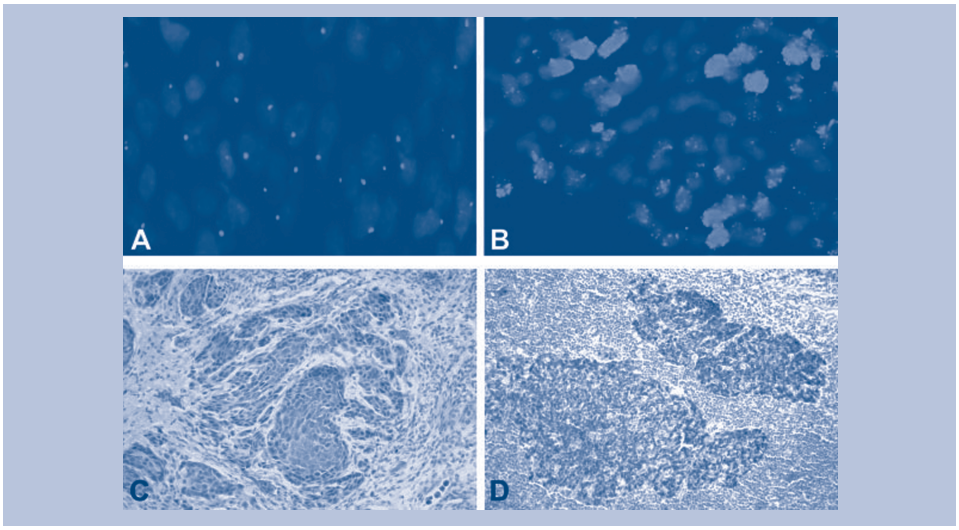

FIGURE 1. REPRESENTATIVE EXAMPLES OF HPV 16 FISH ANALYSIS (A, B) AND $P16^{INK4A}$ IMMUNOSTAINING (C, D) ON PARAFFIN-EMBEDDED TISSUE SECTIONS OF TSCC. (A) Example of one HPV 16-specific punctate signal per nucleus (light) indicating viral integration. Tumor cell nuclei are dark due to DAPI staining (B) Example of a tumor area showing diffuse nuclear staining (light) indicating viral replication (episomal virus copies), nuclei are DAPI counterstained. (C-D) Overexpression of $p16^{INK4A}$ (dark peroxidase-diamino benzidine staining) in two HPV 16-positive TSCC, nuclei counterstained by hematoxylin (light). (see page 156 for color figure)

In order to validate the FISH results, a subset of 25 $p16^{INK4A}$-positive tumors, including 20 FISH-positive cases, was subjected to HPV 16-specific PCR analysis. PCR confirmed the presence of HPV 16 DNA in the 20 FISH positive cases. The 5 remaining TSCC, however, also proved to be negative for 36 other genital/mucosal HPV types (data not shown).

Clinico-pathological features related to the presence of HPV 16

The male/female ratio and the age distribution in the study population were identical in the HPV-positive and HPV-negative subgroup (Table 1). In contrast, statistical

analysis showed a significantly lower prevalence of smoking and drinking habits in patients with HPV-positive carcinomas than in patients with HPV-negative carcinomas (P = 0.002 and 0.011, respectively). In the patients with HPV-positive carcinomas the age distribution was not different between smokers and non-smokers.

The patients with HPV-positive carcinomas presented significantly more often (P = 0.001) with complaints of a swelling in the neck caused by a lymph node metastasis as compared to the patients with HPV-negative carcinomas. However, there was no difference in the number of patients with tumor spread to the regional lymph nodes between the two subgroups. The primary tumor size at time of presentation was found to be significantly smaller in the HPV-positive group than in the HPV-negative group (P = 0.024), suggesting that HPV-positive tumors metastasize at a smaller size than HPV-negative tumors. The HPV-positive tumors, moreover, showed significantly more often a poor or moderate histological degree of differentiation (P = 0.019).

All patients were treated according to the same protocol and therapies did not differ significantly between the HPV-positive and HPV-negative subgroups. Patients with a HPV-negative tumor had a significantly higher chance (P = 0.039) of developing recurrent disease (27%; 9 local and 4 regional recurrences) compared to patients with a HPV-positive tumor (12%; 3 local and 1 regional recurrences). Recurrent disease was seen in 17 patients, and the time of occurrence ranged from 3 to 43 months after the initial diagnosis with a mean time period of 18 months. The mean recurrence-free interval was longer in the patients with HPV-positive carcinomas than in the patients with HPV-negative carcinomas, i.e. 27 vs. 16 months, respectively. Only in the HPV-negative patient group second primary tumors developed in 6 out of 48 cases (13%).

Parameters influencing patient survival

To determine the prognostic value of the presence of HPV in tonsillar carcinomas, we analyzed both overall and disease-specific survival. Two patients died post-operatively, due to bleeding and aspiration, and were excluded from the analysis. Follow-up time ranged from 0 to 141 months, with a mean of 30 months. Fifty-three of 79 (67%) patients died due to their TSCC and 8 (10%) from unrelated causes. Using the Kaplan-Meier algorithm the 5-year overall and disease-specific survival was 34% and 38%, respectively.

Stratification according to HPV status revealed a significantly improved 5-year overall as well as disease-specific survival for the patients with HPV-positive tumors

compared to patients with HPV-negative tumors (log rank test: p = 0.026 and p = 0.023, respectively; see Figure 2A). The disease-specific survival after 5 years for patients with HPV-positive carcinomas was 55%, and 29% for patients with HPV-negative carcinomas (Hazard Ratio (HR) = 2.3; Confidence Interval (CI) = 1.1-4.5; Table 2). In the 51 patients reaching a disease-free state after therapy, the 5-year disease-free survival for patients with HPV-positive and HPV-negative tumors was 73% and 48%, respectively (log rank test: p = 0.038). Because of the strong correlation between HPV 16 FISH status and $p16^{INK4A}$ overexpression, the disease-specific survival curve stratified according to $p16^{INK4A}$ status is comparable with the stratification according to HPV 16 status (log rank test: p = 0.010 and p = 0.023, respectively; compare Figures 2A and 2B).

Apart from absence of HPV 16, other factors were found to be significantly associated with a shorter disease-specific survival in patients with TSCC in a univariate analysis. These include lack of $p16^{INK4A}$ overexpression (HR = 2.3; 95% CI = 1.2-4.3), smoking (HR = 6.0; 95% CI = 1.5-25; see Figure 2C), a combination of smoking and alcohol abuse (HR = 2.8; 95% CI = 1-7.7), a tumor diameter of ≥ 4cm (HR = 2.8; 95% CI = 1.5-5.3), and development of recurrent disease (HR = 15.2; 95% CI = 4.2-54.6) (Table2). Gender, age, complaints at time of diagnosis, alcohol intake, lymph node status and tumor grade were not significantly related to disease-specific survival. Because tobacco use turned out to be the strongest individual predictor for an unfavorable prognosis, we also analyzed the predictive value of the HPV 16 status for survival in smokers, and of tobacco use in the patients with HPV 16-positive TSCC. Interestingly, HPV 16 status had no significant effect on outcome in smokers (Figure 2D). However, 10 of the 12 non-smokers also harbored HPV 16, and these patients had a significantly more favorable disease-specific survival than smokers with HPV-positive TSCC (Figure 2E). In addition, HPV-positive tumors of non-smokers were significantly smaller and less well differentiated than those of smokers (p = 0.026 and 0.013, respectively; data not shown).

Using multivariate analysis (Table 3), patients with HPV-negative TSCCs were found to exhibit a 2 times higher chance of cancer death (95% CI = 0.9-4.2) compared to patients with HPV-positive tumors. Smokers had an even higher risk (5.5 fold) (95% CI = 1.3-23.6) of dying from cancer compared to non-smokers. Patients with a tumor ≥ 4 cm in diameter had a 2.6 times increased risk of cancer death (95% CI = 1.4-4.9). Other factors including gender, age, lymph node metastasis and tumor grade did not significantly influence survival in multivariate analysis.

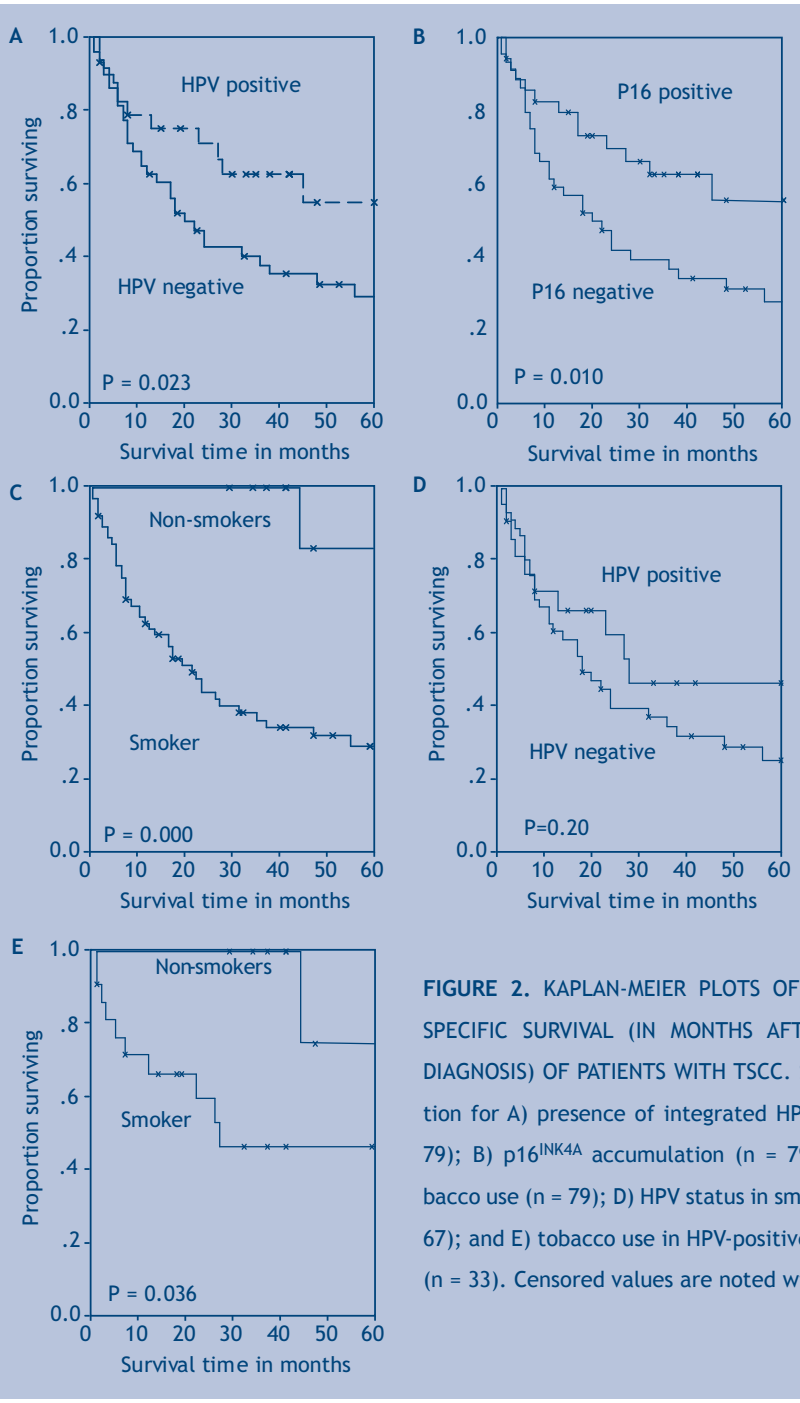

FIGURE 2. KAPLAN-MEIER PLOTS OF DISEASE-SPECIFIC SURVIVAL (IN MONTHS AFTER FIRST DIAGNOSIS) OF PATIENTS WITH TSCC. Stratification for A) presence of integrated HPV 16 (n = 79); B) $p16^{INK4A}$ accumulation (n = 79); C) tobacco use (n = 79); D) HPV status in smokers (n = 67); and E) tobacco use in HPV-positive patients (n = 33). Censored values are noted with ×.

TABLE 2. INFLUENCE OF HPV-RELATED AND CLINICOPATHOLOGIC PARAMETERS ON DISEASE-SPECIFIC SURVIVAL IN 79 PATIENTS WITH TSCC, AS DETERMINED BY UNIVARIATE COX PROPORTIONAL HAZARD REGRESSION ANALYSIS

Characteristic	Total	Death (%)	P value [1]	Unadjusted HR (95%CI)
Integrated HPV 16				
Positive[2]	31	11 (35)	0.015	1 (referent)
Negative	48	34 (71)		2.3 (1.1-4.5)
$P16^{INK4A}$ overexpression				
Positive[2]	35	13 (37)	0.010	1 (referent)
Negative	44	32 (73)		2.3 (1.2-4.3)
Gender				
Male[2]	58	35 (60)	0.095	1 (referent)
Female	21	10 (48)		0.6 (0.3-1.1)
Age				
< 60 years[2]	43	26 (60)	NS	1 (referent)
> 60 years	36	19 (53)		0.8 (0.4-1.4)
Intoxication				
None[2]	8	1 (13)	0.019	1 (referent)
Smoking	23	17 (74)		11.2 (1.5-85)
Alcohol	45	26 (58)		1.3 (0.5-3.4)
Smoking & alcohol	3	1 (33)		2.8 (1.0-7.7)
Smoking				
No[2]	11	2 (18)	0.005	1 (referent)
Yes	68	43 (63)		6.0 (1.5-25.0)
Alcohol intake				
No[2]	31	18 (58)	NS	1 (referent)
Yes	48	27 (56)		1.0 (0.6-1.8)
Complaints at diagnosis				
Local[2]	55	36 (65)	NS	1 (referent)
Metastasis	20	7 (35)		0.5 (0.2-1.2)
Unknown	4			
Lymph node metastasis				
Negative[2]	21	13 (62)	NS	1 (referent)
Positive	58	32 (55)		1.0 (0.5-1.9)
T-classification				
< 4 cm[2]	39	17 (44)	0.001	1 (referent)
> 4 cm	40	28 (70)		2.8 (1.5-5.3)
Tumor grade				
Poor/moderate[2]	28	15 (54)	NS	1 (referent)
Well	48	29 (60)		1.2 (0.6-2.2)
Unknown	3			
Recurrent disease				
No[2]	34	4 (12)	<0.0001	1 (referent)
Yes	17	14 (82)		15.2 (4.2-54.6)
Never disease free[3]	28	27 (96)		

HPV = human papillomavirus; HR = hazard ratio; CI = confidence interval. [1]According to the log rank test. [2]Reference group for HR calculation. For exposure categories, the unexposed were chosen as the reference group. For the other categories the first category was chosen as the reference group. [3]One patient died of other causes

TABLE 3. MULTIVARIATE ANALYSIS, ACCORDING TO COX PROPORTIONAL HAZARD REGRESSION ANALYSIS, OF THE POPULATION CHARACTERISTICS RELATED TO SURVIVAL OUTCOME.

Characteristic	HR	95%CI	P-value
HPV status: negative vs. positive	2.00	0.9-4.2	0.08
Smoking: yes vs. no	5.53	1.3-23.6	0.02
Alcohol use: yes vs. no	0.52	0.3-1.0	0.06
Tumor size: T3-4 vs. T1-2	2.60	1.4-4.9	0.01

HPV = human papillomavirus; HR = hazard ratio; CI = confidence interval

Discussion

In the head and neck region oncogenic HPV 16 appears to be predominantly detected in lesions developing in the oropharynx, in particular the tonsil[6-8,10,12,17,18,44]. In this report we have applied a highly sensitive FISH procedure to 81 TSCC, enabling HPV DNA detection up to the level of a single copy per cell nucleus, and discrimination between replicative (episomal) and integrated virus on the basis of the nuclear staining pattern. Using this approach 41% of these tumors exhibited nuclear HPV signals indicative for viral integration into the cellular genome. These tumors furthermore demonstrated accumulation of the CDK4/6 inhibitor $p16^{INK4A}$. HPV integration was also very strongly associated with specific clinico-pathological characteristics, as well as with absence of extravagant tobacco and/or alcohol consumption. Interestingly, the presence of HPV 16 proved to be a strong independent predictor of favorable outcome in these non-smokers. Tobacco use on the other hand was the most important predictor of a reduced survival rate in patients with TSCC.

HPV 16 is integrated in TSCC

Data concerning the physical status of HPV in TSCC are so far limited and confusing, ranging from virus being present only in the integrated or only in the episomal form[18,24,25,45]. Our FISH results reveal that all HPV 16-positive tumors show integrated virus, predominantly seen as 1 punctate signal per tumor cell nucleus. In 7 HPV-positive cases tumor cells harbored 2 punctate nuclear signals or more signals of different size and intensity. The former FISH signal pattern is indicative for two integration sites in the cellular genome or for a pair of chromosomes with

a single integration site, whereas the latter pattern most probably point to signals composed of integrated HPV DNA together with transcribed viral RNA. It is important to emphasize that RNA can contribute to the observed FISH signal[46], but that the protocol used in this study is optimal for unmasking integrated HPV DNA. Preliminary data on 2 mucosal dysplasia and 4 carcinoma in situ lesions identified adjacent to the 33 TSCC specimens also showed punctate FISH signals indicative for integrated HPV 16. Besides integration, 33% of the TSCC also contained areas with tumor cells showing diffuse nuclear staining indicative for the presence of episomal HPV. Studies on the physical status of HPV using PCR assays, such as the amplification of papillomavirus oncogene transcript (APOT) and the E2/E6 real-time PCR assay, have also shown HPV to be present predominantly in the integrated form[16,25]. These and our findings support the hypothesis that transition of oncogenic HPV DNA from the episomal to the integrated form, as has been shown to be an important factor in uterine cervical tumorigenesis[19,21,47], might also be crucial for progression towards malignancy in TSCC. The finding of mainly episomal HPV 16 in TSCC by Mellin et al.[24] using restriction enzyme cleavage, ligation and PCR (rliPCR) is in contrast with this perspective. However, the difficulty of the rliPCR approach to produce the required extreme long PCR products that indicate viral integration in primary TSCC, as well as the presence of episomal virus in tumor parts or precursor lesions adjacent to or included in the cancer, may explain the data of these authors.

$P16^{INK4A}$ overexpression in TSCC is associated with HPV integration

Approximately 80% of HNSCC show inactivation of $p16^{INK4A}$ as a result of (epi)genetic alterations in this tumor suppressor gene[2,3,48], but in our study about half of the tonsillar carcinomas showed an overexpression of $p16^{INK4A}$. Of the HPV 16-positive carcinomas all but one showed this overexpression, whereas only a few of the HPV 16-negative carcinomas showed accumulation of $p16^{INK4A}$. This is in agreement with earlier reports on oropharyngeal cancers[26,27,45,49]. The strong correlation between HPV 16 positivity and $p16^{INK4A}$ overexpression further supports the statement that the accurate and relatively simple immunohistochemical detection of $p16^{INK4A}$ is a good marker to identify HPV-positive lesions in tonsillar carcinogenesis and may be used in routine practice. The finding of $p16^{INK4A}$ overexpression in HPV-negative tumors may be the result of oncogene-driven cellular senescence[50] or infection with other viruses that downregulate pRb[51,52].

HPV integration correlates with poor tumor differentiation grade and metastatic progression at relatively small tumor size

In our study, the presence of tumor-associated HPV significantly correlated with a poor tumor differentiation grade, which is in agreement with other studies[2,8,53]. HPV-negative carcinomas more often showed to be well differentiated and keratinizing, which was very significantly associated with tobacco use ($p = 0.007$). Whether or not this keratinization process induced by smoking might have a protective effect on HPV infection in, e.g., the oropharynx is unclear.

Patients with HPV 16-positive tumors presented significantly more often with the initial complaint of a swelling in the neck. This is rather surprising since both HPV-positive and –negative tumors showed comparable percentages of detected lymph node metastases. An explanation for this different presentation might be the significantly smaller primary tumor size ($p = 0.024$) at the time of diagnosis, leading to less local problems in the HPV-positive patients. Another explanation may be better health awareness in patients with HPV 16-positive tumors as reflected in significantly less alcohol and tobacco abuse. The significantly smaller HPV-positive tumors were accompanied by slightly more regional metastases compared to the HPV-negative tumors, which might suggest that HPV-positive tumors have a tendency to metastasize early.

Non-smokers exhibit a markedly improved survival rate in HPV-associated TSCC

We found that patients with HPV 16-positive tumors had a significantly better disease-specific and overall survival compared to patients with HPV 16-negative tumors, which is in accordance with most recent studies focusing on oropharyngeal carcinomas and oncogenic HPV types[8,24,29,30,31,54,55]. It has been suggested that this better survival outcome might result from a better response to radiation therapy[56], as a result of induction of apoptosis by intact p53, as well as from a much lower chance of developing a second primary tumor[8], because patients with HPV-positive tumors show often low or no tobacco and alcohol intake and HPV infection tends to be focal. Our data are in agreement with these suggestions, because we noticed a significantly lower tobacco and alcohol intake as well as a lower percentage of (loco) regional recurrences in HPV-positive tumor patients, and found only second primary tumor development in the HPV-negative patient group.

