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Detection of Human Papillomavirus DNA and Oncoprotein Overexpression Are Associated with Distinct Morphological Patterns of Tonsillar Squamous Cell Carcinoma

Sharon P. Wilczynski,* Bryan T.-Y. Lin,* Yuan Xie,* and I. Benjamin Paz†

From the Division of Pathology and Surgery,† City of Hope National Medical Center, Duarte, California*

Human papillomavirus (HPV) DNA has been detected in approximately 15% of squamous cell carcinomas (SCCs) of the head and neck. Recent studies have shown a predilection of HPV for certain anatomical sites, especially the tonsillar region, with viral DNA identified in approximately 60% of SCCs of the Waldeyer's tonsillar ring. This study was undertaken to determine whether there are differences in morphology or in oncogene expression in SCC of the tonsil with and without detectable HPV DNA. Twenty-two SCCs of the tonsil were analyzed for the presence of HPV DNA by polymerase chain reaction (PCR) using both a consensus primer set (My09/My11) and type-specific primers. Viral transcription was established in both primary and metastatic tumors by RNA *in situ* hybridization. The morphology of invasive SCC was classified into three subtypes: well keratinized (K-SCC), intermediate keratinized (I-SCC), and poorly keratinized (P-SCC). Expression of p53, pRB, and cyclin D1 (bcl-1) were studied by immunohistochemistry. In these cases (6 K-SCCs, 2 I-SCCs, and 14 P-SCCs), HPV DNA was detected in 14 (64%), with 11 containing HPV-16 (10 P-SCCs, 1 I-SCCs, and 0 K-SCCs) and 1 each containing HPV-33, HPV-59, and an unclassified HPV type (all P-SCCs). Viral oncoprotein E6/E7 transcription was demonstrated in 7 of 7 HPV-16-positive tumors. Cyclin D1 protein overexpression was detected in the majority of HPV-negative tumors (7 of 8 cases), whereas it was minimal or absent in 13 HPV-positive tumors. Overexpression of p53 protein was detected in 3 HPV-negative K-SCCs. In the HPV-positive tumors, fewer malignant cells expressed pRB and the staining was less intense than in the HPV-negative cancers. HPV DNA and E6/E7 expression, especially HPV-16, is detected in the majority of tonsillar SCCs and is almost exclusively associated with a poorly keratinized tumor histology. Decreased expression of cyclin D1, pRB, and p53 in tumors with HPV DNA is consistent with the known effects of the viral oncoproteins on the cellular protein. The morphology of the HPV-positive tumors suggests that HPV may have a predilection for a population of nonkeratinizing squamous cells or that the virally transformed cells inhibit keratinization of the tumor cells. Well keratinized tonsillar SCCs lack HPV DNA and are associated with overexpression of cyclin D1 protein and/or p53, suggesting that mechanisms that alter the cell cycle regulatory proteins, either by interaction with viral oncoproteins or by changes in the cellular proteins themselves, is critical for tumorigenesis of tonsillar SCC. *(Am J Pathol 1998, 152:145–156)*

Human papillomaviruses (HPVs) are a remarkably diverse group of small double-stranded DNA viruses with 77 types completely cloned and characterized and at least 30 more types partially described.[1–3] A role for HPV in human malignancies has been firmly established in the last 15 years. One of the first tumors found to be associated with these viruses was squamous cell carcinoma (SCC) of the skin that develops in over one-half the patients with epidermodysplasia verruciformis.[4,5] An association of HPV with more common human malignancies was established by demonstrating HPV DNA in over 90% of uterine cervix cancers and also with high frequency in cancers of the vulvar, penis, and anus.[1,6–9]

In the upper aerodigestive tract, HPV is the etiological agent of laryngeal papillomas, but its association with SCCs of the head and neck is more controversial as the presence of HPV DNA in the malignant tumors has been reported over a wide range (2 to 76%; reviewed in Refs. 10 and 11). This variation in detection may depend on the

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Address reprint requests to Dr. Sharon P. Wilczynski, Department of Pathology, City of Hope National Medical Center, 1500 East Duarte Road, Duarte, CA 91010. E-mail: swilczynski@smtplink.coh.org.

