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

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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 metastasis ($p = 0.001$) 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.

Key words: human papillomavirus; $p16^{INK4A}$; tonsillar carcinomas; FISH; viral integration; survival analysis

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 ~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 ~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 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 oropharyngeal 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 immunohistochemical detection of $p16^{INK4A}$ overexpression, which has been postulated as a fast, easy and less expensive alternative for the detection of

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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 our 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.

Material 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 ($\geq$1 cigarette, pipe, and/or cigar per day) or nonsmokers (never smoker ($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 $\sim$10 g alcohol)] or non-drinkers (0–2 whiskey equivalents/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, pretreated with 85% formic acid/0.3% H_2O_2 for 20 min 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 min 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, St. Louis, MO) in 0.02 M HCl. The slides were rinsed 3 times in 0.01 M HCl, acidically dehydrated, air dried, postfixed in 1% formaldehyde in PBS for 15 min 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 50× excess of carrier DNA (salmon sperm DNA). 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, 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 4×SSC containing 5% nonfat dry milk), followed by a tyramide signal amplification (TSA) reaction using rhodamine-labeled tyramide.[18,21,37] Fifty microliter 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 min 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 (*i*) 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 (*ii*) 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 3 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 min to quench endogenous peroxidase activity. Antigen retrieval was performed by microwave heating in 0.01 M 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 3 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 $\geq$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 $\times$ 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] β-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 (5 μl) 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 I 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/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 I). 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 (Fig. 1*a*). 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 (Fig. 1*b*). Interestingly, 32 out of the 33 (97%) HPV-positive TSCC also exhibited strong nuclear and cytoplasmic staining for $p16^{INK4A}$ in the tumor areas harboring cells with nuclear HPV signals (Fig. 1*c* and 1*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 I).

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 I). 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 nonsmokers.

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 2 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

TABLE I – $P16^{INK4A}$ 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/day) or nonsmokers (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 nondrinkers (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]

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 *versus* 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 postoperatively, because of 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.

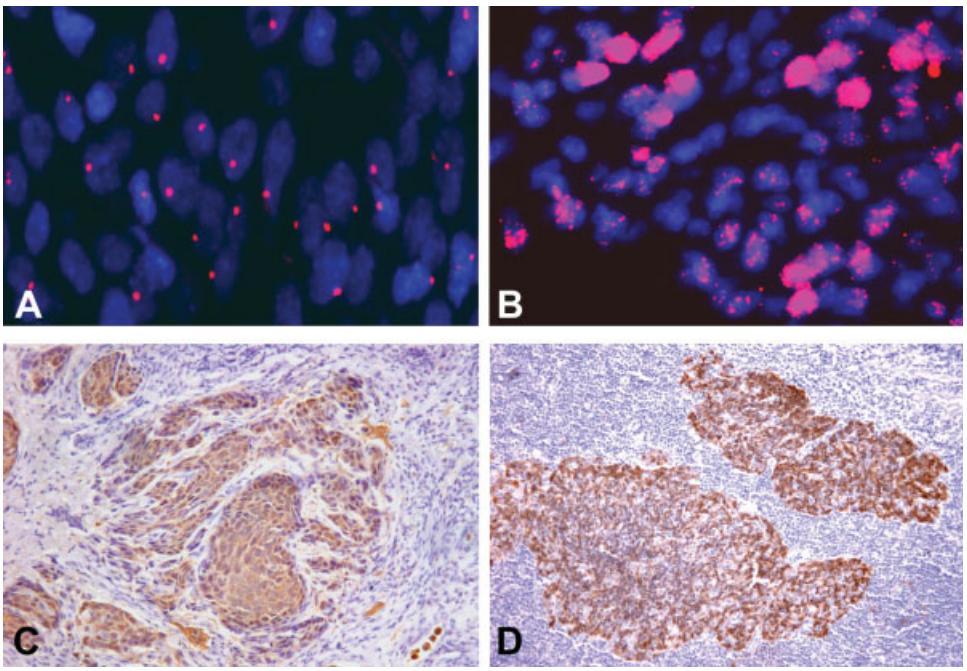

FIGURE 1 – Representative examples of HPV 16 FISH analysis (*a*, *b*) and $p16^{INK4A}$ immunostaining (*c*, *d*) on paraffin-embedded tissue sections of TSCC. (*a*) Example of one HPV 16-specific punctate signal per nucleus (red) indicating viral integration. Tumor cell nuclei are blue due to DAPI staining. (*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 2 HPV 16-positive TSCC, nuclei counterstained by hematoxylin (purple). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

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 Fig. 2*a*). 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 II). 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 2*a* and 2*b*).

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 Fig. 2*c*), a combination of smoking and alcohol abuse (HR = 2.8; 95% CI = 1–7.7), a tumor diameter of $\geq$4 cm (HR = 2.8; 95% CI = 1.5–5.3), and development of recurrent disease (HR = 15.2; 95% CI = 4.2–54.6) (Table II). Gender, age, complaints at time of diagnosis, alcohol intake, lymph node status and tumor grade were not significantly related to disease-specific survival. Because tobacco use turned out to be the strongest individual predictor for an unfavorable prognosis, we also analyzed the predictive value of the HPV 16 status for survival in smokers, and of tobacco use in the patients with HPV 16-positive TSCC. Interestingly, HPV 16 status had no significant effect on outcome in smokers (Fig. 2*d*). However, 10 of the 12 nonsmokers also harbored HPV 16, and these patients had a significantly more favorable disease-specific survival than smokers with HPV-positive TSCC (Fig. 2*e*). In addition, HPV-positive tumors of nonsmokers were significantly smaller and less well differentiated than those of smokers ($p = 0.026$ and 0.013, respectively; data not shown).