Our analyses also showed a significant correlation between $p16^{INK4A}$ overexpression, less or absent smoking, with or without alcohol intake, tumor size < 4 cm, and less recurrent disease on the one hand, and prolonged survival on the other. Because these factors are also significantly correlated to HPV 16 positivity, they could artificially influence the survival data of the HPV 16-positive patients. Multivariate analyses adjusting for HPV, smoking, alcohol consumption and tumor size showed that smoking and tumor size were the most important factors determining survival outcome in the present study population. After these adjustments, HPV 16 positivity showed a strong tendency towards a better prognosis. Because of the relatively small study size and small number of patients in the subcategories of some of the covariates included in the multivariate analysis, additional studies are needed to confirm our results. Unlike earlier reports[29,31,57], we show that gender, age, alcohol consumption and lymph node status do not influence survival outcome. The latter parameter has also been found an unreliable prognostic indicator by others[58,59], which most probably is due to a higher percentage of HPV-positive, non-smoking patients with a better survival in the lymph node-positive group.

From our study we conclude that 1) HPV 16 integration is present in approximately 40% of TSCC, 2) $p16^{INK4A}$ immunostaining is significantly associated with HPV-positive TSCC and may function as a surrogate marker for HPV detection, 3) the presence of HPV in TSCC correlates significantly with low amounts of tobacco and alcohol intake, poor differentiation grade, small tumor size, presence of a swelling in the neck due to local metastases and a decreased recurrence rate, and 4) particularly non-smoking patients with HPV-containing TSCC show a remarkably better disease-specific survival rate.These data are of diagnostic and therapeutic importance, since interventions are dependent on clinical outcome parameters, and an improved understanding of the role of HPV in the carcinogenesis of TSCC may offer strategies for disease prevention.

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Chapter 4

$p16^{INK4A}$ overexpression is frequently detected in tumor-free tonsil tissue without association with HPV

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Abstract

Oncogenic human papillomavirus (HPV) type 16 has been strongly associated with tonsillar squamous cell carcinomas (TSCC) and appears to be of prognostic significance. Because HPV-positive TSCC also accumulate $p16^{INK4A}$, this cyclin-dependent kinase inhibitor has been proposed as a potential biomarker for HPV in clinical diagnosis. Data on HPV prevalence and the value of $p16^{INK4A}$ overexpression to predict its presence in tumor-free tonsil tissue, however, are scarce. We used immunohistochemistry to detect $p16^{INK4A}$ overexpression in sections taken from formalin-fixed, paraffin-embedded tumor-free tonsil tissue of 262 patients. They were treated for non-oncologic reasons (snoring or chronic/recurrent tonsillitis). Genomic DNA isolated from these tissues was subjected to HPV-specific PCR analysis. $p16^{INK4A}$ immunostaining was detected in 28% of the samples in both crypt epithelium (49/177) and germinal centers of the lymphoid tissue (52/187), which was significantly associated with each other ($p < 0.0001$). No staining was observed in superficial squamous cell epithelium. HPV-specific PCR analysis detected HPV 16 and 18 in 2/195 cases (1%), which however were negative in FISH analysis and discrepant in $p16^{INK4A}$ immunostaining. In conclusion, no proof was found for HPV presence in tumor-free tonsil tissue, despite increased $p16^{INK4A}$ expression in a quarter of tonsil tissues. Other mechanisms than HPV infection are therefore implicated in upregulation of $p16^{INK4A}$.

Introduction

On a worldwide scale head and neck squamous cell carcinoma (HNSCC) contributes 6.5% of annual cancer cases[1]. Up to 90% of HNSCC occur in patients with a high intake of tobacco and alcohol[2,3]. Besides tobacco smoking and alcohol consumption, many studies have confirmed that oncogenic human papillomavirus (HPV) is another risk factor for HNSCC, especially oropharyngeal carcinomas, including those arising from Waldeyers ring[4-10]. HPV is generally known for its major role in the development of uterine cervical and anogenital cancer[11,12,13]. However, patients with HPV-associated anogenital cancers also show an increased risk for developing tonsillar squamous-cell carcinoma (TSCC)[14,15]. Recently, an epidemiological study showed that the proportion of potentially HPV-related HNSCC have increased over the last decades, probably because of changing sexual behaviors[16].

HPV-positive HNSCC are more and more considered to be a different tumor entity, because of pronounced clinical and molecular differences with their HPV-negative counterparts. For example, HPV-positive tumors often present as poorly differentiated, aggressively growing tumors already metastasized to the lymph nodes, despite a relatively favorable prognosis[17,18]. At the molecular level these tumors show, amongst others, functional inactivation of the p53 and pRb tumor suppressor proteins by the HPV-derived oncoproteins E6 and E7, resulting in down-regulation of p53, pRb, and cyclin D1 and a strong up-regulation of cyclin-dependent kinase inhibitors $p16^{INK4A}$ and $p21^{CIF1/WAF1}$ as well as the $p14^{ARF}$ protein[9,12,19-22]. HPV-negative tumors, in contrast, often show inactivation of $p16^{INK4A}$, p53 overexpression as a result of gene mutations, cyclin D1 gene amplification and overexpression, as well as EGFR accumulation[23-26]. Because of its very strong association with HPV-positive HNSCC[9,27,28] $p16^{INK4A}$ overexpression has recently been proposed as a surrogate marker for HPV. Moreover, $p16^{INK4A}$ immunostaining and subsequent HPV-specific PCR and/or FISH have been recommended to identify HNSCC with biologically associated HPV[28,29].

The reason for the strong association of HPV with particularly TSCC and thus the susceptibility of tonsils for HPV infection remains unclear, although the easy viral accessibility of the invaginated, monolayered crypt epithelium and the presence of cytokines produced by the lymphoid tissue to stimulate viral transcription and cellular transformation have been suggested as explanations[8,14,30,31]. A recent study by Kim et al.[32] further suggested HPV-positive TSCC to originate predominantly

from the crypts by microscopic detection of specific growth patterns. Data on 1) the prevalence of oncogenic HPV in normal mucosa of tonsils of healthy persons as well as tonsils with carcinomas, 2) the earliest stages of infection and 3) its role in promoting tonsillar carcinogenesis, however, are still scarce.

So far, several studies have reported data on the detection of HPV DNA in normal, tumor-free head and neck mucosa samples (see Table 1). Most often these studies used non-invasive cell sampling methods, such as oral rinsing and cytological scraping or brushing, in order to test for the presence of HPV DNA by means of PCR. A disadvantage of these sampling methods is that they mainly collect superficial epithelial cells and that mostly no additional tests were performed for validation. In addition, a large multicenter study provided evidence that HPV DNA in oral exfoliated cells did not associate with risk for HNSCC or with HPV DNA detection in oral biopsy specimens[33]. Therefore, a more appropriate approach to study HPV presence in tonsil tissue is the use of tissue sections obtained either by biopsy or tonsillectomy in combination with PCR, FISH or immunostaining for $p16^{INK4A}$. Overexpression of this protein has been proposed as a specific biomarker for the presence of HPV in both the uterine cervix as well as the oropharynx[9,10,34,35].

The aim of this study was to assess the prevalence and site of $p16^{INK4A}$ overexpression, as a biomarker for high risk HPV, in tumor-free tonsil tissue of a population presenting in the clinic for non-oncologic reasons. In addition, $p16^{INK4A}$ overexpression was correlated with HPV detection by means of HPV-specific PCR and with clinical data, such as age, gender, alcohol consumption and/or tobacco smoking.

Materials and methods

Study population and tissue selection

Formaldehyde-fixed and paraffin-embedded tonsil biopsies and resection material from 262 patients were selected from the archives of the Department of Pathology, Utrecht Medical Centre (UMC), The Netherlands. These individuals presented subsequently during 1997 and 1998 with chronic tonsillitis or snoring problems and underwent a tonsillectomy. Patients gave their informed consent to use tissue for research purposes and were asked to fill in a small questionnaire, concerning demographic data like age at presentation, gender, and alcohol consumption and

tobacco smoking. The entire study was approved by the medical ethical committee of the UMC Utrecht. Full answered questionnaires were available from 143 (55%) patients. On basis of these data, the male to female ratio was 2:1 (97 male and 46 female patients), the mean age was 29.2 years (range 12-70; n = 256)), 135 patients were classified as daily tobacco smokers (≥ 1 cigarette, pipe, and/or cigar per day) and 122 as non-smokers (never smoker or former smoker, which are those who had stopped smoking more than 10 years before presentation), and 29 patients were classified as drinkers (consumption of ≥ 1 whiskey equivalents per day (1 whiskey equivalent ~10 g alcohol)) and 227 as non-drinkers (no whiskey equivalents per day). A series of 4 µm-thick sections were cut from the specimens to reclassify the tissue on basis of a HE staining, to immunohistochemically visualize $p16^{INK4A}$ and to isolate genomic DNA for beta-globin- and HPV-specific PCR.

Immmunohistochemical staining of $p16^{INK4A}$

Four µm thick sections were deparaffinized in a series of xylol, 100%, 96% and 70% ethanol and incubated for 30 min at room temperature in 2% H_2O_2 in methanol to quench endogenous peroxidase activity. Then the $p16^{INK4A}$ research kit (Dako, Glostrup, Denmark), which contains the mouse monoclonal antibody E6H4 directed against $p16^{INK4A}$ and a peroxidase-conjugated secondary visualization reagent, was used according to the manufacturer's instructions[10]. After diaminobenzidine visualization of peroxidase activity, sections were subsequently counterstained with hematoxylin and mounted with entellan (Merck, Darmstadt, Germany).

Three independent observers (B.K., P.S., E.S.) performed semi-quantitative evaluation of the slides and consensus was acquired. Moderate to strong nuclear and cytoplasmic staining in a small area of the tonsil tissue was considered as a region with $p16^{INK4A}$ overexpression, whereas tissue with only faintly diffuse or no staining was considered to be $p16^{INK4A}$ negative. Controls consisted of tonsillar carcinoma specimens with or without associated $p16^{INK4A}$ overexpression. Two sets of controls were used, i.e. substitution of the primary antibody by a monoclonal mouse IgG2a antibody to Aspergillus Niger, or omission of the primary antibody.

DNA isolation, Polymerase chain reaction, and HPV typing

From 253 of the 262 patient samples enough tissue material was available for genomic DNA extraction and PCR analysis. Tissues were processed with the QIAamp Tissue Kit (Qiagen, Hilden, Germany). Total cellular DNA was eluted with 250 µl of AE-buffer (Qiagen) and 5 µl were used in each polymerase chain reaction (PCR) analysis.

ß-Globin gene PCR was performed with all DNA samples to demonstrate that they contained sufficient DNA of adequate quality and were free of substances inhibitory to PCR (268 bp PCO4/GH20 PCR product)[36]. Negative controls, consisting of water or human placental DNA instead of patient samples, were included in each PCR run. As a result, 195 patient samples were subjected to HPV-specific PCR.

HPV sequences were detected by highly sensitive group-specific nested PCR protocols with degenerate primers A5/A10 and A6/A8 for HPV as described in[37]. PCR products (5µl) were separated on 2% agarose gels and visualized by ethidium bromide staining. For HPV typing internal biotinylated A6/A8-PCR products (270 bp) were hybridized with 37 type-specific digoxigenin-labeled oligonucleotide probes in an enzyme-immunoassay as previously described[38,39].

Twenty-two moderate to strongly $p16^{INK4A}$-positive and 6 negative tonsil samples (n = 28) were subsequently screened by PCR for the presence of adenovirus and cytomegalovirus DNA. Screening for adenoviral DNA was performed with the LightCycler system (Roche, Mannheim, Germany) as previously described[40] and screening for cytomegaloviral DNA was performed with the RealArt CMV PCR Kit (Artus, Hamburg, Germany) according to the manufacturer's recommendation.

Detection of HPV 16 and 18 DNA by FISH

FISH was performed on 4-µm thick tissue sections as described previously[10,41,42]. Briefly, sections were deparaffinized, pretreated with 85% formic acid/0.3% H_2O_2, 1 M NaSCN and 4 mg/ml pepsin, post-fixed in 1% formaldehyde in PBS, dehydrated in an ethanol series and hybridized with digoxigenin-labeled HPV 16- and 18-specific probes (PanPath, Amsterdam, The Netherlands) according to the manufacturer's instructions. After hybridization the preparations were washed stringently in 50% formamide, 2×SSC at 42°C (2 times 5 min). The probes were detected by application of mouse anti-digoxin (Sigma, St. Louis, MO), peroxidase-conjugated rabbit anti-mouse and peroxidase-conjugated swine anti-rabbit (both Dako), and visualized by a peroxidase reaction using rhodamin-labeled tyramide[43,44]. Preparations were mounted in Vectashield (Vector Laboratories, Burlingame, CA) containing 4,6-diamidino-2-phenyl indole (DAPI; Sigma: 0.2 g/ml). Microscope images were recorded with the Metasystems Image Pro System (black and white CCD camera; Sandhausen, Germany) mounted on top of a Leica DM-RE fluorescence microscope equipped with DAPI and rhodamin filters.

Evaluation of nuclear hybridization signals was performed by 3 investigators (E.S., A.H. and B.K.) according to the criteria described by Cooper et al.[45], i.e., punctate and/or diffuse signals throughout the nucleus indicating integrated and episomal HPV DNA, respectively. Controls included hybridizations on 70% ethanol suspensions and formaldehyde-fixed, paraffin-embedded sections of known HPV 16- and 18-positive human cervical carcinoma cell lines (CaSki [ATCC; CRL1550; 500 integrated HPV 16 copies], HeLa [ATCC; CCL2; 20-50 integrated HPV 18 copies] and SiHa [ATCC; HTB35; 1-2 integrated HPV 16 copies]) to guarantee probe specificity, sensitivity and interpretation accuracy[42,46], as well as hybridizations on tissue sections of cervical lesions with proven integration or episomal presence (replication) of HPV genomic DNA. Negative controls consisted of HPV PCR- and FISH-negative cell lines and tissue sections and hybridizations omitting the viral probe. In addition, the number of HPV integration spots per nucleus was scored in the tissue.

Statistical analysis

Clinical patient data were correlated with $p16^{INK4A}$ overexpression in cross-tabulations using the Fisher exact test (two-tailed) and/or chi-square test. A significance level of $P \leq 0.05$ was chosen.

Results

$p16^{INK4A}$ immunostaining

All tonsil samples of 262 patients contained superficial squamous cell epithelium, whereas 187/262 cases harbored lymphoid follicles, 177 reticulated crypt epithelium and 176 both lymphoid follicles and crypt epithelium (Figure 1A). Moderate to strong diffuse cytoplasmic and nuclear $p16^{INK4A}$ immunostaining was identified in crypt epithelium as focal staining generally involving nonkeratinizing cell groups and associated lymphocytes (49/177; 27.7%) (Figures 1 B and D) and in scattered cells within germinal centers of lymphoid follicles (52/187; 27.8%) (Figures 1C and E). In the germinal centers, particularly dendritic cells were positive for $p16^{INK4A}$ (Figure 1E). Interestingly, the outcome of the $p16^{INK4A}$ immunostaining in the crypt epithelium was strongly associated with that in the germinal centers (identical outcome in 139/176 cases; $p<0.0001$). In 31/139 cases both tissue types simultaneously showed $p16^{INK4A}$ accumulation. None of the 262 samples showed any positive staining in

the superficial squamous cell epithelium. No correlation was found when comparing the $p16^{INK4A}$ status with age (comparing groups ≤ or > 26 years old, and ≤ or > 35 years old), gender, and tobacco smoking and alcohol consumption using cross tabulations.

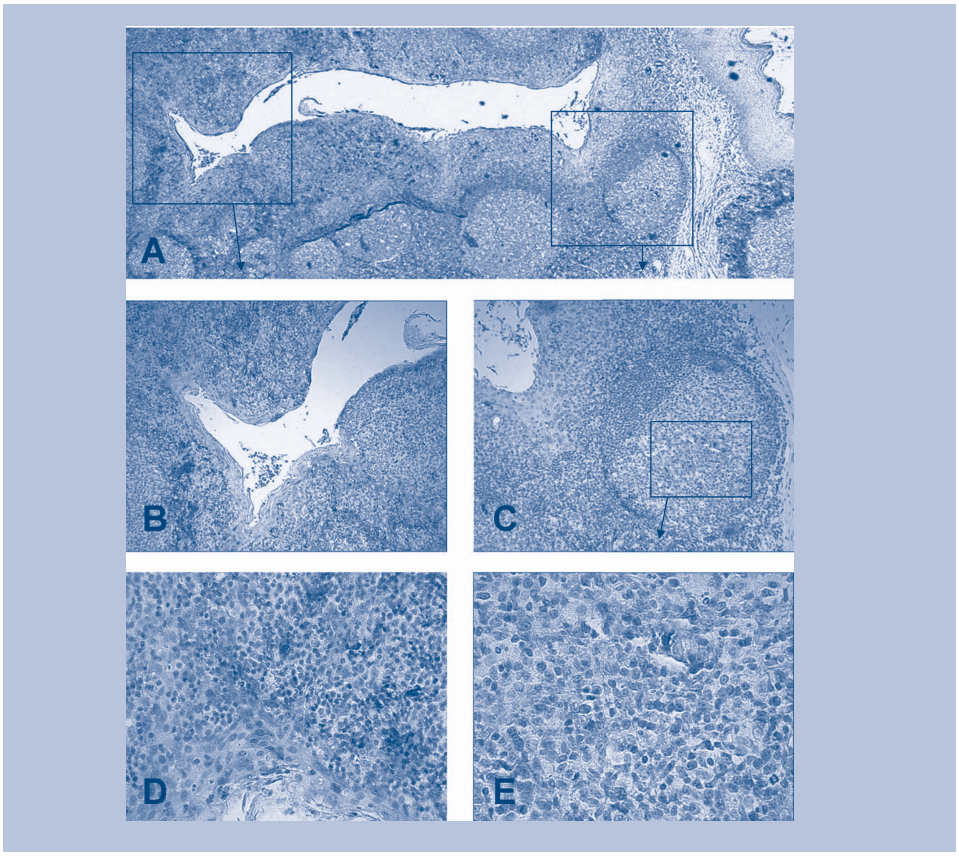

FIGURE 1. REPRESENTATIVE EXAMPLE OF $P16^{INK4A}$ IMMUNOHISTOCHEMISTRY IN A TUMOR-FREE NORMAL TONSIL FROM A PATIENT WITH TONSILLITIS. (**A**) Tissue overview showing strong immunostaining in both the reticulated crypt epithelium as well as in the follicular dendritic cells in one germinal center, whereas the superficial squamous cell epithelium is negative. (**B**) and (**D**) Enlargements of Figure 1A, showing focal and strong diffuse cytoplasmic and nuclear $p16^{INK4A}$ immunostaining in the crypt epithelium involving nonkeratinizing cell groups and associated lymphocytes. (**C**) and (**E**) Enlargements of Figure 1A, showing $p16^{INK4A}$ accumulation in scattered cells within a germinal center of a lymphoid follicle. Nuclei were counterstained (dark) with hematoxylin. (see page 157 for color figure)

HPV detection

Of the 253 DNA samples isolated from tonsil tissue, 195 proved to be positive for ß-globin in the PCR analysis. In 2 of these cases HPV DNAs were identified, i.e. one case with HPV 16 and 18, and one case with HPV 16, 18 and 68 (data not shown). Only the former case was associated with strong $p16^{INK4A}$ overexpression in the crypt epithelium as well as germinal centers. FISH analysis was unable to confirm the presence of oncogenic HPV type 16 and 18 in the 2 PCR-positive tonsil samples. No HPV DNA could be detected in the cell-nuclei of the different tissue areas, including $p16^{INK4A}$-positive areas.

In order to examine if other viral infections underlie upregulation of $p16^{INK4A}$, 28 tonsil samples (22 moderate to strongly $p16^{INK4A}$-positive and 6 negative samples) were analyzed for the presence of adenovirus and cytomegalovirus by PCR. No viral DNA was detected in these samples (data not shown).

Discussion

The palatine tonsils form the boundary between the oropharynx and the oral cavity, and function as the body's first immune defence system against bacteria, viruses and fungi entering through the mouth. These micro-organisms have been implicated in, for example, the induction of tonsillitis, a form of inflammation with reddening and reactive enlargement of lymphoid structures[47]. Besides this benign pathology, a large number of detailed studies have shown that the tonsils are also the preferential site for HPV-induced malignancy[6,7,8,9,10]. Up to 50% of TSCC have been associated with HPV, particularly type 16. Interestingly, these tumors demonstrate overexpression of $p16^{INK4A}$ amongst a variety of molecular and clinical characteristics different from their HPV-negative counterpart. Because this cyclin-dependent kinase inhibitor is mostly inactivated in the latter group, several studies have proposed $p16^{INK4A}$ accumulation as a surrogate biomarker for HPV. Data on the presence of HPV in tumor-free tonsil tissue and on early molecular events during HPV-related carcinogenesis are however scarce. Therefore, we analyzed in this study a large series of tumor-free tonsil tissue samples for $p16^{INK4A}$ overexpression and the presence of HPV-specific DNA. Despite positive $p16^{INK4A}$ immunostaining in 28% of samples, oncogenic HPV was only detected in 2 cases by PCR. Both cases were, in addition, negative in HPV 16/18-specific FISH analysis and only one of them

exhibited $p16^{INK4A}$ overexpression. These data indicate that $p16^{INK4A}$ overexpression occurs frequently in tumor-free tonsil tissue without association with HPV, and thus should be used with caution as surrogate marker.