type of assay used (Southern blot hybridization, polymerase chain reaction (PCR), or *in situ* hybridization) or the source of tumor materials analyzed (paraffin-embedded tissue or frozen tissue).[10,11] In addition, the etiology of these tumors in various geographic and ethnic populations may be different, with studies from Asia frequently showing a higher detection rate at some sites such as the esophagus than those from the United States and Europe.[12–15] However, a strong association has been found between HPV and tonsillar cancers in several series of head and neck tumors from Western nations,[16–18] including a recent report from our laboratory.[18] In our series of 167 SCCs of the esophagus and head and neck, we found 15% of the tumors positive for HPV DNA, but the frequency varied markedly with the primary anatomical site of the tumor. Tonsil cancers had the highest incidence of HPV DNA with 60% of the tumors positive (9 of 15 tumors).[18] As in the genital tract, HPV-16 appears to be the most common genotype identified in malignant tumors in the head and neck region.[11]

The molecular mechanisms underlying head and neck cancers is not clear. Like most malignancies arising in adults, multiple genetic changes are probably involved in their development. The two most consistent alterations identified in head and neck SCC are mutations of the tumor suppressor gene p53 and overexpression of cyclin D1 (bcl-1).[19–22] P53 protein is induced after exposure to DNA-damaging agents and encodes a transcription factor that activates genes that arrest growth of the cells in G1 so that the damaged DNA can be repaired before DNA is replicated in S phase. If the damage is not repaired, p53 may induce apoptosis, or programmed cell death.[23] Cyclin D1 and its interaction with cyclin-dependent kinases, regulates the phosphorylation state of pRB, which in its hyperphosphorylated state, releases E2F, a transcription factor that induces genes necessary for the transition from G1 to S phase.[24] Both of these critical regulatory points in the cell cycle are altered by interaction with oncoproteins of the tumorigenic DNA viruses, including SV40, adenoviruses, and papillomaviruses.[25–27] HPV E7 oncoprotein has a sequence motif that is similar to the pRB binding site of cyclin D1,[27] suggesting that the viral protein substitutes for cyclin D1 in the transition from G1 to S phase. The other oncoprotein of HPV, E6, interacts with p53 and increases its degradation through the ubiquitin-mediated pathway.[26] Presumably, without a functional p53, the HPV-infected cells cannot turn off cell division in the presence of DNA damage, leading to genomic instability and an accumulation of genetic changes that contribute to malignant progression.

This study was undertaken to determine whether head and neck cancers with HPV DNA can be distinguished from tumors that presumably are transformed by other mechanisms, by either the histological features of the tumors or by differences in expression of cellular proteins that interact with the viral oncoproteins. We found that in tonsil SCC the morphology and the expression of cyclin D1, p53, and pRB segregated with the HPV status.

Materials and Methods

Case Selection

Patients with a diagnosis of SCC of the tonsil and treated at the City of Hope National Medical Center, Duarte, CA, between 1982 and 1997 were identified through the surgical pathology files and tumor registry. The charts were reviewed by one author (I. B. Paz) to confirm that the tumor originated in the tonsil. Fifteen of these patients were included in a previous report.[18] Six cases of nonneoplastic tonsils were used as controls for HPV studies and immunohistochemical stains. All patients and controls were from the Southern California region at the time of diagnosis and surgical treatment.

Clinical Data and Statistical Analysis

Clinical information was collected by chart review, and follow-up included age at diagnosis, sex, clinical stage, treatment, tumor recurrence, and survival.

Histological Features

The histopathological features of the tumors were evaluated by light microscopy by two of the authors (B. T.-Y. Lin and S. P. Wilczynski), independently and without knowledge of viral results. Discrepancies were resolved over a two-headed scope. All hematoxylin and eosin (H&E) slide material available for each case was reviewed (1 to 54 slides). Nuclear grade was assigned a value from I to III based on pleomorphism, the presence and size of nucleoli, and chromatin character. The nucleus to cytoplasmic ratio was evaluated by comparing the abundance of cytoplasm to nuclear size and was recorded as low, moderate, or high. The borders between individual tumor cells were described as distinct, less distinct, syncytial, or mixed. The tumor/stroma interface was recorded as blunt, infiltrative, or mixed. The mitotic index is based on the average of mitotic counts in 10 high-power fields (HPF; magnification, ×400) in the most mitotically active area identified: 1, <5/10 HPF; 2, 5 to 20/10 HPF; 3, >20/10 HPF. The following individual features were graded from 1–3: the presence of cytoplasmic bridges, the amount of individual cell keratinization, the extent of extracellular keratin, the presence of tumor necrosis, the frequency of single-cell apoptosis, and the degree of inflammation. The presence of perinuclear clearing and lymphovascular space involvement were also noted.