Using multivariate analysis (Table III), 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 nonsmokers. Patients with a tumor $\geq$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.

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

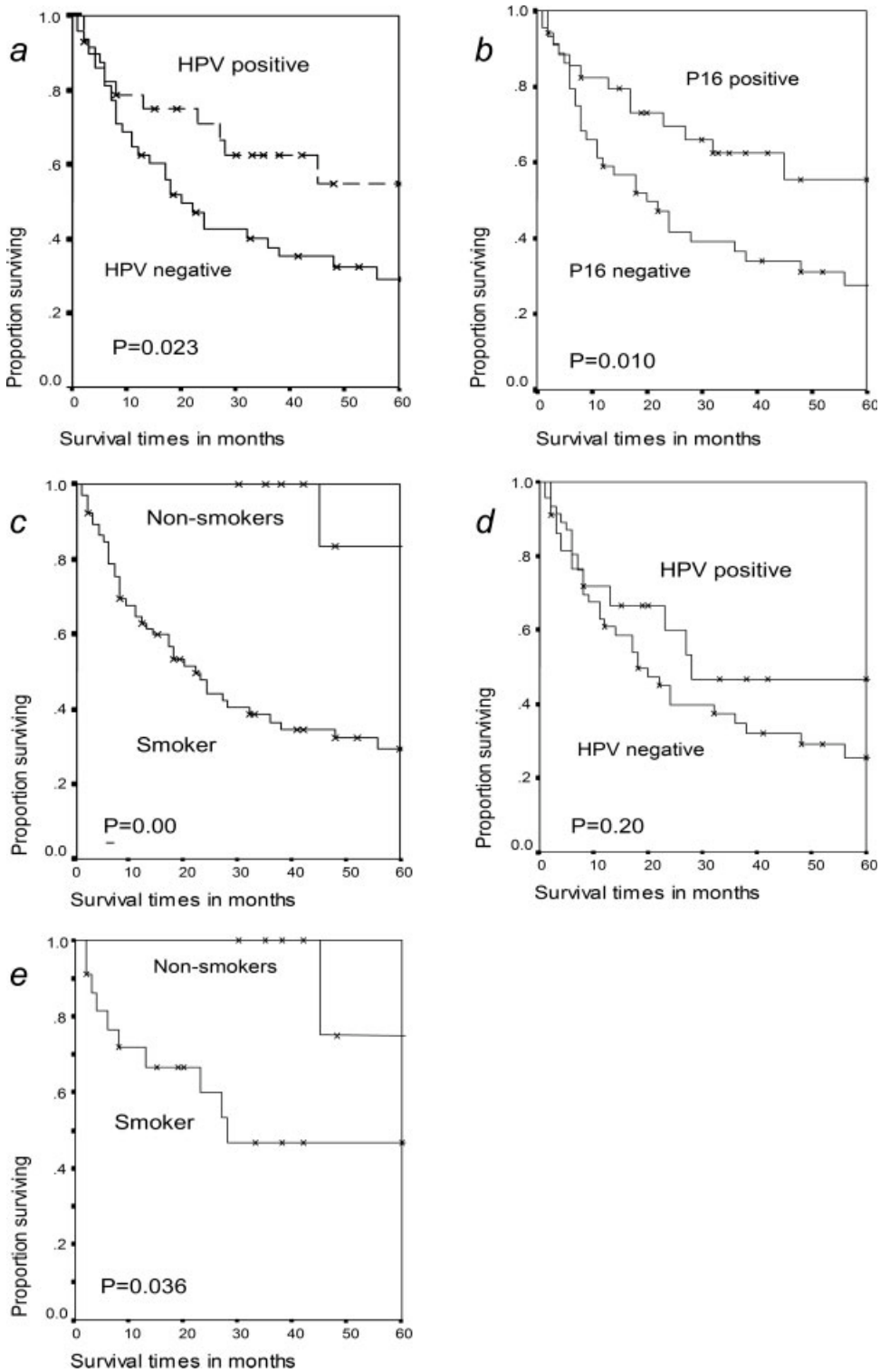

FIGURE 2 – Kaplan–Meier plots of disease-specific survival (in months after first diagnosis) of patients with TSCC. Stratification for (*a*) presence of integrated HPV 16 ($n = 79$); (*b*) $p16^{INK4A}$ accumulation ($n = 79$); (*c*) tobacco use ($n = 79$); (*d*) HPV status in smokers ($n = 67$); and (*e*) tobacco use in HPV-positive patients ($n = 33$). Censored values are noted with x.

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 2 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

TABLE II – 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)	NS	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 and 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 III – 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 *versus* positive	2.00	0.9–4.2	0.08
Smoking: yes *versus* no	5.53	1.3–23.6	0.02
Alcohol use: yes *versus* no	0.52	0.3–1.0	0.06
Tumor size: T3-4 *versus* T1-2	2.60	1.4–4.9	0.01

HPV, human papillomavirus; HR, hazard ratio; CI, confidence interval.

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 oropha-

ryngeal 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.

Nonsmokers 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 (*i*) HPV 16 integration is present in ~40% of TSCC, (*ii*) $p16^{INK4A}$ immunostaining is significantly associated with HPV-positive TSCC and may function as a surrogate marker for HPV detection, (*iii*) 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 (*iv*) 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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