The very low percentage (1%) of oncogenic HPV detected by PCR in this study is in agreement with other reports, describing frequencies of particularly HPV-type 16 and 18 ranging from 0-14% in tumor-free tonsil samples as well as normal tissue adjacent to TSCC (see Table 1). In the recent literature, however, additional tests have been proposed to firmly assess the biological association of the virus to the tissue, e.g. by detecting the presence and physical status of HPV in situ (in situ hybridization), overexpression of $p16^{INK4A}$ (immunohistochemistry), and/or upregulation of E6 or E7 mRNA transcripts (RT-PCR), for example after microdissection[9, 10, 18, 29, 31]. By applying both $p16^{INK4A}$ immunostaining on all and FISH on the two PCR-positive samples, we could not convincingly affirm HPV association in our series of tonsil tissues nor provide evidence for the presence of early stages of HPV-related carcinogenesis. Future studies should take into consideration the use of an appropriate combination of tests per sample to identify a clinically relevant HPV infection, and should focus on the analysis of primary tonsillar dysplasia or TSCC resection margins as more potential sources for this purpose.

In spite of the absence of HPV in tumor-free tonsil tissue, we detected $p16^{INK4A}$ overexpression in more than a quarter of the tonsil samples, i.e. in the reticulated crypt epithelium and in follicular dendritic cells in one or more adjacent germinal centers of the surrounding lymphoid tissue. A marked finding was the highly significant association of immunostaining in both sites. In contrast, no $p16^{INK4A}$ expression was detected in the superficial squamous cell epithelium. No correlation was found between $p16^{INK4A}$-positivity and clinical patient data, such as age, gender, alcohol consumption and/or tobacco smoking. $P16^{INK4A}$ accumulation in tumor-adjacent crypt epithelium has been described previously by Klussmann et al.[9], suggesting the presence of early stages of HPV infection. Our data, and recent data of Begum et al.[27] on tumor-adjacent and contralateral tumor-free tonsil tissue, demonstrate that a meaningful HPV infection in most cases is absent in $p16^{INK4A}$-positive samples. A number of explanations might be considered for this decoupling of $p16^{INK4A}$ accumulation and HPV positivity. First, there is the possibility that infection with other viruses contributes to the observed $p16^{INK4A}$ overexpression by functionally inactivating pRb in a similar way as the HPV oncoprotein E7, pushing infected cells into proliferation. Examples are the major immediate early (IE) proteins of the

herpesvirus CMV and the IE equivalents of small DNA viruses, such as adenovirus[48,49]. PCR analyses of our $p16^{INK4A}$-positive tumor-free tonsil samples, however, revealed no presence of these viruses. This, however, does not exclude infection by other virus types.

Second, $p16^{INK4A}$ accumulation might be the result of cellular senescence and/or aging. On the one hand, a so-called premature senescence could be abruptly induced by functional overactivation of oncogenes including Ras, Raf, MEK and E2F, such as for example have been observed in benign melanocytic Spitz nevi[50]. To the best of our knowledge, evidence for this mechanism to occur in tonsillar lesions has not been described so far. On the other hand, stimuli such as telomere shortening, DNA damage, oxidative stress, and/or sustained mitogen stimulation, may result in so-called replicative senescence[51]. Both p53 and $p16^{INK4A}$ are important mediators in this process, in which p53 responds strongest to these stresses. $p16^{INK4A}$, however, also appears to respond strongly to additional, yet not fully characterized, physiological stresses, leading to so-called physiological aging of tissue cells[52,53]. For example, metabolic stress due to an ad libitum-diet versus a caloric restricted diet was shown to induce tissue aging in combination with $p16^{INK4A}$ upregulation in different rat tissues, including lymph nodes and the spleen. Of particular interest, in addition, were immunostaining results in spleen tissue of aged mice, showing an increase in $p16^{INK4A}$ expression in scattered cells of the follicular germinal centers[53], similar to our results in the tonsillar lymphoid tissue. It is tempting to speculate that $p16^{INK4A}$ accumulation, if observed in tumor-free tonsils, is also related to accelerated, physiological aging, e.g. induced by chronic infection of areas of crypt epithelium by bacteria, viruses or fungi as well as prolonged activation of amongst others interdigitating lymphocytes and antigen-presenting dendritic cells. The fact that our study population consists of patients with chronic tonsillitis and/or snoring problems, and $p16^{INK4A}$ expression did not correlate to patient age, underscores this suggestion.

TABLE 1. FREQUENCIES OF HPV TYPES PRESENT IN NORMAL HEAD AND NECK MUCOSA SSMPLES AS DETERMINED BY PCT

Reference	Sample collection at site of origin	Sample Size	HPV positivity		Type LR[1]	Frequency LR (%)	Type HR[1]	Frequency HR (%)
			n	%				
	Oral cavity							
Bouda M et al. 2000[54]	Tongue and cheek scrapes[2]	16	0	0	6,11	0	16,18,31,33	0
Eike A et al. 1995[55]	Buccal mucosa and tongue border scrapes	61	0	0	6,11	0	16,18	0
Sand L et al. 2000[56]	Oral mucosa biopsies	12	0	0	6,11	0	16,18	0
Ostwald C et al. 1994[57]	Buccal mucosa scrapes	97	1	1	6,11	0	16,18	0
D'Souza G et al. 2007[58]	Oral rinses, oral scrapes	200	n.a.	n.a.	6,42,61,62,66,CP6108	7/200 (4)	51,56,58,68,73, 16	6/200 (3) 8/200 (4)[3]
Smith EM et al. 1998[59]	Oral rinses	205	10	5	12,23,38,72	4/205 (2)	**16**,58	3/205 (2)
Summersgill KF et al. 2001[60]	Oral scrapes, oral rinses	268	16	6	**6**,17, U85662[4]	4/268 (1)	**16,** 18	9/268 (3)
Herrero R et al. 2003[33]	Oral scrapes, oral rinses	613	42	7	6,11,40,42, 43,44	n.a.	16,18,31,33,35,39,45,51, 52,56, 58,59,66,68	n.a.
Lambropoulos AF et al. 1997[61]	Buccal mucosa scrapes	169	16	10	**6**,11	7/169 (4)	**16**,18,33	4/169 (2)
Jimenez C et al. 2001[62]	Oral mucosa biopsies	20	2	10	**6**,13,32	1/20 (5)	**16**	1/20 (5)
Holladay EB et al. 1993[63]	Normal oral biopsies	6	1	16	6,11,40,42,53,54,55,57	0	**16**,18,33,35,39,45, 51,52,56,58,59	1/6 (16)
Sugiyama M et al. 2003[64]	Oral mucosa biopsies	44	16	36	n.a.	n.a.	**16**,18	16/44 (36)
Kojima A et al. 2003[5,6,(5)]	Buccal mucosa scrapes	77	37	48	**1,2**-6,8,10,11,13,20-24-,32,34,36-38, 42,48,50, 57,60,72,75,**76**,77,80	40/52 (77)[6]	**16**,18,31,33,52,58, 59,73	12/52 (23)[6]
Adams V et al. 1999[66]	Biopsies normal tissue adjacent to tumor	6	3	50	6,11,53	0	**16**,18,31,33,35,45,51,56	3/6 (50)

Jalal H et al. 1992[67]	Buccal mucosa, hard palate and dorsum of the tongue scrapes	48	25	52	n.a.	n.a.	16	25/48 (52)
Zhang ZY et al. 2004[68]	Oral mucosa biopsies	40	22	55	n.a.	n.a.	**16**,18	22/40 (55)
Lawton G et al. 1992[69]	"Scrapes Oral rinses Buccal mucosa biopsies"	53 59 49	24 7 25	45 12 51	6,11	11/53(21) 1/59(2) 10/49(20)	16,18,31,33	n.a.
Woods KV et al. 1993[70]	Biopsies normal tissue adjacent to tumor or neck resection	9	6	67	6,11	1/9 (11)	**16**,18	6/9 (67)
Terai M et al. 1999[71]	Oral scrapes	37	30	81	6,**61**	18/37 (49)	16,**18**,59	27/37 (73)
	Tonsil							
Klussmann JP et al. 2001[8]	Tonsillectomies	14	0	0	n.a.	0	n.a.	0
Strome SE et al. 2002[72]	Tonsil biopsies	48	0	0	n.a.	0	n.a.	0
Snijders PJ et al. 1992[73]	Tonsil biopsies	7	0	0	6,11	0	16,18,31,33	0
Sisk J et al. 2006[(7),74]	Tonsillectomies	50	1	2	11	1	n.a.	0
Chen R et al. 2005[75]	"Tonsil scrapes Tonsillectomies"	174 206	1 13	1 6	n.a. n.a.	n.a. n.a.	58 16	1/174 (1) 13/206 (6)
Kim S-H et al. 2007[32]	tonsillectomies	69	8	12	6,11,84	n.a.	**16**	3/69 (4)
Fukushima K et al. 1994[76]	Biopsies normal tonsil, tissue adjacent to tumor	38	5	13	n.a.	n.a.	16,18	5/38 (13)
Watanabe S et al. 1993[77]	Tonsil biopsies	28	4	14	n.a.	n.a.	**16**,18	4/28 (14)

*LR, low risk HPV type; HR, high risk HPV type: n.a., data not available. **1**: Column shows analyzed HPV-types mentioned in study. HPV type in bold represents most frequently found virus DNA. **2**: Scrapes; term used in a general sense, including scrapes, (cyto)brushes, swabs and smears. All techniques retrieve particularly superficial epithelium. **3**: HPV-16 specific real-time PCR. No HPV-16 was detected with consensus primer for 37 types, **4**: Genbank accesion number, **5**: Subject age 3/5 years, **6**: Data relate to # samples instead of subjects, **7**: Subject age 3-12 years*

In conclusion, $p16^{INK4A}$ expression is regularly observed in tumor-free tonsil tissue but is not associated with HPV, and should therefore be used with caution as a biomarker in normal tissue. Other mechanisms than HPV infection are therefore implicated in upregulation of $p16^{INK4A}$.

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Chapter 5

$P21^{Cip1/WAF1}$ expression is strongly associated with HPV-positive tonsillar carcinoma and a favorable prognosis

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Abstract

Human papillomavirus (HPV) is involved in the carcinogenesis of tonsillar squamous cell carcinomas (TSCC). In this study we investigated the expression and prognostic value of key cell cycle proteins in the retinoblastoma (pRb) and p53 pathways in both HPV16-positive and -negative TSCC. Using immunohistochemistry 77 TSCC with known HPV16 status and clinical outcome were analyzed for expression of Ki67, $p16^{INK4A}$, cyclin D1, pRb, $p14^{ARF}$, MDM2, p53, $p21^{Cip1/WAF1}$, and $p27^{KIP1}$. Results were correlated with each other and with clinical and demographic patient data. Survival from TSCC was determined by Kaplan Meier and Cox proportional hazard regression analysis. Nearly all (97%) TSCC showed a high proliferation index as concluded from a nuclear Ki67 expression in 30-90% of the tumor cells. Approximately one-third of these (35%) TSCC harbored HPV16 DNA integrated in the host genome, and showed $p16^{INK4A}$ overexpression, both being considered essential features for HPV association. These TSCC also showed overexpression of $p14^{ARF}$ ($p < 0.0001$) and $p21^{Cip1/WAF1}$ ($p = 0.001$), and downregulation of pRb ($p< 0.0001$) and cyclin D1 ($p = 0.027$) as compared to the HPV-negative cases. Univariate Cox regression analyses revealed a high survival rate for non-smokers ($p = 0.006$), as well as for patients with TSCC classified as T1-2 ($p<0.0001$). Also low expression of cyclin D1 ($p = 0.028$), presence of HPV and overexpression of $p16^{INK4A}$ ($p = 0.01$), $p14^{ARF}$ ($p = 0.02$) or $p21^{Cip1/WAF1}$ ($p = 0.004$) were correlated with survival in a favorable manner. In multivariate Cox regression analyses smoking and tumor size, as well as expression of cyclin D1 and $p21^{Cip1/WAF1}$ were found to be independent prognostic markers. We conclude that HPV-positivity in TSCC strongly correlates with $p21^{Cip1/WAF1}$ and $p14^{ARF}$ overexpression and downregulation of pRb and cyclin D1. In particular $p21^{Cip1/WAF1}$ overexpression is an excellent favorable prognosticator in TSCC.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most prevalent malignancy in the world, contributing 6% of new cancer cases annually worldwide[1,2]. HNSCC has a 5-year survival rate of approximately 50%, which has not improved in the last 2 decades [3]. Well recognized risk factors in the etiology of HNSCC are extensive tobacco smoking and alcohol consumption in ~90% of cases, as well as oncogenic human papillomaviruses (HPVs), predominantly HPV type 16[3,4]. Interestingly, the association of HPV is strongest for tonsillar carcinoma (TSCC) with a prevalence up to 50%[5-8]. The diagnosis of HPV-positivity in HNSCC appears to have significant prognostic implications. In one study these patients had a less than 50% chance of dying from the disease as compared to HPV negative tumors[9].

It has been shown that there are several differences between HPV-positive and -negative HNSCC. Despite the fact that HPV-positivity in HNSCC is an indicator for favorable prognosis, from a clinical point of view these tumors are often poorly differentiated[4,6,10-12] and metastasized to lymph nodes at presentation[10,11]. Furthermore, HPV-positive tumors are often smaller at first diagnosis (diameter ≤ 4cm)[13], and associated with low/no exposure to alcohol and tobacco smoke[10,11]. At the molecular level the functional inactivation of two key tumor suppressor proteins, i.e. p53 and pRb by the HPV-derived oncoproteins E6 and E7, often result in down-regulation of p53, pRb, cyclin D1 and a strong up-regulation of $p16^{INK4A}$ in HPV-positive tumors[5,7,14-16]. HPV-negative tumors, in contrast, often show inactivation of $p16^{INK4A}$, p53 overexpression as a result of gene mutations, cyclin D1 gene amplification and overexpression, as well as EGFR accumulation[3,17-19].

The literature, however, shows conflicting data with respect to HPV-associated characteristics and clinical outcome of HNSCC. First, although many studies describe a significant association between HPV-presence and favorable prognosis, some studies did not find such a correlation[20-22]. Second, HPV has also been identified in HNSCC of smokers, significantly reducing its favorable effect on clinical outcome[13]. Furthermore, despite the fact that regional lymph node metastasis is considered the most important prognostic factor in HNSCC[23], this parameter seems to be unreliable in TSCC[24,25]. Finally, some studies reported overexpression and/or p53 mutations almost exclusively in HPV-negative tumors[20], while others have found that HPV-infection and p53 alterations can coexist[26]. This may have a strong effect on survival, because it has been indicated that tumors with intact p53 are still capable

of inducing apoptosis in response to radiation therapy, which results in a favorable clinical outcome[27].

This study was undertaken to investigate the expression of key cell cycle proteins in the pRb pathway ($p16^{INK4A}$, cyclin D1, $p27^{Kip1}$, pRb) and the p53 cascade ($P14^{ARF}$, MDM2, p53 and $p21^{Cip1/WAF1}$), using a series of 77 TSCC for which the HPV16 status and the clinical follow-up data were available. TSCC show the highest prevalence of oncogenic HPV and are thus ideally suited to search for molecular and clinicopathological differences induced by either HPV, or tobacco smoking and alcohol consumption.

Materials and Methods

Tumor material and patient data

Formaldehyde fixed, paraffin embedded archival biopsy and resection material of primary TSCC from 77 patients, which had been previously examined for HPV16 association[13], was selected from the archives of the Department of Pathology, University Hospital Maastricht, The Netherlands. Demographic data, including age at diagnosis, gender, alcohol and tobacco smoke exposure, treatment modality and date and cause of death were obtained from the medical records. Tumor site, degree of differentiation (i.e., well, moderately, or poorly differentiated) and TNM classification were determined from review of pathological, radiological and surgical reports. All patients were treated by surgery, radiotherapy, chemotherapy or a combination, irrespective of HPV status. The study protocol was approved by the institutional ethical committee, and all patients gave informed consent. Table 1 provides demographic and clinical features of the 77 patients included in this study. Fifty-seven patients were male and 20 patients were female. The mean age at diagnosis was 58.8 (range 39-87) years. Data concerning smoking and alcohol intake were obtained from 76 and 75 patients, respectively. Sixty-four (84%) of 76 patients were smokers (≥ 1 cigarette, pipe, and/or cigar per day) and 46 (61%) of 75 patients were classified as drinkers (consumption of > 2 whiskey equivalents per day (1 whiskey equivalent ~10 g alcohol)). Forty-two (55%) of the patients used both tobacco and alcohol, while only 8 (10%) patients had not been subjected to these intoxications. Thirty-nine (51%) patients had a tumor with a diameter ≥ 4cm, and 55 (71%) had lymph node metastasis at time of diagnosis. Tumor grade was poor or

moderate in 63 (82%) patients, well differentiated in 10 (13%) patients, unavailable in 3 patients (4%), and 1 patient (1%) had a carcinoma in situ. Following primary treatment, 29 (38%) of the patients never became disease-free, 16 (21%) developed a recurrent disease (locoregional, regional or distant) and 32 (42%) patients remained disease-free after primary treatment.

A series of 4 μm-thick sections was cut from the specimens for hematoxylin-eosin staining and detailed histopathological classification (F.J.B.), according to the criteria of the World Health Organization[28]. Furthermore, we applied immunohistochemistry to visualize Ki67, $p16^{INK4A}$, cyclin D1, pRb, $P14^{ARF}$, MDM2, p53, $p21^{Cip1/WAF1}$ and $p27^{Kip1}$ expression.

Immunohistochemical staining

Immunohistochemical protein staining on 4 μm-thick formaldehyde fixed, paraffin embedded tissue sections was performed as described earlier[15]. Briefly, sections were deparaffinized and subsequently pretreated with 2% H_2O_2 in methanol for 30 minutes to quench endogenous peroxidase activity. Antigen retrieval was performed by microwave heating in 0.01M citrate buffer (pH 6.0). The primary antibodies used to detect Ki67, $p16^{INK4A}$, cyclin D1, pRb, $p14^{ARF}$, MDM2, p53, $p21^{Cip1/WAF1}$ and $p27^{Kip1}$ are listed in Table 2. After incubation with a biotinylated secondary antibody, immunohistochemical detection was performed by an avidin-biotinylated peroxidase complex (ABC) procedure (Vectastain-Elite-ABC kit; Vector). Peroxidase activity was detected using 0.5mg/ml diaminobenzidine/ 2% H_2O_2. Sections were counterstained with hematoxylin and mounted in Entellan (Merck, Darmstadt, Germany). In each analysis negative and positive controls were included. Analysis was performed by three independent observers (J.J.M., E.J.M.S. and S.M.H.C.) and in case of interobserver variations, consensus was reached by combined examination of the slides. Both staining intensity (-, +/-, +, ++, +++) and the percentage of stained tumor cells were scored. Evaluation criteria for positive scoring of each of the cell cycle proteins are listed in Table 2.

Statistical analysis

The study population consisted of 77 patients with a TSCC diagnosed between 1992 and 2001. TSCC were considered to be HPV-associated if they showed HPV16 presence by in situ hybridization analysis, and in addition overexpression of $p16^{INK4A}$

as detected by immunohistochemistry. Factors associated with HPV status were selected on cross-tabulations, which were analyzed by the use of the two-tailed Fisher exact test and/or Chi-square test. The maximum significance levels are indicated for all analyses ($p \leq 0.05$). Disease-specific survival curves were calculated using the Kaplan-Meier method. Survival was calculated from the date of diagnosis until patient's death or until the last date the patient was known to be alive (this ranged from 16-141 months). Patients that died of other causes than tonsillar carcinoma were considered censored observations in the disease-specific survival analyses. Disease-free survival was calculated from the date of diagnosis until the date of recurrence (local, regional or distant, whichever occurred first). Patients without recurrence were censored at the date of the last follow-up or the date of death. The statistical significance of differences between survival times was determined by the log rank test in univariate analysis. Multivariate analyses were performed using the Cox proportional hazards model. Variables in the multivariate model included: HPV-association, T-classification, smoking, cyclin D1-, $p14^{ARF}$- and $p21^{Cip1/WAF1}$ expression. Variables remained in the model if their p-values were below 0.10. All calculations were performed by use of the SPSS Base System version 12.0.1.

Results

Cell cycle protein expression

Seventy-seven TSCC were examined for expression of the cell cycle proteins Ki67, $p16^{INK4A}$, Cyclin D1, pRb, $P14^{ARF}$, MDM2, p53, $p21^{Cip1/WAF1}$ and $p27^{Kip1}$ using the primary antibodies and evaluation criteria stated in Table 2. Representative immunostaining results in normal epithelium and tumor tissues are shown in Figure 1. The frequency of tumors exhibiting expression of the respective proteins is presented in Figure 2A. Seventy-five out of 77 (97%) TSCC showed strong, nuclear Ki67 expression in 30-90% of the tumor cells (Figure 1A). Also nuclear pRb staining was often observed in TSCC (62 out of 77 cases, 81%), although two subsets were identified with a difference in staining intensity (i.e., tumors showing < or ≥ intense immunostaining than present in the adjacent squamous epithelium. Figure 1D versus 1J, respectively). A significant association was seen between overexpression of pRb (a higher nuclear staining intensity in tumor cells than in the adjacent normal squamous epithelium) and p53 accumulation ($p = 0.017$). Both overexpression of p53 and $p21^{Cip1/WAF1}$

correlated independently with accumulation of MDM2 (Figure 1K) (p = 0.030 and p = 0.004, respectively), but not with each other (data not shown). In addition, there was a significant association between overexpression of $p16^{INK4A}$ and overexpression of both $p14^{ARF}$ ($p < 0.0001$) and $p21^{Cip1/WAF1}$ (p = 0.027) (Figure 1B, C and F). In case of $p14^{ARF}$ overexpression in the tumor, a diffuse nuclear staining pattern was more often observed than nucleolar staining (Figure 1C, above versus below, respectively). Furthermore, $p16^{INK4A}$ accumulation strongly correlated with both downregulation of pRb ($p < 0.0001$) and cyclin D1 (p =0.035) (Figure 1D and E).