HPV Analysis

For 16 cases, fresh-frozen tumor tissue was available from the original biopsy or radical resection specimen for DNA extraction: 13 from the primary tumor, 1 from a lymph node metastasis, and 2 from neck recurrences. In six cases, only paraffin-embedded tissue was available for HPV analysis, including four primary tumors, a neck recurrence, and a lung metastasis. DNA extraction, PCR

primers used, HPV analysis, and assignment of type were as previously described.[18,28–30] Briefly, β-globin primers were used to confirm the presence of amplifiable DNA in the extracted specimens. The DNA extracted from fresh tissue was amplified with consensus primers to the L1 region MY09/MY11 as described by Ting and Manos,[29] and primers were modified for paraffin material as described.[30] PCR conditions and type-specific primers for HPV-16, -18, and -6 have been described.[18,28] DNA extracted from the fresh tissue was also tested for HPV with a second set of consensus primers (IU/IWDO) in the E1 gene.[31] The amplified PCR product was electrophoresed in 2% agarose gels, stained with ethidium bromide, visually detected, and transferred to nylon membrane (MagnaNT, MSI, Westborough, MA) and probed with ^{32}P-labeled HPV-6, -16, or -18 DNA as previously described.[18,28] Selected cases were also hybridized with probes for HPV-31, -33, -34, -35, -44, -45, and -56.

In situ hybridization with HPV-16 riboprobes was performed as previously described.[32,33] Briefly, 6-μm sections were deparaffinized in xylene and rehydrated through graded ethanol and air dried. After proteinase K digestion (30 minutes at 1 μg/ml; Boehringer Mannheim, Indianapolis, IN), the tissue was acetylated in 0.25% acetic acid anhydride and then dehydrated though graded ethanols. [^{35}S]UTP-labeled HPV-16 riboprobes encompassing the open reading frames of E6 and E7 genes in both the sense and antisense orientation were prepared from pBluescript plasmids and reduced to approximately 150 bp by alkaline hydrolysis. The sections were hybridized with the antisense or sense strand riboprobe for 16 hours at 45°C in hybridization solution (50% formamide, 10% dextran sulfate, 10 mmol/L Tris/HCl, pH 7.4, 2X SSC, 1 mmol/L EDTA, 500 g/ml *Escherichia coli* tRNA, and 1X Denhardt's solution). After hybridization, the slides were washed in 4X SSC for 20 minutes, incubated with RNAse A (10 μg/ml) at 37°C for 30 minutes followed by an additional 30-minute wash with 0.1X SSC at 55°C, and dehydrated through graded ethanol containing 300 mmol/L ammonium acetate, coated with emulsion (Kodak, New Haven, CT), and exposed for 2 weeks at 4°C. Developed slides were lightly counterstained with hematoxylin and photographed under darkfield illumination.

Immunohistochemical Studies

Immunostaining for cyclin D1, pRB, and p53 was performed according to standard procedures for heat-induced epitope retrieval as previously described.[34] P53 antibody (clone DO-7, Novocastra, Newcastle, UK) was used at a dilution of 1:100 with multitumor block containing colon adenocarcinomas as positive controls. Cyclin D1, clone DCS-6 immunostains were performed according to the specification of the manufacture (Novocastra) at a dilution of 1:20 for primary antibody. Mantle cell lymphoma from the tongue and intestine were used as positive controls for cyclin D1 staining. The anti-pRB clone 3C8 (QED Bioscience, San Diego, CA) was used at a dilution of 1:500. All tumors were evaluated for pRB expression in areas of the slide that showed positive endothelial cells as an internal control, and only nuclear staining was considered positive. Sections from a retinoblastoma served as the negative control. The immunostains were reviewed by two of the authors (B. T.-Y. Lin and S. P. Wilczynski) independently with discrepancies reconciled over a double-headed scope. The number of positive tumor cells were recorded as follows: none, rare (less than 1%), few (<10%), some (10 to 30%), many (30 to 75%), and most (>75%). Also recorded were the intensity of staining (1–4) and the distribution of positive cells as focal (clusters of tumor cells positive), scattered (isolated positive cells), or diffuse.

Results

Clinical Features

There were 16 males and 6 females, with a mean age of presentation of 51.9 (range, 35 to 73) years. The initial diagnosis of invasive SCC in all cases was made on biopsy. Most patients had advanced disease, with 16 of the 22 patients presenting at stage IV. Fifteen patients received a radical neck lymph node dissection with or without adjuvant radiotherapy, and in seven cases chemotherapy was administered before surgery. Ten patients recurred, eight in the neck and one patient each with metastasis to the lung or liver. Thirteen patients are alive and free of disease, seven patients have died of their tonsil cancer, one patient died of a probable primary lung cancer, and the final patient is alive with local recurrence.

