A SUBSET OF HEAD AND NECK SQUAMOUS CELL CARCINOMAS EXHIBITS INTEGRATION OF HPV 16/18 DNA AND OVEREXPRESSION OF p16INK4A AND p53 IN THE ABSENCE OF MUTATIONS IN p53 EXONS 5–8


1Research Institute GROW, Department of Otorhinolaryngology and Head and Neck Surgery, University Hospital Maastricht, Maastricht, The Netherlands
2Research Institute GROW, Department of Molecular Cell Biology, University of Maastricht, Maastricht, The Netherlands
3Research Institute GROW, Department of Pathology, University Hospital Maastricht, Maastricht, The Netherlands
4Department of Pathology, Josephine Nefkens Institute, Erasmus University Medical Center, Rotterdam, The Netherlands

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 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, 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 been concentrated on the role of HPV in the etiology of uterine cervical cancer.12 Particularly the high-risk oncogenic HPV, 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 E7.12 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 cancers10,12 but also in half of the HNSCC.8 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 spe-
specific genetic alterations, 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 hybridization (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 but also allows for the distinction between the integrated or replicative (episomal) state of HPV 16/18 on a basis of a punctate or a diffuse hybridization signal, respectively. 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 hyberplasia, 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.

A series of 4-μm-thick sections were cut from the specimens for (i) hematoxylin-eosin staining and detailed histopathologic classification (F.J.B.), including determination of the absence or presence of basaloid features; (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. Formaldehyde-fixed, paraffin-embedded sections were deparaffinized, pretreated with 85% formic acid/0.3% H\(_2\)O\(_2\) 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 post-fixed in 1% formaldehyde in PBS for 15 min at room temperature and dehydrated in an ascending ethanol series. The digoxigenin-labeled 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. In short, 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 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\(_2\)O\(_2\) 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 (CaSkii [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. 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., i.e., punctate 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. Briefly, sections were deparaffinized and subsequently pretreated with 0.3% H\(_2\)O\(_2\) 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\(_2\)O\(_2\). 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. Normal expression levels of p16\(^{INK4A}\) in squamous epithelium are under...
the detection limit of immunohistochemistry. Strong nuclear and
cytoplasmic p16INK4A 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, dCTP
and dTTP each, 0.8 μM θ1.2-FdATP (Amersham, Uckington,
England), 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
non-denaturing 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 I) was excised from an addition-
ally prepared Sybr Green-stained SSCP polyacrylamide gel, ream-
plified, 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 χ² test. A significance level of p ≤ 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 proce-
dures 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 pres-

---

**TABLE I – CLINICOPATHOLOGIC DATA, HPV 16/18 AND p16INK4A, pRb and p53 STATUS IN 16 OROPHARYNGEAL CARCINOMAS AND 1 TONGUE CARCINOMA**