Staining of normal tonsillar squamous epithelium was seen in the basal or parabasal cell layers for Ki67, cyclin D1, pRb, $p21^{Cip1/WAF1}$ and p53, varying from low to high intensities (Figure 1A, B, D-G). This squamous epithelium, present in the individual sections, was used as an internal positive control. In $p14^{ARF}$- and $p27^{Kip1}$ stained sections, adjacent lymphocyte infiltration often showed a strong cytoplasmatic and nuclear staining, respectively, and therefore served as the internal positive control (Figure 1L).

Correlations between HPV status, expression of cell cycle proteins and clinicopathological variables

The correlations between each cell cycle marker and HPV status are shown in Table 1 and Figure 2b. HPV-associated TSCC showed significantly more often overexpression of $p14^{ARF}$ ($p < 0.0001$) and $p21^{Cip1/WAF1}$ (p = 0.008), and downregulation of pRb ($p < 0.0001$) and cyclin D1 (p = 0.027) than HPV-negative tumors. P53 accumulation tended to be associated with absence of HPV (p = 0.079).

The male/female ratio and the age distribution were similar for the HPV-positive and HPV-negative subgroups. Smoking and alcohol abuse were seen significantly more often in the HPV-negative patient group ($p < 0.0001$ and p = 0.024, respectively). HPV-positive tumors tended to be smaller than HPV-negative tumors (p = 0.065). Male patients were more often diagnosed with a T3-4 tumor (p = 0.006) and were more often smokers (p = 0.011).

TABLE 1. CLINICOPATHOLOGICAL FEATURES AND CELL CYCLE PROTEIN EXPRESSION IN RELATION TO HPV STATUS

Characteristic	All TSCC n = 77 (%)	HPV-positive TSCC n = 27 (35%)	HPV-negative TSCC n = 50 (65%)	Fisher exact P-value
Cell cycle proteins				
Ki67				
Positive	75 (97%)	26 (96%)	49 (98%)	NS
Negative	2 (3%)	1 (4%)	1 (2%)	
$p16^{INK4A}$				
Positive	29 (38%)	27 (100%)	2 (4%)	<0.0001
Negative	48 (62%)	0 (0%)	48 (96%)	
cyclin D1 > 5%				
Positive	33 (43%)	7 (26%)	26 (52%)	0.027
Negative	44 (57%)	20 (74%)	24 (48%)	
cyclin D1 > 50%				
Positive	12 (16%)	2 (7%)	10 (20%)	NS
Negative	65 (84%)	25 (93%)	40 (80%)	
pRb > 20%				
Positive	62 (81%)	22 (81%)	40 80%)	NS
Negative	15 (20%)	5 (19%)	10 (20%)	
pRb intensity in tumor ≥ intensity in adjacent squamous epithelium				
Positive	41 (53%)	4 (15%)	37 (74%)	< 0.0001
Negative	36 (47%)	23 (85%)	13 (26%)	
$p14^{ARF}$				
Positive	34 (44%)	20 (26%)	14 (18%)	<0.0001
Negative	43 (56%)	7 (9%)	36 (47%)	
MDM2				
Positive	23 (30%)	10 (37%)	13 (26%)	NS
Negative	54 (70%)	17 (63%)	37 (74%)	
p53				
Positive	39 (51%)	10 (37%)	29 (58%)	NS
Negative	38 (49%)	17 (63%)	21 (42%)	
$p21^{Cip1/WAF1}$				
Positive	32 (42%)	17 (63%)	15 (30%)	0.008
Negative	43 (56%)	10 (37%)	33 (66%)	
Unknown	2 (3%)		2 (4%)	
$p27^{KIP1}$				
Positive	25 (33%)	9 (33%)	16 (32%)	NS
Negative	49 (64%)	18 (66%)	31 (62%)	
Unknown	3 (4%)		3 (6%)	
Clinicopathological variables				
Gender				
Female	20 (26%)	9 (33%)	11 (22%)	NS
Male	57 (74%)	18 (67%)	39 (78%)	
Age (years)				
<60	41 (53%)	13 (48%)	28 (56%)	NS
≥60	36 (47%)	14 (52%)	22 (44%)	

Characteristic	All TSCC n = 77 (%)	HPV-positive TSCC n = 27 (35%)	HPV-negative TSCC n = 50 (65%)	Fisher exact P-value
Death due to TSCC				
Yes	41 (53%)	8 (30%)	33 (66%)	0.002
No	33 (43%)	18 (67%)	15 (30%)	
Unknown	3 (4%)	1 (4%)	2 (4%)	
Death due to any cause				
Yes	48 (62%)	10 (37%)	38 (76%)	<0.0001
No	26 (34%)	16 (59%)	10 (20%)	
Unknown	3 (4%)	1 (4%)	2 (4%)	
Smoking[1]				
Yes	64 (83%)	17 (63%)	47 (94%)	<0.0001
No	12 (16%)	10 (37%)	2 (4%)	
Unknown	1 (1%)		1 (2%)	
Alcohol[2]				
Yes	46 (60%)	12 (44%)	34 (68%)	0.024
No	29 (38%)	15 (56%)	14 (28%)	
Unknown	2 (3%)		2 (4%)	
Smoking and/or alcohol				
Yes	68 (88%)	20 (74%)	48 (96%)	0.002
No	8 (10%)	7 (26%)	1 (2%)	
Unknown	1 (1%)		1 (2%)	
Smoking and alcohol				
Yes	42 (55%)	9 (33%)	33 (66%)	0.003
No	33 (43%)	18 (67%)	15 (30%)	
Unknown	2 (3%)		2 (4%)	
TNM-classification				
Stage 0-3	39 (51%)	13 (48%)	26 (52%)	NS
Stage 4	37 (48%)	14 (52%)	23 (46%)	
Unknown	1(1%)		1 (2%)	
T-classification				
<4cm (T 1-2)	37 (48%)	17 (63%)	20 (40%)	NS
≥4cm (T 3-4)	39 (51%)	10 (37%)	29 (58%)	
Unknown	1 (1%)		1 (2%)	
Tumor grade[3]				
Poor/ moderate	63 (82%)	24 (89%)	39 (78%)	NS
Well	10 (13%)	2 (7%)	8 (16%)	
Unknown	4 (5%)	1 (4%)	3 (6%)	
Lymph node metastasis				
Positive	55 (71%)	22 (81%)	33 (66%)	NS
Negative	21 (27%)	5 (19%)	16 (32%)	
Unknown	1 (1%)		1 (2%)	
Recurrent disease				
Yes	16 (21%)	4 (15%)	12 (24%)	NS
No	32 (42%)	16 (59%)	16 (32%)	
Never disease free	29 (38%)	7 (26%)	22 (44%)	

TSCC = tonsillar squamous cell carcinoma; HPV = human papillomavrus; NS = not significant. [1]Patiens were classified as daily tobacco smokers (≥ 1 cigarette, pipe, and/or cigar per day). Or non-smokers (never smokers or patients who had stopped smoking more than 10 years before the diagnosis of TSCC). [2]Patients were classified as drinkers (consumption of > 2 whiskey equivalents per day (1 whiskey equivalent ~ 10g alcohol). [3]Tumor grade was scored as well-, moderately-, or poorly differentiated according to the criteria of the World Health Organization

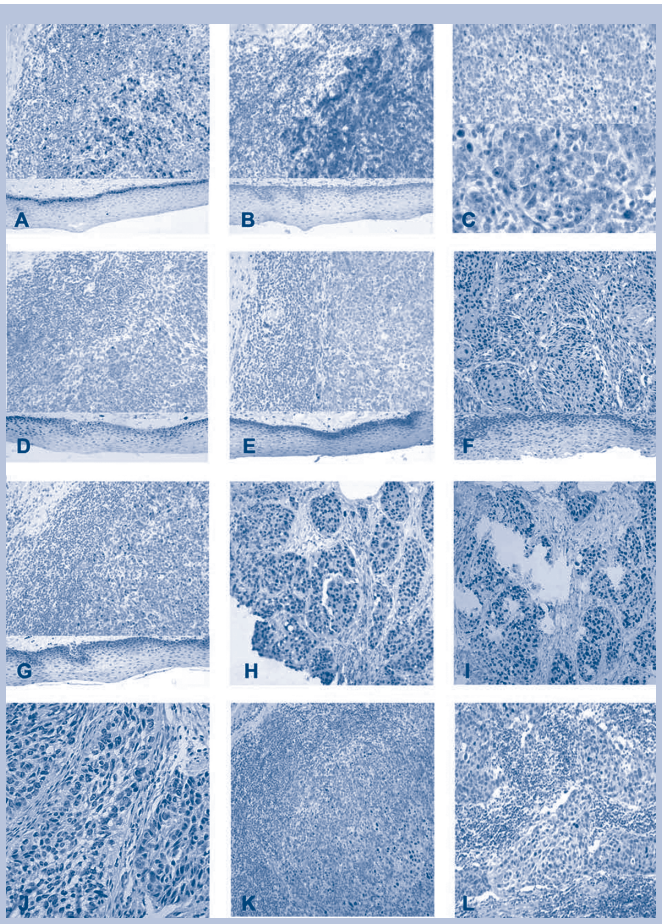

FIGURE 1. REPRESENTATIVE EXAMPLES OF IMMUNOHISTOCHEMICAL STAINING ON TISSUE SECTIONS FOR (A) KI67, (B) $P16^{INK4A}$, (C) $P14^{ARF}$, (D, J) PRB, (E, I) CYCLIN D1, (F) $P21^{CIP1/WAF1}$, (G,H) P53, (K) MDM2 AND (L) $P27^{KIP1}$. Sections are from HPV-positive TSCC (A-G), HPV-negative TSCC (H-L) and adjacent squamous epithelium (A, B, D-G; lower image). Evaluation criteria as described in Materials and Methods section. (A) Strong nuclear Ki67 staining in tumor cells and parabasal epithelial cells; (B) Strong and diffuse $p16^{INK4A}$ staining in tumor cells, predominantly cytoplasmatic; epithelium negative; (C) Strong and diffuse nuclear $p14^{ARF}$ staining (higher image). Some tumors show a rather nucleolar-like immunostaining (lower image). (D) Weak/no nuclear pRb staining in tumor cells in comparison with adjacent normal squamous epithelium. (E) Weak/no nuclear cyclin D1 staining in tumors cells; parabasal epithelial cells weakly positive; (F) Strong nuclear $p21^{Cip1/WAF1}$ staining in tumor cells; some (para)basal epithelial cells weakly positive; (G) Low frequency of tumor cells positive for nuclear p53; some (para)basal epithelial cells weakly positive; (H) High frequency of tumor cells positive for nuclear p53; (I) High frequency of tumor cells positive for nuclear cyclin D1; (J) Strong nuclear pRb staining in tumor cells; (K) Low frequency of tumor cells showing strong and nuclear MDM2 staining; (L) Weak to strong nuclear immunostaining for $p27^{Kip1}$ in tumor cell areas as well as in adjacent lymphocyte areas. Magnification: 40x (A-C (higher image), D-I, K-L), 120x (J) and 200x (C (lower image)). (see page 160 for color figure)

TABLE 2. PRIMARY ANTIBODY CHARACTERISTICS AND USED EVALUATION CRITERIA

Antibody characteristics and tissue pretreatment:								Evaluation criteria for positivity:	References
Antigen	Antibody clone	Monoclonal/ polyclonal	Raised in	Isotype	Dilution	Tissue pretreatment[a]	Company[b]	If % tumor cells with nuclear staining (+, ++, +++)[c]	
Ki67	Ki-67	Mono	Mouse	IgG	1:50	3x5 min	Dako A/S	> 20%	47, 49, 61
$p16^{INK4A}$	E6H4	Mono	Mouse	IgG	1:25	40 min	Dako A/S	> 25% and/or > 25% cytoplasmatic staining	10, 15, 62
cyclin D1	SP4	Mono	Rabbit	IgG	1:50[d]	3x5 min	Labvision	1) > 5%, and 2) > 50%	47, 63, 64
pRb	84-B3-1	Mono	Mouse	IgM	1:50[d]	3x5 min	Novocastra	1) Intensity in tumor ≥ intensity in adjacent squamous epithelium, and 2) > 20%	7, 16, 63
$P14^{ARF}$		Poly	Rabbit	IgG	1:100	3x5 min	Labvision	> 30% and/or any nucleolar staining	61
MDM2	IF2	Mono	Mouse	IgG	1:50[e]	10 min	Zymed	> 10%	50
p53	DO-7	Mono	Mouse	IgG	1:50	3x5min	Dako A/S	> 10%	7, 49, 50, 61, 64
$p21^{Cip1/WAF1}$	70	Mono	Mouse	IgG	1:100	3x5min	BD Biosciences	> 10%	47, 48, 49, 50
$p27^{Kip1}$	1B4	Mono	Mouse	IgG	1:20	3x5min	Monosan	> 10%	48, 49

[a]Microwave heating in 0.01M citrate buffer (pH 6.0). [b]Companies: - Dako A/S: Glostrup, Denmark- Labvision: Fremont, USA- Novocastra: Newcastle upon Tyne, UK - Zymed: South San Francisco, USA- BD Biosciences: San Jose, USA- Monosan: Uden, The Netherlands[c] + positive, ++ strongly positive, +++ extremely positive [d]Primary antibody incubation overnight instead of one hour period[e]Detection of primary antibody by Powervision (Dako) instead of avidin-biotinylated peroxidase complex

TABLE 3. INFLUENCE OF HPV-RELATED AND CLINICOPATHOLOGIC PARAMETERS ON DISEASE-SPECIFIC SURVIVAL IN 74 PATIENTS WITH TSCC, AS DETERMINED BY UNIVARIATE COX PROPORTIONAL HAZARD REGRESSION ANALYSIS

Variable	Total	Death	P value[1]	Unadjusted HR (95% CI)
Cell cycle proteins				
Ki67				
Positive	72	40 (56%)	NS	1.5 (0.2-10.9)
Negative	2	1 (50%)		1 (referent)
$p16^{INK4A}$				
Positive	28	10 (36%)	0.029	0.5 (0.2-0.9)
Negative	46	31 (67%)		1 (referent)
cyclin D1 > 5%				
Positive	31	20 (65%)	0.060	1.8 (1.0-3.4)
Negative	43	21 (49%)		1 (referent)
cyclin D1 > 50%				
Positive	11	9 (82%)	0.028	2.2 (1.1-4.7)
Negative	63	32 (51%)		1 (referent)
pRb > 20%				
Positive	61	35 (57%)	NS	1.5 (0.6-3.5)
Negative	13	6 (46%)		1 (referent)
pRb intensity in tumor ≥ intensity in adjacent squamous epithelium				
Positive	40	25 (62%)	NS	1.5 (0.8-2.9)
Negative	34	16 (47%)		1 (referent)
$p14^{ARF}$				
Positive	33	12 (36%)	0.020	0.5 (0.2-0.9)
Negative	41	29 (71%)		1 (referent)
MDM2				
Positive	22	10 (45%)	NS	0.8 (0.4-1.7)
Negative	52	31 (60%		1 (referent)
p53				
Positive	37	22 (59%)	NS	1.2 (0.7-2.2)
Negative	37	19 (51%)		1 (referent)
$p21^{Cip1/WAF1}$				
Positive	30	10 (33%)	0.004	0.4 (0.2-0.8)
Negative	43	31 (72%)		1 (referent)
Unknown	1			
$p27^{KIP1}$				
Positive	25	14 (66%)	NS	1.2 (0.6-2.3)
Negative	47	26 (55%)		1 (referent)
Unknown	2			

Variable	Total	Death	P value[1]	Unadjusted HR (95% CI)
Clinicopathological Variables				
Gender				
Male	55	33 (60%)	0.084	1 (referent)
Female	19	8 (42%)		1,9 (0,9-4,2)
Age (years)				
< 60	41	24 (58%)	NS	1 (referent)
≥ 60	33	17 (52%)		0.8 (0.4-1.5)
Smoking[2]				
No	11	2 (18%)	0.006	1 (referent)
Yes	63	39 (62%)		5.8 (1.4-24.1)
Alcohol[3]				
No	29	16 (55%)	NS	1 (referent)
Yes	45	25 (56%)		1.1 (0.6-2.0)
Smoking and/or alcohol				
No	8	1 (12%)	0.015	1 (referent)
Yes	66	40 (61%)		7.9 (1.1-57.7)
Smoking and alcohol				
No	32	17 (53%)	NS	1 (referent)
Yes	42	24 (57%)		1.3 (0.7-2.3)
TNM-classification				
Stage 0-3	38	20 (53%)	NS	1 (referent)
Stage 4	36	21 (58%)		1.5 (0.8-2.8)
T-classification				
< 4cm (T 1-2)	35	14 (40%)	<0.001	1 (referent)
≥ 4cm (T 3-4)	39	27 (69%)		3.1 (1.6-6.0)
Tumor grade[4]				
Poor/ moderate	61	37 (61%)	NS	1 (referent)
Well	10	3 (30%)		0.4 (0.1-1.4)
Unknown	3			
Lymph node metastasis				
Positive	54	29 (54%)	NS	1.0 (0.5-1.9)
Negative	20	12 (60%)		1 (referent)
Recurrent disease				
Yes	16	13 (81)	<0.0001	1 (referent)
No	32	3 (9%)		14.1 (3.9-51.1)
Never disease free	26	25 (96%)		
HPV-association				
Yes	26	8 (31%)	0.010	0.4 (0.2-0.8)
No	48	33 (69%)		1 (referent)

HR = hazard ratio; NS = not significant; HPV = human papillomavrus. [1]P-values based on the Log Rank test. [2]Patiens were classified as daily tobacco smokers (≥1 cigarette, pipe, and/or cigar per day) or non-smokers (never smokers or patients who had stopped smoking more than 10 years before the diagnosis of TSCC). [3]Patients were classified as drinkers (consumption of >2 whiskey equivalents per day (1 whiskey equivalent ~ 10g alcohol). [4]Tumor grade was scored as well-, moderately-, or poorly differentiated according to the criteria of the World Health Organization

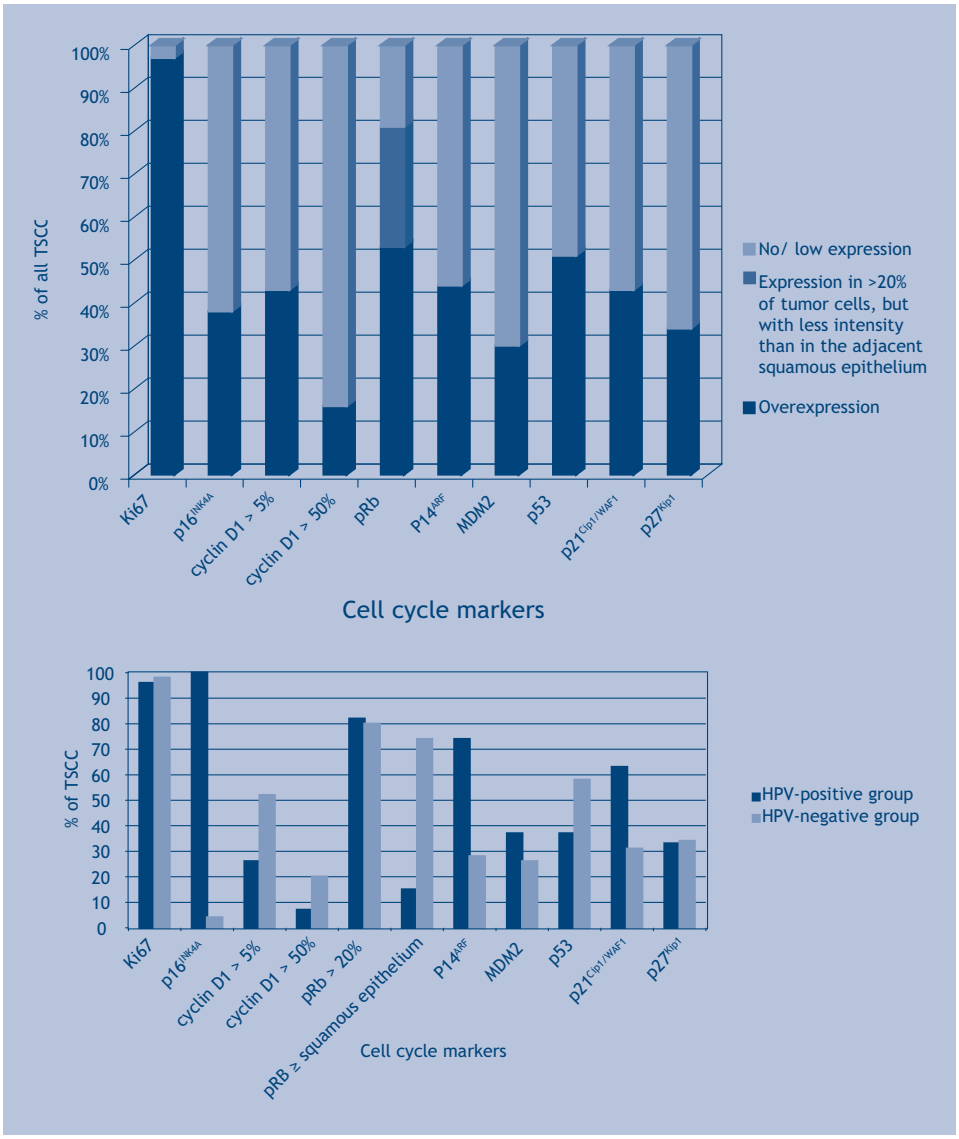

FIGURE 2. (A) CELL CYCLE PROTEIN EXPRESSION IN TSCC ACCORDING TO THE EVALUATION CRITERIA PRESENTED IN TABLE 2. (B) Fraction of HPV-positive and HPV-negative tonsillar squamous cell carcinoma (TSCC) showing expression of individual cell cycle markers. See table 2 for the used criteria for positivity of each individual cell cycle marker.