HPV Studies

The results are summarized in Table 1. HPV DNA was detected in 14 patients by PCR (14/22, 64%). Similar results were obtained using both consensus primer sets complementary to either sequences in L1 (MY09/MY11) or the E1 (IU/IWDO) gene. Figure 1 shows a Southern blot of the PCR product amplified with the MY09/MY11 primers using DNA extracted from paraffin blocks of tonsil cancers and hybridized with ^{32}P-labeled HPV-16 DNA. No tumor amplified with type-specific primers for HPV-6 or HPV-18. The majority of the HPV-positive cases were HPV-16 (11/14 HPV-positive cases). One case contained HPV-33 DNA, and in another HPV-59 was identified by restriction fragment length polymorphism analysis of the PCR L1 product (data not shown). The HPV type has not been further characterized in the last HPV-positive tumor. The consensus PCR product from this tumor does not hybridize to HPV-31, -33, -34, -35, -44, -45, or -56 probes, nor could we type it by restriction fragment length polymorphism analysis of the L1 PCR product. DNA extracted from six non-neoplastic tonsils were negative for HPV by the PCR analysis.

Eleven tonsil cancers were analyzed for HPV-16 E6/E7 message by *in situ* hybridization with antisense riboprobes specific for HPV-16 E6/E7 expression. Seven cases positive for HPV-16 DNA by PCR expressed

Table 1. HPV Detection in Tonsil Carcinomas

Case	Tissue extracted	Specimen type*	HPV detection: PCR: MY09/MY11 visual, hybrid	PCR: IU/IWDO visual, hybrid	PCR: Type-specific hybrid	SB†	ISH‡	Final HPV assignment
1	Primary tonsil cancer	F	Neg, Neg	Neg, Neg	Neg	ND	ND	Neg
2	Primary tonsil cancer	F	Neg, Neg	Neg, Neg	Neg	ND	ND	Neg
3	Lymph node metastasis	F	Neg, Neg	Neg, Neg	Neg	ND	ND	Neg
4	Tonsil recurrence	F	Neg, Neg	Neg, Neg	Neg	ND	ND	Neg
5	Primary tonsil cancer	F	Neg, Neg	ND	Neg	ND	ND	Neg
6	Tonsil recurrence	P	Neg, Neg	ND	Neg	ND	Neg	Neg
7	Primary tonsil cancer	P	Neg, Neg	ND	Neg	ND	Neg	Neg
8	Neck recurrence	F	Pos, 16	Pos, 16	16	16	ND	HPV-16
9	Primary tonsil cancer	F	Pos, 16	Pos, 16	16	16	Pos	HPV-16
10	Primary tonsil cancer	F	Pos, 16	Pos, 16	16	16	ND	HPV-16
11	Primary tonsil cancer	F	Pos, 16	Pos, 16	16	ND	ND	HPV-16
12	Primary tonsil cancer	F	Pos, 16	Pos, 16	16	16	Pos	HPV-16
13	Primary tonsil cancer	P	Pos, 16	ND	16	ND	ND	HPV-16
14	Primary tonsil cancer	F	Pos, 16	Pos, 16	16	16	Pos	HPV-16
15	Primary tonsil cancer	F	Pos, 16	Pos, 16	16	16	Pos	HPV-16
16	Primary tonsil cancer	F	Pos, 16	Pos, 16	16	16	Pos	HPV-16
17	Primary tonsil cancer	F	Pos, Neg	Pos, Neg	Neg	ND	ND	HPV-59§
18	Lung metastasis	P	Pos, 16	ND	16	ND	Pos	HPV-16
19	Primary tonsil cancer	P	Pos, 16	ND	16	ND	Pos	HPV-16
20	Primary tonsil cancer	F	Pos, Neg	Pos, 33	Neg	ND	Neg	HPV-33
21	Primary tonsil cancer	P	Pos, Neg	ND	Neg	ND	Neg	HPV-X
22	Primary tonsil cancer	F	Neg, Neg	Neg, Neg	Neg	ND	ND	Neg

*Tissue extracted from fresh (F) or paraffin (P) material.
†DNA extracted from tumors were digested with restriction enzymes *Pst*I and *Bam*HI, Southern blotted (SB), and hybridized to probes for HPV-16. The data have previously been reported.[18]
‡*In situ* hybridization (ISH) for HPV-16 antisense riboprobes complementary to E6/E7 transcript.
§Type determined by restriction enzyme digestion pattern of the L1 PCR product. HPV-X could not be further typed.
Neg, negative; Pos, positive; ND, not done.