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age at diagnosis</th>
<th>Gender</th>
<th>Smoking behaviour1</th>
<th>Alcohol consumption2</th>
<th>Tumor site</th>
<th>Tumor classification3</th>
<th>Nuclear HPV FISH pattern4</th>
<th>Immunohistochemistry5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>T N M</td>
<td>punctate spot no. Diffuse</td>
<td>p53 p16INK4A pRb</td>
</tr>
<tr>
<td>HPV positive</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>46</td>
<td>Male</td>
<td>25</td>
<td>3U</td>
<td>Tongue</td>
<td>3 0 0</td>
<td>HPV 16 1 and &gt;1 HPV 16</td>
<td>Pos Pos 3+</td>
</tr>
<tr>
<td>2</td>
<td>54</td>
<td>Female</td>
<td>0</td>
<td>0</td>
<td>Base of tongue</td>
<td>4 3 0</td>
<td>HPV 16 2 and &gt;1 HPV 16</td>
<td>Pos Pos n.a.</td>
</tr>
<tr>
<td>3</td>
<td>48</td>
<td>Male</td>
<td>0</td>
<td>0</td>
<td>Tonsil + LN</td>
<td>2 1 0</td>
<td>HPV 16 1 and &gt;1 HPV 16</td>
<td>Neg Pos ±</td>
</tr>
<tr>
<td>4</td>
<td>64</td>
<td>Female</td>
<td>0</td>
<td>0</td>
<td>Tonsil + LN</td>
<td>2 2b 0</td>
<td>HPV 16 1 and &gt;1 HPV 16</td>
<td>Neg/pos Pos 2+</td>
</tr>
<tr>
<td>5</td>
<td>71</td>
<td>Male</td>
<td>0</td>
<td>0</td>
<td>Tonsil</td>
<td>2 2b 0</td>
<td>HPV 16 1 and &gt;1 HPV 16</td>
<td>Pos Pos ±</td>
</tr>
<tr>
<td>6</td>
<td>54</td>
<td>Female</td>
<td>0</td>
<td>0</td>
<td>Tonsil R</td>
<td>1 0 0</td>
<td>HPV 16 1 and &gt;1 HPV 16</td>
<td>Pos Pos +</td>
</tr>
<tr>
<td>7</td>
<td>56</td>
<td>Female</td>
<td>0</td>
<td>0</td>
<td>Tonsil</td>
<td>2 2b 0</td>
<td>HPV 16 1 and &gt;1 HPV 16</td>
<td>Pos Pos 2+</td>
</tr>
<tr>
<td>8</td>
<td>54</td>
<td>Male</td>
<td>0</td>
<td>0</td>
<td>Tonsil</td>
<td>2 2b 0</td>
<td>HPV 16 1 and &gt;1 HPV 16</td>
<td>Pos Pos ±</td>
</tr>
<tr>
<td>9</td>
<td>69</td>
<td>Male</td>
<td>0</td>
<td>0</td>
<td>Tonsil</td>
<td>3 1 0</td>
<td>HPV 16 1 and &gt;1 HPV 16</td>
<td>Pos Pos ±</td>
</tr>
<tr>
<td>10</td>
<td>54</td>
<td>Male</td>
<td>33</td>
<td>7U</td>
<td>Tonsil</td>
<td>4 2b 0</td>
<td>HPV 16 1 and &gt;1 HPV 16</td>
<td>Pos Pos ±</td>
</tr>
<tr>
<td>HPV negative</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>84</td>
<td>Male</td>
<td>20</td>
<td>0</td>
<td>Base of tongue</td>
<td>3 2b 0</td>
<td>HPV 16 1 and &gt;1 HPV 16</td>
<td>Pos Neg Neg</td>
</tr>
<tr>
<td>12</td>
<td>58</td>
<td>Female</td>
<td>15</td>
<td>15U</td>
<td>Base of tongue</td>
<td>1 2b 0</td>
<td>HPV 16 1 and &gt;1 HPV 16</td>
<td>Pos Neg +</td>
</tr>
<tr>
<td>13</td>
<td>49</td>
<td>Male</td>
<td>0</td>
<td>0</td>
<td>Base of tongue</td>
<td>3 2c 0</td>
<td>HPV 16 1 and &gt;1 HPV 16</td>
<td>Pos Neg Neg</td>
</tr>
<tr>
<td>14</td>
<td>47</td>
<td>Male</td>
<td>10</td>
<td>15U</td>
<td>Tonsil</td>
<td>2 2b 0</td>
<td>HPV 16 1 and &gt;1 HPV 16</td>
<td>Pos Neg 2+</td>
</tr>
<tr>
<td>15</td>
<td>49</td>
<td>Male</td>
<td>48</td>
<td>15U</td>
<td>Tonsil</td>
<td>4 3 0</td>
<td>HPV 16 1 and &gt;1 HPV 16</td>
<td>Pos Neg 3+</td>
</tr>
<tr>
<td>16</td>
<td>51</td>
<td>Male</td>
<td>16</td>
<td>3U</td>
<td>Tonsil</td>
<td>3 2b 0</td>
<td>HPV 16 1 and &gt;1 HPV 16</td>
<td>Neg Neg 2+</td>
</tr>
<tr>
<td>17</td>
<td>61</td>
<td>Male</td>
<td>25</td>
<td>4U</td>
<td>Tonsil</td>
<td>2 2b 0</td>
<td>HPV 16 1 and &gt;1 HPV 16</td>
<td>Neg Neg +</td>
</tr>
</tbody>
</table>

1The number of cigarettes smoked per day.–2The units of alcohol beverages consumed per day (1 unit = 12 g alcohol).–3Classification
according to the pTNM classification system.27–4FISH 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).–5Protein staining in tumor cells was
scored as follows: p16INK4A: 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.–Former smoker: stopped smoking more than 10 years ago.–Prominently in the right tonsil.–Prominently in the left tonsil.
HPV INTEGRATION IN HNSCC