Indicators for disease-specific patient survival

To determine whether or not cell cycle protein expression patterns and clinicopathological parameters can be used as indicators of prognosis, we correlated these with the disease-specific survival data of patients with TSCC (Table 3). Two patients died post-operatively, due to bleeding and aspiration, and from one patient no follow-up data was available. These patients were excluded from the analyses. Follow-up time ranged from 0 to 141 months, with a mean of 30 months. Forty-one (55%) of 74 patients died as a consequence of TSCC. The survival after 5 years was 31% for patients with a HPV-negative tumor and 69% for patients with a HPV-positive carcinoma (Hazard ratio (HR) = 0.4; 95% Confidence Interval (CI) = 0.2-0.8). Besides absence of HPV, the following parameters were also significantly associated with a shorter disease-specific survival according to Univariate Cox regression analysis: 1) smoking (HR = 5.8; 95% CI = 1.4-24.1), 2) a tumor diameter ≥ 4cm (HR = 3.1; 95% CI = 1.6-6.0), 3) development of recurrent disease (HR = 14.1; 95% CI = 3.9-51.1), 4) no/ low expression of either $p14^{ARF}$ (HR = 2.2; 95% CI = 1.1-4.3) or $p21^{Cip1/WAF1}$ (HR = 2.7; 95% CI = 1.3-5.5), and 5) positive cyclin D1 immunostaining in > 50% of tumor cells (HR = 2.2; 95% CI = 1.1-4.7). Gender, age at diagnosis, alcohol use, tumor grade, TNM-stage, lymph node status and the remaining cell cycle markers were not related to disease-specific survival.

The parameters that were significantly correlated with disease-specific survival in the univariate analysis, i.e. HPV-status, tumor size, smoking, and immunostaining of $p14^{ARF}$, $p21^{Cip1/WAF1}$ and cyclin D1, were included in the multivariate Cox regression analysis. Development of recurrent disease was not included for multivariate analysis, because this factor cannot be predicted at time of diagnosis, so the clinical impact is of less importance. Table 4 shows that 4 of the 6 parameters, i.e. tobacco consumption, tumor diameter ≥ 4cm, no/ low $p21^{Cip1/WAF1}$ immunostaining or strong cyclin D1 immunostaining, were the most optimal indicators of cancer-specific death, with tumor size and $p21^{Cip1/WAF1}$ immunostaining being the most significantly correlated. In Figure 3 the Kaplan-Meier curves for these 4 parameters are shown.

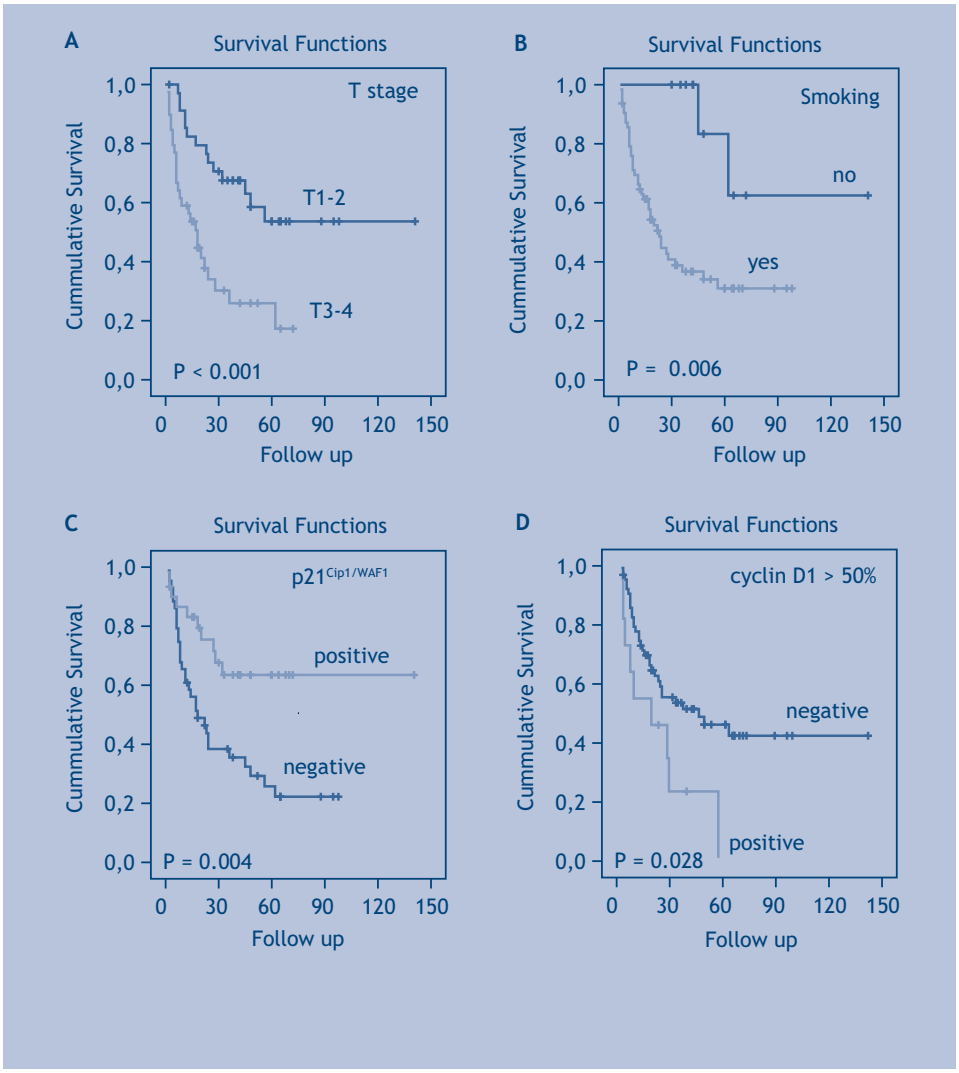

FIGURE 3. KAPLAN-MEIER SURVIVAL CURVES ACCORDING TO (**A**) TUMOUR SIZE, (**B**) SMOKING STATUS, (**C**) $P21^{CIP1/WAF1}$ EXPRESSION, AND (**D**) STRONG CYCLIN D1 EXPRESSION.

TABLE 4. MULTIVARIATE ANALYSIS, ACCORDING TO COX PROPORTIONAL HAZARD REGRESSION ANALYSIS, OF THE PATIENT AND TUMOR CHARACTERISTICS RELATED TO DISEASE-SPECIFIC MORTALITY

Characteristic	HR	95% CI	P-value
Smoking: yes vs. no	4.06	0.95-17.26	0.058
Tumor size: T3-4 vs. T1-2	2.58	1.32-5.07	0.006
$p21^{Cip1/WAF1}$: positive vs. negative	0.38	0.18-0.79	0.009
cyclin D1: > 50% vs. ≤ 50%[1]	2.19	1.02-4.69	0.044

HR = hazard ratio; CI = confidence interval. [1]Nuclear staining

Discussion

In this study we examined the expression of cell cycle-related constituents in a series of 77 TSCC with the goal to determine their role in HPV-dependent and HPV-independent carcinogenesis. Furthermore, their prognostic value was evaluated by correlating the results with clinical follow-up data. Our results show that HPV16-positive tumors exhibit $p14^{ARF}$ and $p21^{Cip1/WAF1}$ overexpression and downregulation of pRb and cyclin D1 in contrast to HPV16-negative tumors. Secondly, tumor size and $p21^{Cip1/WAF1}$ positivity are the strongest independent indicators for a favorable outcome in patients with TSCC.

All TSCC, except one HPV-positive and one HPV-negative tumor, showed a high expression of Ki67, indicating that almost all tumors contained a high percentage of proliferative cells, which is in agreement with other studies on HNSCC[29]. In addition, high expression levels of inhibitors of apoptosis, such as $BclX_L$ and survivin, have been reported in HNSCC[30-33]. Expression of pRb was detected in 81% of TSCC. Approximately half of all tumors showed strong nuclear expression in the tumor cells, with expression levels equal to or higher than the adjacent normal squamous epithelium, whereas pRb downregulation was observed in the remaining cases. In our study p53 overexpression was observed in little more than half of all TSCC, which is consistent with other studies[7,34]. Immunostaining of all other cell cycle proteins was evident in less than 50% of tumors.

We noticed that the tumors with the low expression levels of pRb showed overexpression of $p16^{INK4A}$. Accumulation of this latter protein has been strongly associated with the presence of oncogenic HPV in oropharyngeal carcinomas[7,10,14,15],

which was also evident in the underlying study. These HPV16-positive TSCC furthermore showed downregulation of cyclin D1, next to accumulation of $p14^{ARF}$ and $p21^{Cip1/WAF1}$. In addition, p53 expression was less profound in these HPV-positive tumors.

In HPV-associated tumors the oncoprotein E7 interacts with pRb, resulting in its degradation. As a result $p16^{INK4A}$ is upregulated and cyclin D1 downregulated[35]. This is in agreement with Andl et al.[5], who suggested that E7 might overcome the need for cyclin D1 in the G1 phase of the cell cycle, because it interacts with the cyclin D1-binding site on pRb[7,36]. Indeed, high expression levels of cyclin D1 were predominantly observed in HPV-negative TSCC, most probably pointing to cyclin D1 gene amplification in these cases.

The $p14^{ARF}$ gene is a target for the transcription factor E2F, which promotes its expression particularly in the HPV-positive tumors, as has become evident in this study. In the HPV-negative tumors both $p14^{ARF}$ as well as $p16^{INK4A}$ were often undetectable, which is in accordance with the fact that their expression is downregulated in most HNSCC due to gene inactivation at the 9p21 locus[37,38]. In normal tissue cells $p14^{ARF}$ upregulation might lead to p53 upregulation, but in HPV-positive TSCC this is usually counteracted by means of functional inactivation of wild-type p53 by the viral E6 protein. In contrast and as observed in our study, p53 is frequently upregulated in HPV-negative tumors due to mutations in the TP53 gene as a result of exposure to tobacco and/or alcohol[10,16,20,27,39].

Although $p21^{Cip1/WAF1}$ is known to be a downstream effector of p53[40,41], it was surprising to find overexpression in HPV-positive tumors harboring low or no detectable levels of p53. Such observations have also been reported by Milde-Langosch et al.[42] in HPV-associated uterine cervical tumors and suggest that also p53-independent mechanisms may lead to $p21^{Cip1/WAF1}$ accumulation as previously described[43,44]. Indeed, in HPV-positive cancer cells $p21^{Cip1/WAF1}$ expression appears to be inducible[45], although it remains unclear why under these conditions E7 cannot inactivate $p21^{Cip1/WAF1}$ by direct interaction[14].

Despite the association of $p21^{Cip1/WAF1}$ overexpression with HPV-positivity in most TSCC, we found $p21^{Cip1/WAF1}$ protein accumulation in some HPV-negative tumors, which is in accordance with the study of Li et al.[46]. These authors examined 67 TSCC for HPV involvement but did not find $p21^{Cip1/WAF1}$ expression being associated with HPV-positivity. An explanation for this paradox might be on the one hand the use of a different $p21^{Cip1/WAF1}$ -specific primary antibody and different criteria to assess

p21$^{Cip1/WAF1}$ upregulation (> 20% of positive nuclei versus > 10%) in comparison with our and other studies[47-50], and on the other hand the use of only PCR to determine the presence of HPV. In order to assess a firm association between virus and tumor cells, namely, it is recommended to carry out additional tests, such as p16^{INK4A} immunostaining and/or FISH, which has been applied in our study[51-52].

In the univariate and multivariate statistical analyses we found that p21$^{Cip1/WAF1}$ overexpression was a highly significant indicator of favorable prognosis in TSCC independent of HPV status. Expression of p21$^{Cip1/WAF1}$ has also been associated with a favorable survival in patients with tongue squamous cell carcinoma, and in patients with ovarian, superficial bladder, gastric, colorectal and esophageal cancers[35,47,53,54]. In one other study on TSCC and two other studies on laryngeal squamous carcinoma no correlation was reported[46,48,55] and in one study on HNSCC derived from all head-and-neck localizations even a negative correlation with survival was described[19], which may be explained by the heterogeneous tumor population. Other strong indicators for a favorable prognosis in our study include tumor diameter < 4cm, low/ no expression of cyclin D1 and low/no tobacco smoking. In accordance with most other studies on HPV-related oropharyngeal carcinomas, we also found a favorable disease-specific survival in patients with HPV16-positive TSCC[12,38,56-60], and p14^{ARF} overexpression[61], although with less significance than the indicators described above. We noticed that lymph node status, which is generally considered the most important prognostic factor in HNSCC[23], had little value in our series of TSCC, which is in concordance with other studies[24,25].

In summary we can conclude that HPV16-positive TSCC exhibit overexpression of p14^{ARF} and p21$^{Cip1/WAF1}$, as well as downregulation of pRb and cyclin D1, while strong immunostaining for p21$^{Cip1/WAF1}$ appears to be one of the most potent indicators for favorable prognosis in these tumors.

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Chapter 6

General Discussion

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General discussion

The aim of the studies described in this thesis was to obtain a better insight into the role of oncogenic human papillomaviruses (HPVs) in the carcinogenesis of head and neck squamous cell carcinomas (HNSCC). In this chapter our results will be discussed and related to previous reports in the literature.

Clinical characteristics of HPV-positive carcinomas

The main clinical and molecular characteristics that seem to differ strongly between HPV-positive and HPV-negative tumors are listed in Table I. We and others have shown that HPV-positive HNSCC are predominantly localized to the oropharynx[1-11], and that integration of HPV type 16 is involved in almost all cases. The virus has also been detected in lymph-node metastases of these tumors[7] as well as recently found in dysplasia in the tumor resection margins (Klussmann and Speel, unpublished results). These findings answer the first question raised in Chapter 1. HNSCC containing high-risk HPV appear in general to have a poor differentiation as compared to the often moderate- to well-differentiated HPV-negative tumors[3,11-13]. Furthermore, HPV-positive tumors more often show a characteristic basaloid morphology (meaning an invasive growth pattern as small solid foci) than HPV-negative tumors[6,7,12-14].

We found that patients with HPV 16-positive tumors presented significantly more often with the initial complaint of a swelling in the neck. This is rather surprising since both HPV-positive and HPV–negative tumors showed comparable percentages of detectable lymph node metastases[11,14]. An explanation for this different presentation might be the significantly smaller primary tumor size (diameter < 4 cm) at the time of diagnosis as found in our patient series with tonsillar squamous cell carcinomas (TSCC) and also described in previous literature[11,15], leading to less local complaints in the HPV-positive patients. Another explanation may be a better health awareness in patients with HPV 16-positive tumors as reflected by significantly less alcohol and tobacco (ab)use. The significantly smaller HPV-positive primary tumors were accompanied by slightly more regional metastases compared to the HPV-negative tumors, which might suggest that HPV-positive tumors have a tendency to metastasize early. In our study we noticed that the lymph node status, which is generally considered the most important prognostic factor in HNSCC[16], had little value in the series of TSCC, which is in concordance with earlier studies[17].

A significant correlation between tumor-associated HPV and strongly reduced or absent exposure of patients to the known risk factors of tobacco smoking and alcohol consumption has been reported in our and several other studies[6,7,11,15]. In this respect it was hypothesized that smoking may have a protective effect on HPV infection in the oral cavity and oropharynx due to an increased keratinization of the mucosa. This increased keratinization may protect the mucosa from minor trauma, a situation suggested to be required for HPV infection of the basal cell layer. The finding that tumors of patients with a high tobacco intake are less often infected with HPV seems to support this hypothesis[2,6]. Patients with HPV-containing tumors were reported to be several years younger[18-21] and more often male[14,20]. In our and other studies, however, this tendency could not be confirmed[6,11,22].

With respect to the clinical course and prognosis of HPV-related tonsillar squamous cell carcinomas (TSCC), varying observations have been reported. Although most authors described a survival advantage of HPV-positive TSCC patients when compared to HPV-negative cases[3,10,14,20,23-28], others showed the opposite result[29] or could not show a difference in survival outcome[30,31]. In addition, it has been suggested that the clinical course of the disease might be gender-specific, since males with a HPV-positive tumor had a better prognosis than males with a HPV-negative tumor, whereas this correlation could not be detected in women[14].

Despite the fact that patients with HPV-positive TSCC show a favorable prognosis, our study revealed that tobacco smoking is an even stronger prognostic indicator[11].These findings answer the third question raised in Chapter 1, and furthermore implicate that oncogenic processes in the tonsillar epithelium of non-smokers differ from those occurring in smokers, the former being related to HPV 16 infection.

Molecular characteristics of HPV-positive carcinomas

In addition to clinical features, we and others have described molecular features that are associated with HNSCC harboring high-risk HPV types (Table I). Besides the presence of HPV DNA sequences, tumor cells in most instances show upregulated E6 and E7 oncoprotein expression due to viral integration to the host cell genome, thereby disrupting amongst others the E2 gene sequence and the ability of the E2 protein to down-regulate E6 and E7 transcription. As the HPV E6 oncoprotein promotes

degradation of p53, it is expected that p53 mutations are not a prerequisite in HPV-induced tumorigenesis and that in HPV-positive tumors wild-type p53 expression levels are too low to be detected immunohistochemically. Compared to the reported mutation frequency of approximaly 50% for the p53 gene in all HNSCC taken together[12], significantly lower percentages of HPV-containing tumors exhibit p53 mutations in exons 5-8. However, large discrepancies in the number of HPV-positive HNSCC exhibiting simultaneous p53 mutations have been described, ranging from 13% to 46%[32-36]. In two of these studies[32,33] it was observed that when E6 expression was also taken into account as an additional indicator of a causal relationship between HPV and HNSCC, a maximum of 8% of HPV-positive tumors harbored p53 mutations. This is in accordance with our FISH study in which we reported an inverse relationship between p53 mutations and the presence of HPV DNA[7]. Despite the lack of p53 mutations in exons 5-8, we[7] and others[23,37] also observed an unexpected accumulation of p53 in HPV-containing HNSCC. The molecular mechanisms that may underlie this stabilization and/or overexpression of p53 protein are still unclear. Possible explanations include: (i) the presence of mutations occurring outside exons 5-8; (ii) upregulation of the wild type p53 protein by genotoxic insults, e.g. ultraviolet radiation, or hypoxia[38]; (iii) non-mutational p53 stabilization by mdm2 or viral proteins such as large T-cell antigen of SV40; and/or (iv) the lack of functional E6 expression e.g. due to a mutation in the gene sequence[32]. The above data answer the second question raised in Chapter 1.

In HPV-positive tumors pRb expression appears to be reduced due to the activity of the E7 oncoprotein[7,33,39]. As described previously, this will result in upregulation of $p16^{INK4A}$ expression, previously reported to be a specific surrogate marker for oncogenic HPV-containing (pre)neoplastic lesions of the uterine cervix. Also a downregulation of cyclin D1 synthesis has been observed under these circumstances. We and others[40,41] also found cyclin D1 downregulation in HPV16-positive TSCC. On the other hand, high expression levels of cyclin D1 were predominantly observed in HPV-negative TSCC, most probably pointing to amplification of the cyclin D1 gene at chromosome 11q13 in these cases[12,42].

Whereas $p14^{ARF}$ and $p16^{INK4A}$ are often undetectable in HPV-negative tumors due to gene inactivation at the 9p21 locus, we found $p14^{ARF}$ and $p16^{INK4A}$ overexpression in our series of HPV 16-positive tumors[11,41]. The $p16^{INK4A}$ overexpression in HPV-positive oropharyngeal cancers has been reported earlier[7,43,44]. As $p16^{INK4A}$ overexpression is highly correlated with HPV status, it is tempting to speculate that

p16^{INK4A} overexpression may be used as an additional primary screening method to identify HPV-positive lesions in routine testing[7,11,33,43].

TABLE I. CLINICAL AND MOLECULAR DIFFERENCES BETWEEN HNSCC WITH OR WITHOUT HIGH-RISK HPV.

	HPV-positive	HPV-negative
Clinical characteristics		
Preferred location	oropharynx	all sites
Differentiation	poor	well
Basaloid appearance	yes	no
Size at diagnosis (D=4 cm)	smaller	larger
Disease stage(TNM)	more advanced	less advanced
Age (about 60 years)	slightly younger	slightly older
Tobacco (ab)use	low	high
Alcohol (ab)use	low	high
5 years disease-free survival	55%-85%	30%-55%
Second primary tumors	less	more
Local recurrences	less	more
Molecular characteristics		
HPV DNA	yes	no
E6/E7 expression	yes	no
P16 overexpression	yes	no
P14 overexpression	yes	no
P21 overexpression	yes	no
PRb down regulation	yes	no
Cyclin D1 down regulation	yes	no
P16 inactivation	no	yes
P53 mutations	no	yes
Cyclin D1 amplification	no	yes
EGFR overexpression	no	yes
Nuclear survivin	no	yes

Although p21$^{Cip1/WAF1}$ is known to be a downstream effector of p53, several studies describe an overexpression in HPV-positive tumors harboring low or no detectable levels of p53[41,45]. This finding suggests that also p53-independent mechanisms may lead to p21$^{Cip1/WAF1}$ accumulation. In our recent study[41], we found that p21$^{Cip1/WAF1}$ overexpression was a highly significant indicator of favorable prognosis in TSCC, independent of HPV status. This association has already been described for patients with tongue squamous cell carcinoma, and in patients with ovarian, superficial bladder, gastric, colorectal and esophageal cancers[46,47]. The above data answer the fifth question raised in Chapter 1.