TABLE II - HPV 16 AND p53 STATUS ACCORDING TO PRIMARY TUMOR SITE

<table>
<thead>
<tr>
<th></th>
<th>p53 +1</th>
<th>p53 -</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HPV +</td>
<td>HPV -</td>
</tr>
<tr>
<td>Oral cavity (n = 19)</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Oropharynx (n = 16)</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Hypopharynx (n = 5)</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Larynx (n = 7)</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Total (n = 47)</td>
<td>8</td>
<td>22</td>
</tr>
</tbody>
</table>

*p53 accumulation in ≥30% of tumor cell nuclei.

concomitant replication of the virus in specific areas of the tumor (Fig. 1g). Seven of the 10 HPV-positive tumors showed 1 HPV integration site per nucleus (Fig. 1e), 2 tumors harbored areas with more than 1 integration site per nucleus (Fig. 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 no. 2, Table I). Only in 2 of the 10 HPV-positive tumors did all tumor cells contain the viral DNA (tumor nos. 1 and 3, Table I), 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 nos. 3 and 4, Table I and Fig. 1a–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 I and II, and Fig. 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 (Fig. 1f). Only the analysis of tumor DNA from patient no. 1 (Table I) 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 (Fig. 1g). Thus, in our study no p53 mutations could be identified in HPV-positive HNSSC, despite the fact that these tumors often accumulate wild-type p53.

Correlation between HPV status, clinicopathologic parameters and risk factors

To assess the level of pRb and p16INK4A expression in HNSCC associated with HPV, immunohistochemical staining was performed on tissue sections of the 17 tumors listed in Table I. Representative examples are presented in Figure 2c–f. A remarkable correlation was found between p16INK4A overexpression and the presence of HPV because all 10 HPV-positive HNSSC exhibited strong nuclear and cytoplasmic staining in the tumor areas harboring cells with integrated HPV (Fig. 2e), while the HPV-negative cancers were also negative for p16INK4A (Fig. 2f). 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%; Fig. 2f). Accordingly, in 63% of carcinomas pRb expression was reduced or negative, including 6 of 9 HPV-positive cases (Table I and Fig. 2f). These data are in agreement with the results of Wiest et al.32 and demonstrate that besides a wild-type p53 status HPV-positive HNSSC are characterized by p16INK4A overexpression, as well as reduced pRb levels in most cases.

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
and/or alcohol consumption.\textsuperscript{8} Especially the transition has caused confusion with respect to the frequency of HPV-positive tonsillar carcinoma no. 15 showing no p16\textsuperscript{INK4A} staining (e) but strong nuclear pRb staining in the tumor cells (f).

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 basoloid characteristics (i.e., case nos. 3, 7, 8 and 9 in Table I). 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.

\section*{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,\textsuperscript{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.\textsuperscript{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\textsuperscript{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,\textsuperscript{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\%.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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\textsuperscript{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 harbored 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.\textsuperscript{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.\textsuperscript{15,25} If this hypothesis also holds for head and neck mucosal lesions, one would expect predominantly diffuse HPV staining patterns in premalignant 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,\textsuperscript{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,\textsuperscript{9,39} further epidemiologic studies should determine HPV prevalence in tonsils of the healthy population in relation to sexual behavior.\textsuperscript{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;\textsuperscript{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 intense antigenic stimulation;\textsuperscript{36,42} (iii) the presence of cytokines produced by lymphoid tissue may affect HPV transcription and cellular transformation.\textsuperscript{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.9,10,13–16. 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%.9,9,10,18,21,32,44 In 2 of these studies,9,32 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 p16INK4A 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 a pathologic 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 p16INK4A 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;18 (ii) upregulation of the wild-type p53 protein by genotoxic insults, e.g., ultraviolet radiation or hypoxia;46 (iii) nonmutational p53 stabilization by mdm2 and/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, 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,9 tumor size and gender could neither be substantiated in the current study, nor by earlier investigations.5,36

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 p16INK4A 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.

ACKNOWLEDGEMENTS

The authors thank Ms. S.M.H. Claessen and Ms. M. Schepers (Department of Molecular Cell Biology, University of Maastricht) and Mr. H.F. Sleddens (Department of Pathology, Erasmus University Medical Center Rotterdam) for outstanding technical support.

REFERENCES

16. Münger K, Schellfr M, Hultbrecht JM, Howley PM. Interactions of
HPV E6 and E7 oncoproteins with tumor suppressor gene products.