In addition to $p16^{INK4A}$ and $p21^{CIP1/WAF1}$ as potential prognostic markers for oropharyngeal cancers, the expression of the epidermal growth factor receptor (EGFR) has been in focus as a molecular prognosticator[20,48]. EGFR overexpression appeared to be inversely related to the presence of HPV[20,49]. Overexpression of EGFR has been associated with local recurrence after radiation and a poor prognosis with respect to disease-free and overall survival in oropharyngeal cancers[20,48-50].

A recent study by Preuss et al.[51] has shown an inverse correlation between cytoplasmatic and nuclear survivin localization and HPV-associated carcinomas. Survivin is an inhibitor of apoptosis, with a bifunctional role in apoptosis and in cell division. Overexpression of particularly nuclear survivin has been correlated with poor prognosis, cancer progression and drug resistance[51,52]. However, this finding remains controversial as a recent paper described that nuclear survivin expression predicts a favorable effect on overall survival in oral squamous cell carcinoma[53]. This positive correlation with a better survival was most pronounced in patients treated by radiotherapy, and therefore it was concluded that survivin can be used as a marker to predict a good response to radiotherapy.

Recent comparative genomic hybridization and gene expression profiling studies have reported additional molecular differences between HPV-positive and negative HNSCC, that most likely underlie differences in tumor development and progression and may have prognostic significance[42,54-56]. Marked findings in HPV-positive tumors included the overexpression of several cell cycle regulators ($p16^{INK4A}$, p18 and CDC7), transcription factors (TAF7L, RFC4, RPA2 and TFDP2), and genes on chromosome 3q. Chromosome 3q loci indeed were found gained or amplified in up to two third of HPV-positive oropharyngeal carcinomas, which may underlie this upregulation of gene expression. However, because 3q gain is also the most frequent chromosomal alteration detected in HPV-negative tumors, more studies are needed to indicate which genes of interest are pivotal in HPV-related and -unrelated HNSCC carcinogenesis. HPV-positive tumors furthermore showed to harbor less chromosomal alterations and amplifications as their HPV-negative counterparts, as well as more often 16q loss. Particularly the absence of amplifications and the presence of 16q loss proved to be strong indicators of favorable outcome.

In conclusion, these data provide strong evidence that HPV-positive HNSCCs, particularly oropharyngeal carcinomas, represent a different tumor entity.

Oropharyngeal carcinogenesis

From the above we can conclude that there are two separate routes leading to oropharyngeal cancers: one related to the traditional risk factors such as tobacco smoking and alcohol (ab)use, and the other related to infections with oncogenic HPVs (Figure 1). In the HPV-unrelated route, excess of alcohol or tobacco smoke exposure results in deregulation of the p53 and pRb pathways in a multistep process of progression from healthy cell through hyperplasia, dysplasia to finally carcinoma. Predominant molecular alterations include inactivation of $p16^{INK4A}$ and $p14^{ARF}$ gene expression, p53 mutations and upregulation of EGFR and cyclin D by amongst others gene amplification. In the HPV-related route the oncogenic HPV E6 and E7 proteins act to inactivate p53 and pRb pathways, with subsequent upregulation of $p16^{INK4A}$, $p14^{ARF}$ and $p21^{Cip1/WAF1}$ and downregulation of cyclin D1 expression. In Chapter 3 however we describe that there are also tumors in which a combination of the pathways might have taken place. In these cases the human epithelium was exposed to tobacco and alcohol, and meanwhile an infection with an oncogenic HPV has occurred. In these tumors it is unclear which oncogenic events eventually lead to the development from normal epithelium to carcinoma. These tumors may or may not arise via a separate pathway, which needs to be investigated further. In Figure 1 we represent this pathway as an extension of the classical route of carcinogenesis of HPV-negative lesions.

Due to the fact that the total mucosa of the head and neck region is exposed to the carcinogenic effects of tobacco smoking and alcohol use (field cancerization), secondary primary tumors are frequently found in the patients with HPV-negative tumors. In patients with HPV-positive tumors however, we have shown that second primary tumors are uncommon, probably because HPV infection is a rather locally occurring event (Table 1). Why in particular the tonsils are susceptible to HPV infection is unclear at present. Possible explanations can be that: (i) tonsillar epithelium, like the uterine cervix epithelium, is easily accessible and appears to undergo metaplastic processes[57]; (ii) tonsils contain deep invaginations of the mucosal surface (crypts) which create an extensive monolayered epithelial surface which may facilitate viral access to basal cells and intensive antigenic stimulation[6,58]; and (iii) the presence of cytokines produced by lymphoid tissue may affect HPV transcription and cellular transformation[57,59].

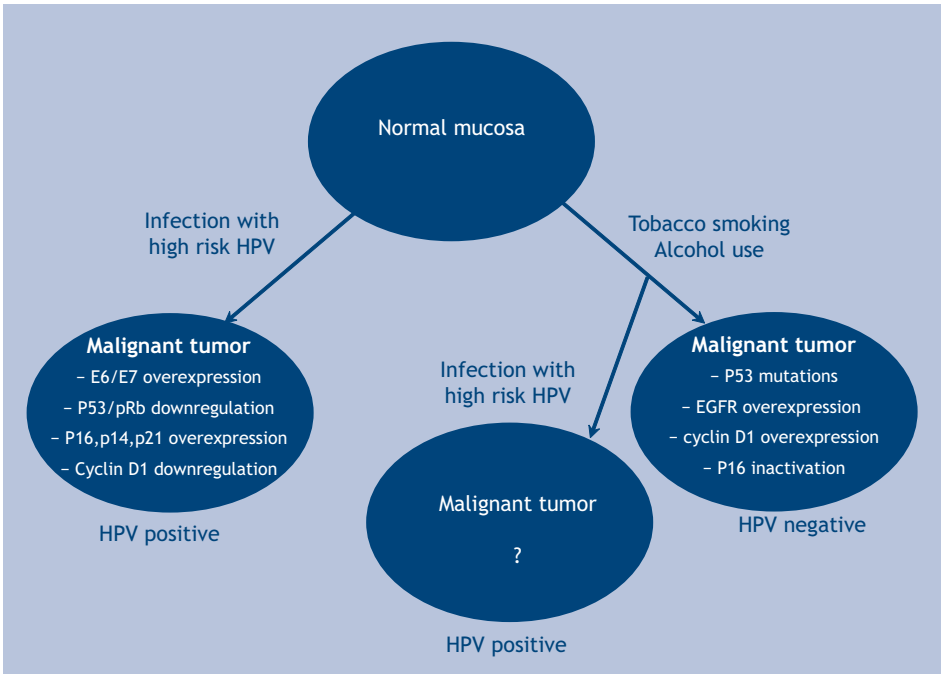

FIGURE 1. PROPOSED MODEL FOR OROPHARYNGEAL CARCINOGENESIS. The model shows three pathways leading to malignancy. In the first pathway the normal mucosa is infected with a high risk human papillomavirus (HPV) leading to expression of E6 and E7 proteins. These proteins cause downregulation of pRb and p53, and as a consequence, overexpression of $p16^{INK4A}$, $p14^{ARF}$, and $p21^{CIP1/WAF1}$ and downregulation of cyclin D1. In the second pathway the mucosa is exposed to tobacco smoke and alcohol leading to $p16^{INK4A}$ inactivation, p53 mutations, as well as EGFR and cyclin D1 overexpression. Finally, in the third pathway the mucosa is exposed to tobacco smoke and alcohol next to an infection with a high risk HPV.

HPV infections of the anogenital tract are known to represent a sexually transmitted disease. However, the route by which HPV reaches the mucosa of the head and neck in adults is not known with certainty. HPV may be acquired through infection at birth and may remain latent for years for unknown reasons. On the other hand it has been reported that husbands of patients with uterine cervical cancer had a higher risk of developing both tonsillar cancer and cancer of the tongue[60], and that people with a history of oral sex had a fourfold higher chance of HPV infection than those without[39,61]. This finding suggests that oral-genital contact may be a mode of transmission of HPV. Further studies should seek to determine the prevalence

of HPV in the tonsils of the healthy population in relation to sexual behavior. If the situation in the head and neck region resembles that of the uterine cervix, only a small proportion of patients with HPV infection will develop virus-associated malignancies[39]. This indicates that additional factors are involved in the progression of an infected cell to a transformed cell with invasive potential. Besides the accumulation of genomic alterations data from immunocompromised patients and immuno-manipulated animal models indicate that an impaired immune response is another important factor that leads to tumor progression[42,62].

Detection of HPV in TSCC

In our studies we particularly used the fluorescence in situ hybridisation (FISH) technique to detect the presence of HPV. This technique allows the visualization of one copy of HPV DNA in tumor cells, and at the same time the determination of the physical status, i.e. presence of the virus in an integrated versus episomal fashion. HPV 16 proved to be predominantly integrated in TSCC. Our preliminary results moreover show a significant correlation with results of the amplification of papillomavirus oncogene transcript (APOT) assay, an alternative PCR approach to detect integrated HPV[11,63]. Also Koskinen et al.[64] demonstrated integrated HPV DNA in > 50% of HPV-positive HNSCC as assessed by a real-time PCR approach comparing the presence of HPV E2 and E6 gene sequences. Although very specific and sensitive, the FISH technology is more time-consuming and expensive than PCR technologies, and therefore not readily suitable for routine practice.

As described earlier $p16^{INK4A}$ overexpression, as demonstrated by immuno-histochemistry, is significantly correlated with HPV 16 DNA integration into the tumor cell genome[11]. $P16^{INK4A}$ is a cyclin-dependent kinase inhibitor that prevents pRb phosphorylation and blocks cell cycle progression at the G1 to S check point. Loss of $p16^{INK4A}$ expression by deletion, mutation, or hypermethylation is common in HNSCC. In contrast, functional inactivation of pRb by HPV E7 protein results in overexpression of $p16^{INK4A}$, which makes it a surrogate marker for HPV. Since $p16^{INK4A}$ immunohistochemistry is a relatively fast, easy, and cheap approach, it is a good alternative to predict the detection of HPV infection in tonsillar cancer for use in routine practice. The immunohistochemical detection procedure of $p16^{INK4A}$ overexpression, however, may remain negative in cases where the gene

is inactivated, or $p16^{INK4A}$ may become positive in cases where HPV DNA could not be detected, for example due to infection with other viruses or as a result of senescence and physiological aging[65,66]. This seems to be the case in normal, tumor-free tonsil tissue, in which $p16^{INK4A}$ overexpression was unable to predict the presence of HPV67. These findings answer the fourth question raised in Chapter 1.

A frequently used method to detect HPV infections is the polymerase chain reaction (PCR)[68]. In most of the PCR-based HPV detection methods, a broad spectrum of HPV types is amplified by consensus primers, followed by detection with specific probes. Recent studies have shown that oncogenic HPV testing can also be done through detection of the viral mRNA, by reverse transcriptase PCR, even in routinely fixed paraffin embedded tissues[69]. It was hypothesized that the presence of viral E6/E7 mRNA has a better positive predictive value for malignant transformation than the presence of viral DNA, because the E6/E7 mRNA represents an active infection with cell-transforming potential, whereas the viral DNA may also be present in clinically irrelevant conditions. However, tumors with high levels of E6/E7 mRNA transcripts also showed punctate FISH patterns and $p16^{INK4A}$ overexpression, further underscoring the value of these approaches to detect meaningful HPV association to the tumor[69]. PCR-based methods have a very high analytical sensitivity, with only slight variations between the methods used. On the other hand it appeared that these PCR-based techniques have a relatively low clinical specificity.

Since none of the HPV detection methods have the ideal sensitivity and specificity for routine practice, we advise to use combinations of these detection methods. Testing of $p16^{INK4A}$ overexpression by immunohistochemistry appears the method of choice for primary screening purposes. In case of $p16^{INK4A}$ overexpression a sensitive PCR based assay can be applied to confirm the presence of HPV and determine which type of HPV is present. Finally, a FISH technique will provide information concerning the physical status of the virus and a direct association with the tumor cells.

Prevention of HPV related TSSC

The increasing incidence of oropharyngeal cancers might be due to changes in sexual behavior of adolescents in the last decades[70]. As mentioned above, a recent study by D'Souza et al.[61] has shown that a high lifetime number of vaginal-sex partners and a

high lifetime number of oral-sex partners was associated with oropharyngeal cancer. There is some evidence that health education programs that promote abstinence, conscientious condom use, or both, could reduce the risk of cervical, and most probably also oropharyngeal cancer at the population level[71]. However, strict condom use is not completely protective against HPV transmission because the male anogenital skin is not completely covered[72]. Thus, the development of HPV L1 virus-like-particle (VLP) vaccines is a potentially major advance in prevention of cervical cancer. These vaccines are based on the self-assembly of recombinant L1 protein into non-infectious capsids that contain no genetic material. Intramuscular injection of the vaccine induces high titers of neutralizing antibody, more than 50 times the titers induced by natural infection[73]. In populations of young adult women without known exposure to HPV-16 and 18, VLP vaccines have shown near perfect efficacy against HPV infection and related cytological and histological endpoints of the cervix for up to 5 years[74]. Whether or not these vaccines also protect against HPV-related oropharyngeal cancer is not clear until now. Important questions do, however, remain with respect to the duration of protection and the total effect on cancer incidence, prevention of infection in men, and the reduction of transmissibility of HPV from men to their partner. Furthermore, it remains a question whether prevention of infection with HPV 16 or HPV18 alters the natural history of other carcinogenic HPV-types and the number of cervical cancers they cause[75]. Besides these uncertainties concerning the effectiveness of the VLP vaccines in uninfected individuals it has even been suggested that activation of the innate immune mechanisms, that would usually protect against viral infection, may have a more important role in causing disease progression in individuals where the high risk HPV DNA integration has already taken place[76]. The mechanism underlying this disease progression process includes the loss of the episome-derived E2 protein. Recent work has suggested that the episome-derived E2 protein inhibits transcription of the integrated HPV-DNA, thereby creating an important intermediate stage in cervical carcinogenesis where transcriptionally silent HPV integration co-exists with viral episomes[76,77].

Another cause of the increasing number of patients with tonsillar carcinomas might be the decreased number of tonsillectomies performed at younger age in some countries. In the Netherlands for instance the rate of pediatric tonsillectomies with or without adenotomy decreased rapidly between 1974 and 1985[78]. In other countries, such a decrease could not be found. It is unclear whether the increased incidence of tonsillar carcinomas in the Netherlands is a result from a decreased rate

of tonsillectomies. Well performed population based studies linking tonsillectomy rates and incidence rates of tonsillar carcinomas over many years should shed further light on this subject.

Therapy of oropharyngeal cancers

About 50% of patients with oropharyngeal cancers survive more than 5 years after first diagnosis. Mortality rates of patients with HNSCC have, however, not improved substantially over the past decades, despite advances in diagnosis and therapy[79]. In order to improve outcome it is necessary to identify patients who will respond to a particular therapy. Surgery affects structures important for food uptake, swallowing and speach. Organ-sparing therapies, such as radiation or chemotherapy, can lead to improved outcomes in patients who respond to such therapy. Different studies are undertaken to improve the understanding of the biology of resistance to chemo- or radiation therapy in order to develop effective alternative treatments.

We found that patients with HPV 16-positive tumors had a significantly better disease-specific and overall survival compared to patients with HPV 16-negative tumors. It has been suggested that this better survival outcome might result from a better response to radiation- and chemotherapy[80,81]. An explanation for this better response may be the fact that although the pRb and p53 pathways are compromised in HPV-positive tumors they retain some function, such that under the pressure of chemotherapy or radiotherapy p53-mediated apoptotic pathways may still function. Another reason for the better survival outcome in patients with HPV-positive tumors might be the significantly lower chance of developing a second primary tumor[3], because patients with HPV-positive tumors show often low or no tobacco and alcohol intake and HPV infection tends to be focal. Our data are in agreement with these suggestions, because we noticed a significantly lower tobacco and alcohol intake in HPV-positive tumor patients, as well as a lower percentage of (loco)regional recurrences, and found only second primary tumor development in the HPV-negative patient group[11].

In order to further identify patients that will respond to a particular therapy, different molecular biomarkers, sex and smoking status have been examined for their correlation to treatment outcome.

The presence of p53 mutations was not linked to treatment response or survival

in different studies[21,49]. It appeared that overexpression of the anti-apoptotic proteins Bcl-2 and Bcl-xL is associated with chemotherapy and radiation resistance[28]. The combination of a low p53 and low Bcl-xL expression however was correlated to a better overall and disease specific survival, which is in accordance with the observed apoptotic process in HNSCC tumor cells in response to chemotherapy in *in vitro* studies[49].

Kumar et al.[20,21] also reported a poor response to organ-sparing therapy in smoking female patients, with a high EGFR expression in the tumor. It appeared that EGFR expression was significantly higher in smokers, which suggests that smoking may contribute to up-regulation of EGFR expression, through increased hypoxia in the tumor tissue.

This finding suggests that non-smoking male patients with HPV-positive tumors, with low EGFR, low p53 and low Bcl-xL expression may benefit the most from chemo- and radiation therapy. However, before these findings are implemented in treatment protocols, more extensive studies must be performed.

Furthermore, alternative treatment strategies must be developed for those patients that benefit to a more limited extent from chemotherapy, radiation therapy and surgery.

Possible future therapies

To improve the therapeutic outcome of HPV-related HNSCC the use of antiviral drugs, such as the acyclic nucleoside phosphonate cidofovir, is being investigated for clinical applications[82-84]. Local injection of cidofovir in immunocompetent patients with recurrent respiratory papillomatosis resulted in complete disappearance or partial remission of the lesions, which appears to be based on the induction of apoptosis. This process was associated with accumulation of the tumor suppressor proteins p53 and pRb and the cyclin-dependent kinase inhibitor $p21^{Cip1/WAF1}$ [85]. Administration of cidofovir to HPV-positive cancer cell lines showed a down-regulation of E6 and E7, with subsequent reactivation of p53 and pRb, and induction of $p21^{Cip1/WAF1}$ [83]. Combining cidofovir with irradiation both in vivo and in nude mice xenografts resulted in a marked radiosensitization of HPV-positive cells[83]. In another study where a naturally HPV-16 transformed HNSCC cell line was used, administration of cidofovir showed only a marginally reduced HPV-16 gene transcription and a modest

p53 restorative effect. In combination with irradiation the effect of cidofovir was enhanced[84]. These studies may provide the basis for a new anticancer strategy, in which cidofovir is used to enhance the antitumor effect of radiation therapy in HPV-related cancers. More studies will be required to evaluate its therapeutic potential and to optimize its clinical usefulness.

As a future perspective, targeted use of EGFR inhibitors in patients with high tumor EGFR expression could improve survival outcome of HNSCC patients. The combined use of specific monoclonal antibodies and tyrosine kinase inhibitors appears very promising in this respect[86,87]. Such monoclonal antibodies bind to the extracellular domain of EGFR, preventing ligand binding, aggregation or internalization of the EGFR, thereby interrupting the signaling cascade. Tyrosine kinase inhibitors bind to the intracellular domain of EGFR and inhibit the downstream effects of EGFR ligand binding. Blocking EGFR signaling has been shown to effectively reduce HNSCC aggressive cell phenotypes, to inhibit angiogenesis, and induce cancer cell death (apoptosis) in a subset of patients[86]. However, because EGFR overexpression appears to be inversely related to the presence of HPV in oropharyngeal carcinomas[48], EGFR-directed therapy might be of less value in the treatment of these HPV-positive HNSCC.

Another therapeutic target might be the insulin-like growth factor type 1 receptor (IGF-1R), since it was shown that the expression of this receptor is increased in HNSCC and that IGF-1R signaling significantly influences proliferation, motility and tumorigenicity[88].

Survivin, an inhibitor of apoptosis, has become of interest in cancer research since it is often upregulated in different types of malignant lesions[51]. Targeting survivin may provide a novel therapy and several clinical trials targeting survivin with immunotherapy and antagonists are under way, which might lead to a broadly applicable therapy[89].

Another new treatment modality mentioned in the literature is the use of resveratrol, a polyphenol with reported antiangiogenic effects found at high concentrations in grapes and red wine. This agent may be particularly effective in HPV-positive tumors since it appears that HPV 16 oncoproteins promote tumor angiogenesis. For example, an *in vitro* study has shown that resveratrol could abolish capillary and tubule formation initiated by the E6 and E7 oncoproteins in HPV 16 positive cervical cancer cells[90].

Finally, targeted inhibition of the E6 and E7 oncogenes in HPV-related cancers is a rational approach toward the development of additional cancer therapy. Evidence from *in vitro* studies has shown that inhibition of these viral oncogenes by gene therapy approaches, such as antisense RNA and small interfering RNA, results in loss of the transformed phenotype of the cells[91,92]. The loss of E6 and E7 resulted in massive apoptosis in HPV 16-related cancer cells by activating cellular p53, p21 and pRb[93]. These studies suggest that gene therapy against the viral oncogenes E6 and E7 could be advantageous in promoting better outcomes for patients with HPV-related cancers. Since there is no effective delivery mechanism for anti HPV gene therapy thus far, the challenge now is to turn these research findings into meaningful clinical applications.

Concluding remarks and future perspectives

We can conclude that HNSCC associated with high-risk HPV types show substantial differences in histology, risk factors, expression of cell cycle proteins and prognosis in comparison with HPV-negative tumors. This supports the view that HPV-positive HNSCC can be considered a different tumor entity with a favorable response to radio- and chemotherapy, which improves the prognoses of patients with HPV-positive tumors compared to those with HPV-negative tumors.

If the HPV vaccination therapy for prevention of cervical neoplasia would be applied to both uninfected boys and girls, the annual increase in the incidence of oropharyngeal cancers might be stopped in the near future. Studies concerning the therapeutic properties of HPV vaccines may lead to the development of additional therapeutic possibilities to eradicate tumor cells and/or dysplastic lesions as well as circulating micrometastases (minimal residual disease) still present after tumor resection. Screening of HNSCC, and particularly oropharyngeal and tonsillar cancers, for the presence of integrated HPV DNA and/or $p16^{INK4A}$ overexpression may help to optimize treatment protocols and to provide more accurate prognostic information.

In addition, studies should be started to elucidate questions with respect to: (i) the prevalence of high-risk HPV in, for example, the tonsils of healthy people; (ii) the transmission and natural course of HPV infection in the oropharynx; and (iii) the molecular mechanisms underlying the progression of HPV-associated HNSCC.

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Chapter 7

Summary

Chapter 1 reviews epidemiological aspects and risk factors of head and neck cancer. Head and neck cancer represents 6,5% of all annual cancer cases worldwide. This incidence is increasing in Western Europe and the US during the last decades. Besides the well known risk factors such as smoking, extensive alcohol use and betel quid chewing it has become clear that also oncogenic human papilloma viruses (HPVs) may play a role in the carcinogenesis of head and neck cancer. An overview of the HPVs is provided as well as an overview of the oncogenes that are expressed when the viral DNA is integrated in the host cell genome.

The overall aim of this thesis was to obtain more insight into the role of HPVs in the tumorigenesis of head and neck cancer, and into the clinical effects of HPV infection.

Oncogenic HPVs cause cancer by inactivation of the cell cycle regulators p53 and pRb by expression of viral oncoproteins E6 and E7. This means that p53 mutations are not a prerequisite in HPV-induced tumor development. However, a discrepancy existed in the literature with respect to the percentage of head and neck squamous cell carcinomas (HNSCC) harboring oncogenic HPV and the fraction of these tumors showing p53 mutations. The different results may be due to the detection method used, the anatomic location of tumors, the type of HPV detected and/or the number of tissue samples analyzed in the various studies. Because HNSCC are thought to arise via a multistep process with histologically distinct precursor phenotypes the relationship between the presence of HPV16/18 and p53 alterations in premalignant lesions of the head an neck mucosa, HNSCC and their metastases was examined. This study is presented in **Chapter 2**. For the detection of HPV the fluorescence in situ hybridization (FISH) protocol, combined with tyramide signal amplification was applied to paraffin-embedded tissue sections. Clinical data, as well as alcohol and tobacco intake were related to the presence of HPV in the tumors. It was found that HPV 16/18 DNA could not be detected in the premalignant lesions. Ten of the 47 HNSCC exhibited HPV 16-specific FISH signals and only in one case a simultaneous infection with HPV 18 was observed. Of these 10 HPV positive HNSCC, 9 were derived from the oropharynx, indicating a highly significant correlation between HPV positivity and localization in the oropharynx and especially in the tonsils. From the patients of which lymph node metastases were available for analysis, both the metastasis as well as the primary tumor displayed identical punctate nuclear HPV FISH signals, indicating stable integration of the virus prior to and during tumor

metastasis. Interestingly, 64% of the HNSCC showed accumulation of p53, including 8 of the 10 HPV positive carcinomas. In the HPV positive carcinomas with p53 overexpression, p53 mutations in exon 5-8 could not be detected, suggesting an overexpression of the wild-type p53. This study also showed a significant correlation between on the one hand low or absent exposure to tobacco and alcohol and HPV integration on the other.

Since it became known that oncogenic HPVs are of importance in the pathogenesis of HNSCC and especially oropharyngeal carcinomas, 81 tonsillar squamous cell carcinomas (TSCC) were examined for the presence of HPV 16, as described in **Chapter 3**. Integration of oncogenic HPV DNA into the human cellular genome is considered an important step in malignant transformation. After integration and disruption of (part of) the viral E2 gene, an upregulation of the oncoproteins E6 and E7 is detected. The HPV E6 protein can interact with the cellular wild-type p53 tumor suppressor protein, inducing p53 degradation via the ubiquitin-mediated pathway. The E7 protein inactivates pRb, resulting in release of the transcription factor E2F and upregulation of $p14^{ARF}$ and $p16^{INK4A}$. With respect to viral integration in oropharyngeal cancers however, the literature is controversial. In this study we used the FISH method for the detection of HPV 16. This technique allows the visualization of 1 copy of HPV DNA in tumor cells and at the same time the determination of the physical status (integrated versus episomal) of the virus on basis of the nuclear staining pattern. Furthermore, it was examined if $p16^{INK4A}$ overexpression as detected by immunohistochemistry correlates with HPV 16 positivity, in order to investigate whether or not this method can reliably distinguish between HPV-positive and HPV-negative TSCC. The results were correlated to clinical and demographic data. HPV 16 DNA integration was detected in 41% of the TSCC. All but one of the 33 HPV-positive tumors, and only 5 out of 48 HPV-negative tumors showed overexpression of $p16^{INK4A}$. This implicates that $p16^{INK4A}$ overexpression may be considered a reliable HPV biomarker. The presence of HPV furthermore correlated significantly with low tobacco and alcohol intake, poor differentiation grade, small tumor size, presence of a local metastases and a decreased (loco) regional recurrence rate. Statistical analysis showed that patients with HPV- positive tumors had a statistically better overall and disease specific survival rate. The 5 years disease-specific survival for patients with HPV-positive and HPV-negative carcinomas was 55% and 29%, respectively. Apart from absence of HPV 16, also lack

of $p16^{INK4A}$ overexpression, smoking, a combination of smoking and alcohol abuse, a tumor diameter of > 4 cm and development of recurrent disease were associated with a shorter disease-specific survival in patients with TSCC. Multivariate analysis of all data, however, revealed that tobacco smoking was the strongest prognostic indicator. Smokers had a 5.5 times higher chance of cancer death compared to non-smokers, and patients with HPV-negative TSCCs were found to exhibit a 2 times higher chance of cancer death compared to patients with HPV-positive tumors. These data indicate that oncogenic processes in the tonsils of non-smokers differ from those in smokers, the former being related to HPV 16 infection.

Since $p16^{INK4A}$ overexpression has been proposed as a potential biomarker for infection with HPV in squamous cell carcinomas it was investigated if $p16^{INK4A}$ overexpression could also predict the presence of HPV in tumor-free tonsillar tissue. For this purpose paraffin-embedded tumor-free tonsils of 262 patients were tested for $p16^{INK4A}$ overexpression by immunohistochemistry and HPV presence was tested by HPV-specific PCR analysis and FISH. In **Chapter 4** a detailed prescription of this study is given. $P16^{INK4A}$ overexpression was found in 28% of the tissue samples, whereas HPV DNA could not be detected by PCR and FISH analysis. Therefore it is concluded that $p16^{INK4A}$ overexpression is not a suitable marker to predict the presence of HPV in tumor-free tonsils. Other mechanisms than HPV infection seem therefore also to be implicated in upregulation of $p16^{INK4A}$.

In **Chapter 5** the expression of key cell cycle proteins in the pRb en p53 pathways was examined in a series of 77 TSCC, with the goal to determine their role in HPV-dependent and HPV-independent carcinogenesis. Furthermore, their prognostic value was evaluated by correlating the results with clinical follow-up data. Is was shown that HPV16-positive tumors exhibit $p14^{ARF}$ and $p21^{Cip1/WAF1}$ overexpression, next to downregulation of pRb and cyclin D1, which was not found in HPV16-negative tumors. Statistical analysis revealed a favorable survival rate for non-smokers, as well as for patients with T1-2 tumors or tumors showing low expression of cyclin D1, presence of HPV and overexpression of $p16^{INK4A}$, $p14^{ARF}$ or $p21^{Cip1/WAF1}$. In multivariate regression analyses smoking and tumor size, as well as expression of cyclin D1 and $p21^{Cip1/WAF1}$ were found to be independent prognostic markers. In particular $p21^{Cip1/WAF1}$ overexpression appeared to be the strongest indicator for a favorable outcome in patients with TSCC.

Finally, in **Chapter 6** an overall discussion of the results is presented and findings are related to the recent literature. On basis of the results described in this thesis a diagnostic sequence for routine practice is suggested and treatment protocols are proposed.

Chapter 7

Samenvatting

In **Hoofdstuk 1** wordt een overzicht gegeven van de epidemiologische aspecten van hoofd- halskanker en tevens wordt ingegaan op de risicofactoren die kunnen lijden tot het ontstaan van hoofd-halskanker. Hoofd-halskanker vertegenwoordigd 6,5% van alle nieuwe kankergevallen wereldwijd. Deze incidentie neemt de laatste jaren toe in West Europa en de Verenigde Staten. Naast de bekende risicofactoren zoals roken, overmatig alcoholgebruik en het kauwen van betelnoten is het duidelijk geworden dat ook oncogene humaan papilloma virussen (HPVs) een rol kunnen spelen bij het ontstaan van hoofd-halskanker. Er wordt een overzicht gegeven van het HPV, evenals van de oncogenen die tot expressie kunnen komen wanneer het virale DNA in het genoom van de gastheer integreert. Het doel van dit promotie-onderzoek was meer inzicht te verkrijgen in de rol die het HPV speelt bij het tot stand komen van hoofd-halskanker.

De oncogene HPVs veroorzaken kanker ten gevolge van inactivatie van de celcyclus regulerende eiwitten p53 en pRb door middel van de virale oncogene eiwitten E6 en E7. Dit betekent dat p53 mutaties niet noodzakelijk zijn bij HPV-geïnduceerde tumorontwikkeling. Met betrekking tot het percentage hoofd-halstumoren dat oncogeen HPV bevat en tegelijk mutaties heeft in het p53 eiwit bestaat echter onduidelijkheid in de literatuur. Deze onduidelijkheid wordt mogelijk veroorzaakt door het gebruik van verschillende detectie methoden, de anatomische locatie van de tumor, het type HPV en/of het aantal tumoren dat onderzocht werd in de verschillende onderzoeken. Aangezien de ontwikkeling van hoofd-halstumoren verloopt via meerdere stappen met histologisch te onderscheiden voorloper stadia werd getracht de relatie tussen de aanwezigheid van HPV type 16 en 18 enerzijds en p53 veranderingen anderzijds in voorloperleasies van de hoofd-hals mucosa, in hoofd- halscarcinomen en in hun metastasen vast te stellen. Dit onderzoek wordt beschreven in **Hoofdstuk 2**. Voor de detectie van HPV in paraffine coupes werd de fluorescente in situ hybridisatie (FISH) techniek gecombineerd met tyramide signaal versterking. De aanwezigheid van HPV in de tumoren werd gecorreleerd aan klinische gegevens en gegevens over het drink- en rookgedrag. In de premaligne laesies die werden onderzocht kon geen HPV 16/18 DNA gedetecteerd worden. In 10 van de 47 hoofd-halscarcinomen werd een HPV 16 specifiek FISH signaal gevonden en slechts in één geval werd tegelijkertijd een infectie met HPV 18 waargenomen. Van deze 10 HPV-positieve hoofd-halscarcinomen bleken er 9 afkomstig uit de oropharynx, hetgeen betekent dat er een duidelijk significante correlatie is tussen

HPV positiviteit en lokalisatie in de oropharynx en met name in de tonsillen. Bij de patiënten waarbij zowel de primaire tumor en de lymfeklier-metastase werd onderzocht, bleek dat zowel de tumor als de metastase hetzelfde nucleaire HPV-signaal toonden. Dit betekent dat het virus stabiel in het genoom is geïntegreerd alvorens de tumor zich verspreidt. In 64% van de hoofd- halscarcinomen, waaronder 8 van de 10 HPV-positieve carcinomen, werd een stapeling van p53 gevonden. In de HPV-positieve carcinomen met p53 overexpressie werden geen mutaties gevonden in exon 5-8 van p53, hetgeen suggereert dat er een overexpressie is van het wild-type p53. Gegevens uit dit onderzoek toonden eveneens een significante correlatie tussen HPV-integratie en een beperkt gebruik van alcohol en tabak.

Aangezien de oncogene HPVs een rol spelen bij het ontstaan van hoofd-halscarcinomen, met name in oropharyngeale carcinomen, werden 81 tonsilcarcinomen onderzocht op de aanwezigheid van HPV16. Dit onderzoek wordt beschreven in **Hoofdstuk 3**. Integratie van het oncogene HPV DNA in het menselijke cellulaire genoom wordt als een belangrijke stap in de maligne transformatie beschouwd. Na integratie en verstoring van (een gedeelte van) het virale E2 eiwit wordt er een verhoogde hoeveelheid van de oncogene eiwitten E6 en E7 gedetecteerd. Het HPV E6 eiwit kan een verbinding aangaan met p53, waardoor dit eiwit wordt afgebroken. Het E7 eiwit inactiveert het pRb, waardoor de transcriptie-factor E2F vrijkomt en er een verhoogde expressie van $p14^{ARF}$ en $p16^{INK4A}$ wordt gezien. Met betrekking tot de virale integratie bij oropharyngeale carcinomen bestaan tegenstrijdige berichten in de literatuur. In deze studie is de FISH-methode gebruikt voor het aantonen van HPV 16. Deze techniek maakt het mogelijk om slechts 1 kopie van het HPV DNA in de tumorcellen in beeld te brengen en tegelijkertijd is het mogelijk om te bepalen wat de fysieke toestand van het virus is (geïntegreerd ten opzichte van episomaal), dit op basis van het kleuringspatroon in de celkernen. Daarnaast werd onderzocht of $p16^{INK4A}$ overexpressie, zoals aangetoond met behulp van immunocytochemie, correleert met HPV 16 positiviteit, met als doel te onderzoeken of deze detectiemethode betrouwbaar is om een onderscheid te maken tussen HPV-positieve en HPV-negatieve tonsilcarcinomen. De resultaten werden gecorreleerd aan klinische en epidemiologische gegevens. HPV 16 integratie werd gedetecteerd in 41% van de tonsilcarcinomen. Alle behalve één van de 33 HPV-positieve tumoren en slechts 5 van de 48 HPV-negatieve tumoren toonden overexpressie van $p16^{INK4A}$. Dit impliceert dat $p16^{INK4A}$ overexpressie gezien kan worden als een betrouwbare

biomarker voor de aanwezigheid van HPV. Deze aanwezigheid van HPV correleerde verder significant met een laag alcohol- en tabakgebruik, een slechtere differentiatie, kleinere tumor diameter, de aanwezigheid van locale metastasen en een verlaagde kans op terugkeer van de tumoren. Statistische analyse van de gegevens toonde aan dat patiënten met HPV-positieve tumoren een betere ziekte-specifieke en totale overlevingskans hebben. De 5-jaars ziekte-specifieke overleving voor patiënten met HPV-positieve en HPV-negatieve carcinomen bedroeg respectievelijk 55% en 29%. Naast afwezigheid van HPV 16 bleek ook afwezigheid van $p16^{INK4A}$ overexpressie, roken, een combinatie van roken en alcohol gebruik, een tumor diameter van meer dan 4 cm en de ontwikkeling van terugkerende ziekte geassocieerd met een kortere ziekte-specifieke overleving bij patiënten met tonsilcarcinomen. Multivariantie analyse van alle gegevens toonde echter aan dat roken de belangrijkste prognostische factor was. Rokers hadden een 5,5 keer grotere kans om aan kanker te overlijden dan niet-rokers. Patiënten met HPV-negatieve tonsilcarcinomen bleken 2 keer zoveel kans te hebben om aan kanker te overlijden dan patiënten met HPV-positieve tonsiltumoren. Deze gegevens tonen aan dat de oncogene processen in de tonsil bij niet-rokers verschillen van het proces bij rokers, waarbij het proces bij de niet-rokers gecorreleerd is aan HPV 16 infecties.

Aangezien $p16^{INK4A}$ overexpressie gezien kon worden als marker voor de aanwezigheid van HPV in carcinomen, werd onderzocht of $p16^{INK4A}$ overexpressie ook de aanwezigheid van HPV in tumorvrij tonsilweefsel kon voorspellen. Met dit doel werden tumorvrije tonsillen onderzocht van 262 patiënten. De aanwezigheid van $p16^{INK4A}$ overexpressie werd bekeken met behulp van immunocytochemie. De aanwezigheid van HPV werd getest met behulp van HPV-specifieke PCR analyse en FISH. In **Hoofdstuk 4** wordt een gedetailleerde beschrijving van deze studie gegeven. $P16^{INK4A}$ overexpressie werd gevonden in 28% van de tumorvrije tonsillen en HPV kon met behulp van PCR analyse worden aangetoond in 2 tonsillen, terwijl FISH deze aanwezigheid van HPV niet kon bevestigen. Uit deze gegevens werd dan ook geconcludeerd dat $p16^{INK4A}$ overexpressie geen betrouwbare marker is om de aanwezigheid van HPV te voorspellen in tumorvrije tonsillen. Andere mechanismen dan HPV infecties moeten daarom verantwoordelijk zijn voor deze $p16^{INK4A}$ overexpressie.

In **Hoofdstuk 5** wordt het onderzoek beschreven naar de expressie van verschillende celcyclus-eiwitten in de pRB- en p53-routes van een serie van 77 tonsilcarcinomen.

Met dit onderzoek werd bepaald wat de rol is van de verschillende eiwitten in de HPV-onafhankelijke en HPV-afhankelijke ontwikkeling van carcinomen. Daarnaast werd de prognostische waarde van de verschillende eiwitten bepaald door de resultaten te correleren met de klinische gegevens. De HPV-16 positieve tumoren toonden een overexpressie van $p14^{ARF}$ en $p21^{Cip1/WAF1}$ en een verlaagde expressie van pRb en cycline D1, dit in tegenstelling tot HPV-16 negatieve tumoren. Statistische analyse toonde een betere overlevingskans voor niet-rokers en voor patiënten met T1-2 tumoren of tumoren met een lage expressie van cycline D1, de aanwezigheid van HPV en overexpressie van $p^{16INK4A}$, $p14^{ARF}$ of $p21^{Cip1/WAF1}$. Multivariante analyse toonde dat roken, tumor grootte, expressie van cycline D1 en $p21^{Cip1/WAF1}$ onafhankelijke prognostische factoren zijn. Met name $p21^{Cip1/WAF1}$ overexpressie bleek de belangrijkste voorspeller van een gunstige prognose bij patiënten met tonsilcarcinomen.

In **Hoofdstuk 6** worden tenslotte in een algehele discussie onze bevindingen gerelateerd aan tegenwoordige kennis uit de literatuur. Er wordt een diagnostisch protocol voorgesteld dat toegepast zou moeten worden in het routine onderzoek. Tevens worden verschillende behandelmogelijkheden beschreven. Daarnaast wordt aangegeven welke richting het toekomstig onderzoek in de komende jaren zou kunnen inslaan.

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Curriculum Vitae

Harriët Hafkamp, auteur van dit proefschrift, werd op 10 juni 1971 geboren te Hilversum. Met haar oudere zus Martine groeide zij op in het ouderlijk gezin te Kortenhoef. In 1989 behaalde zij haar V.W.O. diploma aan het Comenius College te Hilversum. Datzelfde jaar startte zij met de studie Biomedische Wetenschappen aan de Rijksuniversiteit Leiden. In 1991 startte zij tevens met de studie Geneeskunde eveneens aan de Rijksuniversiteit Leiden. De opleiding Biomedische Wetenschappen werd afgesloten met onderzoek naar de seroprevalentie van *Strongyloides Stercoralis* aan de Anton de Kom Universiteit van Paramaribo, Suriname onder begeleiding van Prof. Dr. A.M. Polderman en Prof. Dr. B.F.J. Oeseburg. In 1997 werden beide studies afgerond en begon zij als AGNIO te werken op de afdeling KNO van het Academisch Ziekenhuis Maastricht. In dit jaar begon zij met het onderzoek dat heeft geleid tot deze dissertatie. Van april 1998 tot april 2003 werd zij opgeleid tot Keel-, Neus-, en Oorarts in het Academisch Ziekenhuis Maastricht, onder leiding van Prof. Dr. J.J. Manni. De B-opleiding werd gevolgd in het Atrium Medisch Centrum te Heerlen, onder leiding van Dr. T.D. Zijlker. Na het afronden van de opleiding bleef zij werkzaam als staflid KNO in het Academisch Ziekenhuis Maastricht. Sinds september 2004 is de auteur toegetreden tot de KNO maatschap van het Reinier de Graaf gasthuis te Delft. De auteur woont samen met Roland Hage en samen hebben zij een prachtige zoon, Hugo (2007).

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List of abbreviations

ABC	avidin-biotinylated peroxidase complex
APOT	amplification of papillomavirus oncogene transcripts
Cdk	cyclin-dependent kinase
CI	confidence interval
CT	chemotherapy
DAPI	6-diamidino-2-phenyl indole
EDTA	ethylene diamine tetra-acetic acid
EGFR	epidermal growth factor receptor
FISH	fluorescence in situ hybridisation
HNSCC	head and neck squamous cell carcinoma
HPV	human papillomavirus
HR	hazard ratio
IGF-1R	insulin-like growth factor type 1 receptor
ISH	in situ hybridization
NA	not available
NS	not significant
OR	odds ratio
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RT	radiation therapy
SD	standard deviation
SSC	saline sodium citrate
SSCP	single-strand conformation polymorphism
TSA	tyramide signal amplification
TSCC	tonsil squamous cell carcinoma
UPT	unknown primary tumor

CHAPTER 2, FIGURE 1. (a–k) RESULTS OF HPV 16/18 FISH ANALYSIS AND (l–n) SSCP ANALYSIS OF CELL LINES (a–d), TISSUE SECTIONS (e–k) AND EXTRACTED GENOMIC DNA (l–n) FROM SQUAMOUS CELL CARCINOMAS OF THE CERVIX (a–d) AND HEAD AND NECK REGION (e–n). (a,b) Hela cell nuclei (blue) showing the expected 5 HPV 18 integration sites (punctate signals,red) as visualized by conventional (a) and TSA (b) probe detection. (c,d) SiHa cell nuclei (blue) showing the expected 1–2 HPV 16 integration sites (punctate signals, red) as visualized by conventional (c) and TSA (d) detection. (e–g) Example of HPV 16-positive tongue carcinoma (patient no. 1, Table 1) showing different tumor areas harboring nuclei (blue) with 1 integration site (e), 2 integration sites (f) and episomal appearance of the virus (g) (red punctate (e,f) and diffuse (g) signals, respectively). (h– k) Primary tonsillar carcinoma and corresponding metastasis of patient nos. 3 (h,i) and 4 (j,k) demonstrating clonal association of HPV 16 during tumor spread as 1 integration site (red) per nucleus (blue). (l–n) p53 exon 6 II PCR-SSCP results of 9 of the 10 HPV-positive HNSCC patients. (l) Lanes 1–9 correspond to patient nos. 9, 5, 4, 6, 8, 3, 1, 7 and 2 in Table 1, respectively. The arrow indicates an aberrantly migrating fragment in tumor 1. (m) Comparison of tumor and normal DNA from patient 1 showing the aberrant fragment in both tissue samples. (n) Identification of an exon 6 codon 213 polymorphism (arrow) after sequencing the exon 6 II PCR products of tumor 1. (see page 37)

CHAPTER 2, FIGURE 2. IMMUNOHISTOCHEMICAL STAINING FOR P53 (a,b), $P16^{INK4A}$ (c,e) AND PRB (d,f). (a,b) HPV-positive HNSCC no. 4 (a) and 1 (b) (Table 1) showing distinct nuclear staining for p53 protein in <30% and 100% of tumor cells. (c,d) HPV-positive tonsillar carcinoma no. 8 (Table 1) showing strong nuclear and cytoplasmic staining for $p16^{INK4A}$ (c) but only weak nuclear staining for pRb (d) in the tumor cells. (e,f) HPV-negative tonsillar carcinoma no. 15 showing no $p16^{INK4A}$ staining (e) but strong nuclear pRb staining in the tumor cells (f). (see page 38)

CHAPTER 3, FIGURE 1. REPRESENTATIVE EXAMPLES OF HPV 16 FISH ANALYSIS (A, B) AND P16^{INK4A} IMMUNOSTAINING (C, D) ON PARAFFIN-EMBEDDED TISSUE SECTIONS OF TSCC. (A) Example of one HPV 16-specific punctate signal per nucleus (red) indicating viral integration. Tumor cell nuclei are blue due to DAPI staining (B) Example of a tumor area showing diffuse nuclear staining (red) indicating viral replication (episomal virus copies), nuclei are DAPI counterstained. (C-D) Overexpression of p16^{INK4A} (brown peroxidase-diamino benzidine staining) in two HPV 16-positive TSCC, nuclei counterstained by hematoxylin (purpel). **(see page 59)**

CHAPTER 4, FIGURE 1. REPRESENTATIVE EXAMPLE OF P16^{INK4A} IMMUNOHISTOCHEMISTRY IN A TUMOR-FREE NORMAL TONSIL FROM A PATIENT WITH TONSILLITIS. (A) Tissue overview showing strong immunostaining in both the reticulated crypt epithelium as well as in the follicular dendritic cells in one germinal center, whereas the superficial squamous cell epithelium is negative. (B) and (D) Enlargements of Figure 1A, showing focal and strong diffuse cytoplasmic and nuclear p16^{INK4A} immunostaining in the crypt epithelium involving nonkeratinizing cell groups and associated lymphocytes. (C) and (E) Enlargements of Figure 1A, showing p16^{INK4A} accumulation in scattered cells within a germinal center of a lymphoid follicle. Nuclei were counterstained (purpel) with hematoxylin. (see page 80)

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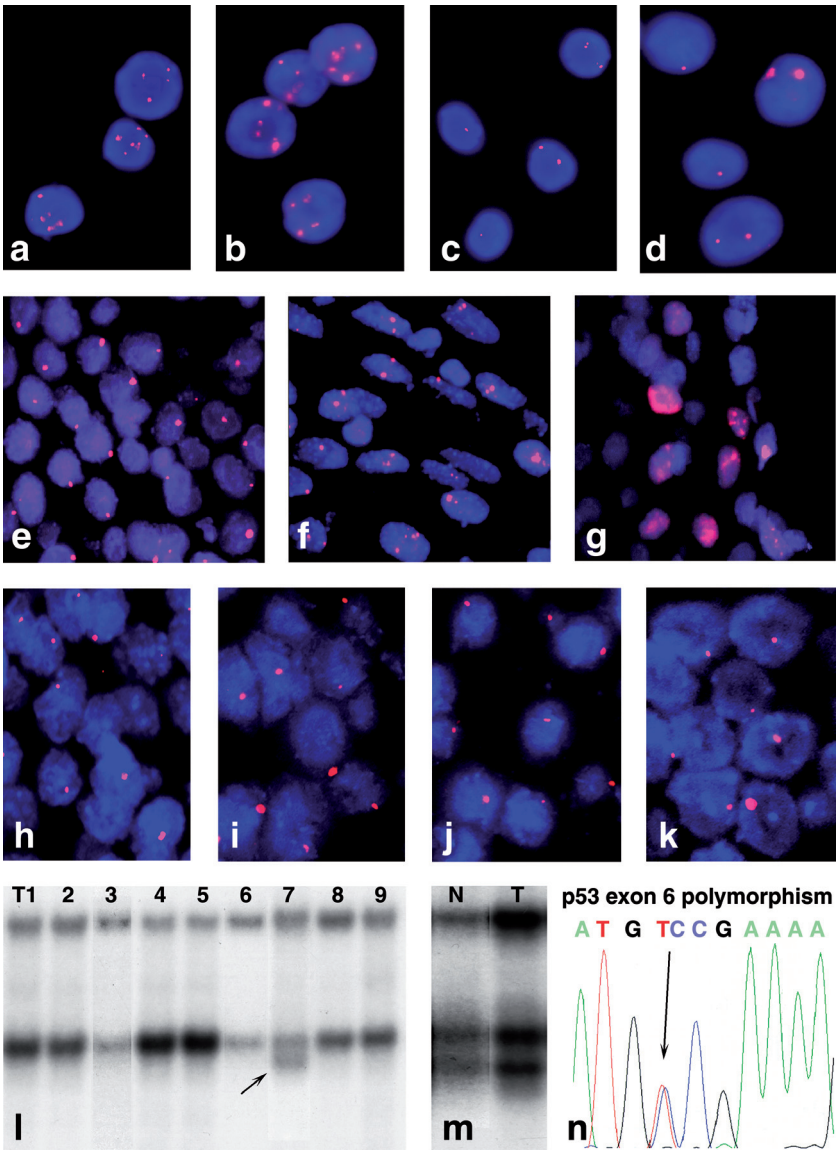
CHAPTER 5, FIGURE 1. REPRESENTATIVE EXAMPLES OF IMMUNOHISTOCHEMICAL STAINING ON TISSUE SECTIONS FOR (A) KI67, (B) $P16^{INK4A}$, (C) $P14^{ARF}$, (D, J) PRB, (E, I) CYCLIN D1, (F) $P21^{CIP1/WAF1}$, (G,H) P53, (K) MDM2 AND (L) $P27^{KIP1}$. Sections are from HPV-positive TSCC (A-G), HPV-negative TSCC (H-L) and adjacent squamous epithelium (A, B, D-G; lower image). Evaluation criteria as described in Materials and Methods section. (A) Strong nuclear Ki67 staining in tumor cells and parabasal epithelial cells; (B) Strong and diffuse $p16^{INK4A}$ staining in tumor cells, predominantly cytoplasmatic; epithelium negative; (C) Strong and diffuse nuclear $p14^{ARF}$ staining (higher image). Some tumors show a rather nucleolar-like immunostaining (lower image). (D) Weak/no nuclear pRb staining in tumor cells in comparison with adjacent normal squamous epithelium. (E) Weak/no nuclear cyclin D1 staining in tumors cells; parabasal epithelial cells weakly positive; (F) Strong nuclear $p21^{Cip1/WAF1}$ staining in tumor cells; some (para)basal epithelial cells weakly positive; (G) Low frequency of tumor cells positive for nuclear p53; some (para)basal epithelial cells weakly positive; (H) High frequency of tumor cells positive for nuclear p53; (I) High frequency of tumor cells positive for nuclear cyclin D1; (J) Strong nuclear pRb staining in tumor cells; (K) Low frequency of tumor cells showing strong and nuclear MDM2 staining; (L) Weak to strong nuclear immunostaining for $p27^{Kip1}$ in tumor cell areas as well as in adjacent lymphocyte areas. Magnification: 40x (A-C (higher image), D-I, K-L), 120x (J) and 200x (C (lower image)). (see page 100)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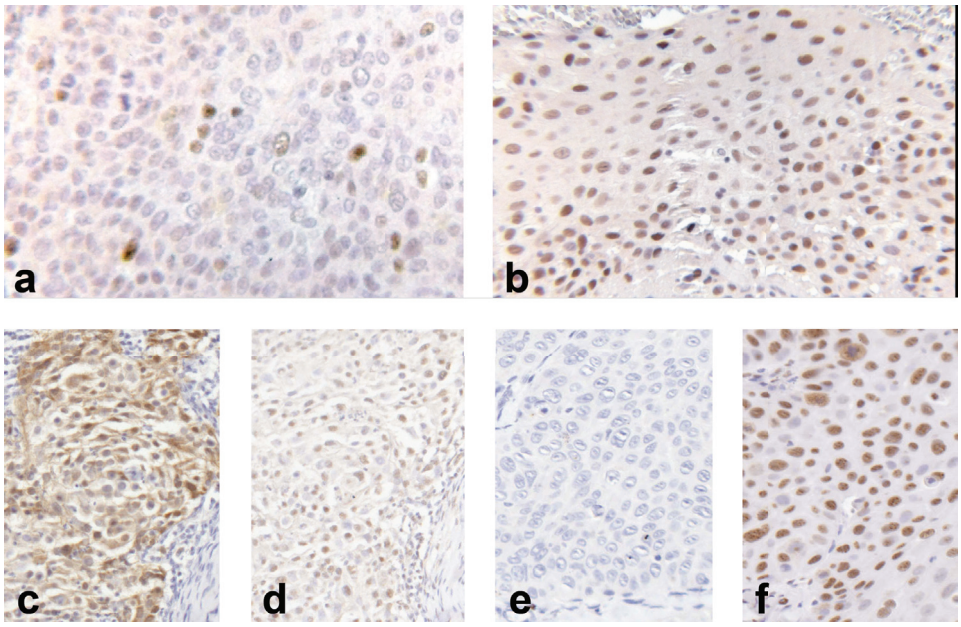
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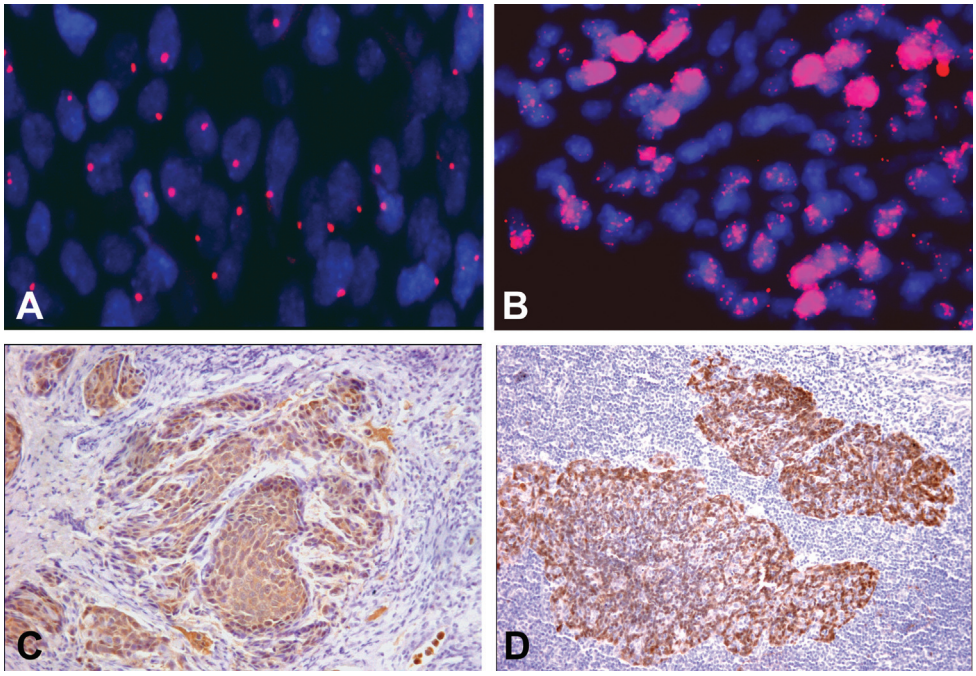
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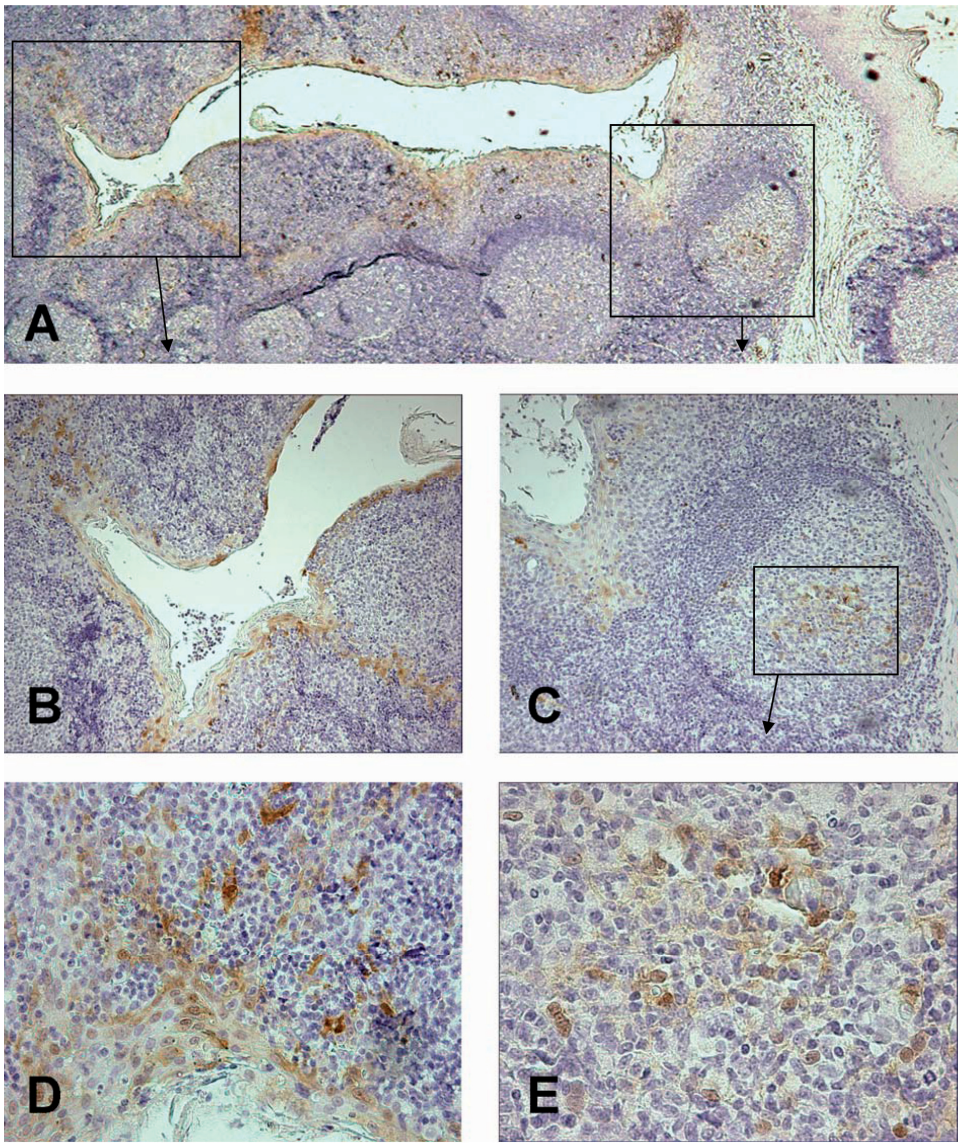
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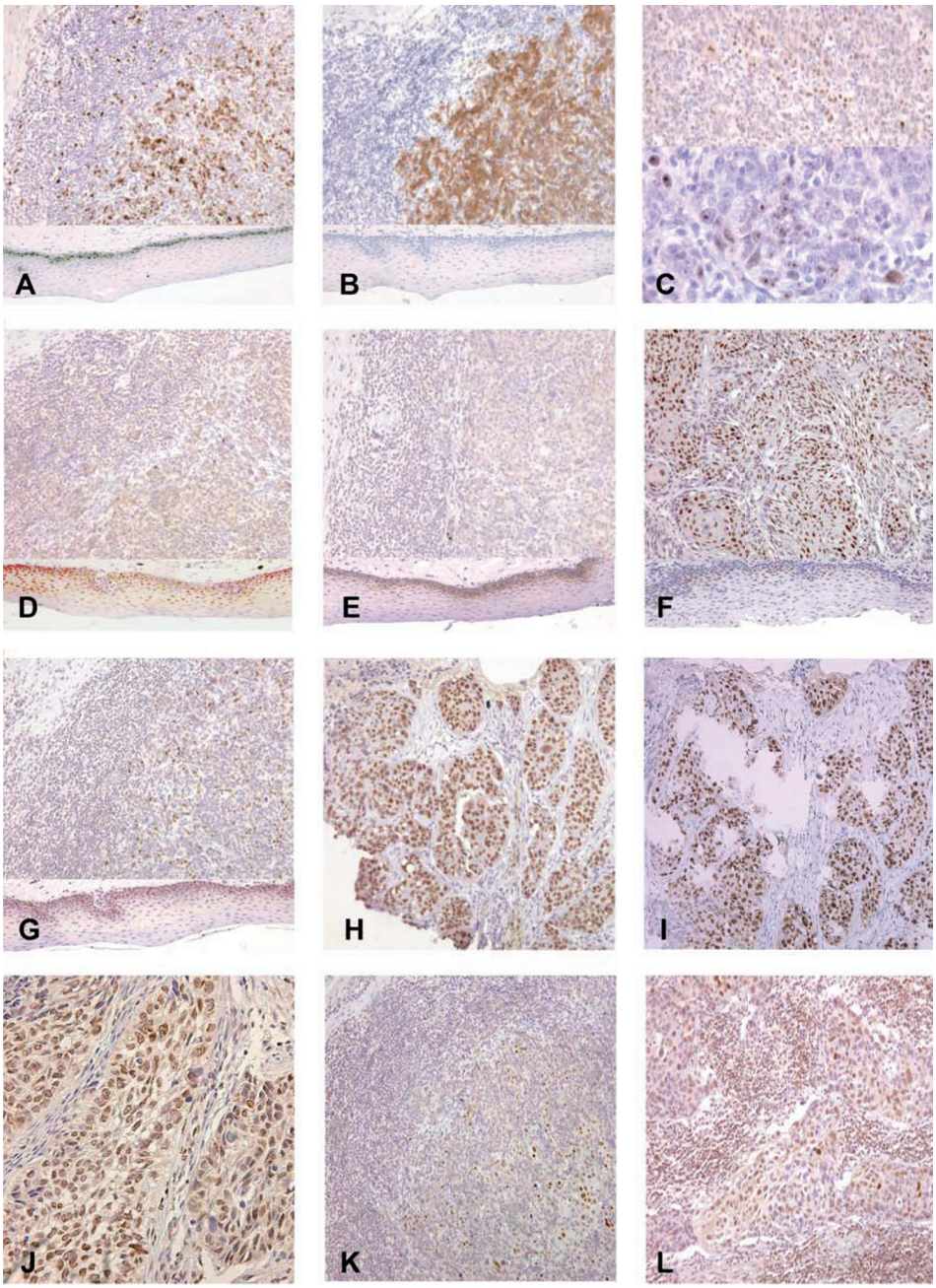
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