HPV-16 message, and viral gene expression was detected in both primary and metastatic tumor (Figure 2, A–C). With the sense probe, only minimal signal was seen attributed to hybridization with viral DNA (Figure 2D), and no expression was seen in two HPV-negative cases nor in the tumors with HPV-33 and the unclassified HPV type.

Histopathology

The histological features of the tumors are summarized in Table 2. The morphological features that best characterized the tumors were related to keratinization and included the presence of intracellular bridges, extracellular

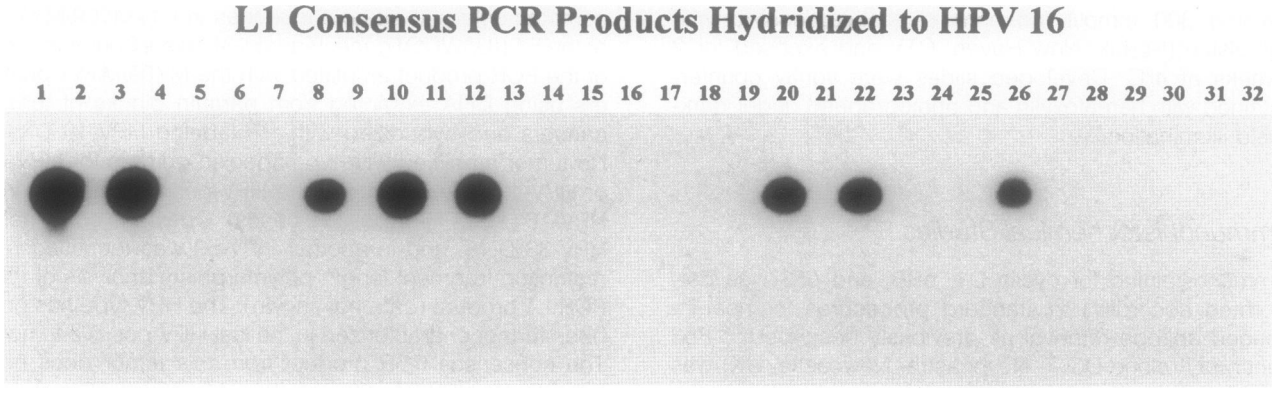

Figure 1. DNA extracted from paraffin blocks of tonsil cancers, amplified with HPV L1 consensus primers, Southern blotted, and hybridized to HPV-16 ^{32}P-labeled probe. **Lane 1**, mixture of HPV DNA from 6, 16, and 18 plasmids; **lane 2**, DNA extracted from paraffin-embedded HeLa cells, HPV 18 positive; **lane 3**, DNA extracted from paraffin-embedded SiHa cells, HPV 16 positive; **lane 4**, DNA extracted from HPV-6-positive condyloma; **lane 5**, DNA extracted from HPV-18- and HPV-6-positive condyloma; **lane 6**, DNA from paraffin-embedded HPV-negative cervical cancer; **lanes 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27,** and **29**, DNA from paraffin-embedded normal liver sectioned between each case and extracted in sequence; **lane 8**, DNA extracted from paraffin-embedded HPV-16-positive cervical cancer; **lane 10**, DNA extracted from paraffin-embedded HPV-16-positive cervical cancer; **lane 12**, DNA extracted from paraffin-embedded HPV-16-positive cervical cancer; **lane 14**, DNA extracted from paraffin-embedded primary tonsil cancer, case 7; **lane 16**, DNA extracted from paraffin-embedded recurrent tonsil cancer, case 7; **lane 18**, DNA extracted from paraffin-embedded tonsil cancer, case 20; **lane 20**, DNA extracted from paraffin-embedded tonsil cancer, case 19; **lane 22**, DNA extracted from paraffin-embedded tonsil cancer, case 13; **lane 24**, DNA extracted from paraffin-embedded tonsil cancer, case 6; **lane 26**, DNA extracted from paraffin-embedded lung metastasis from a tonsil cancer, case 18; **lane 28**, DNA extracted from paraffin-embedded SCC of retromolar region; **lane 30** , DNA from normal placenta; **lane 31**, primers only, no DNA; **lane 32**, no primers, no DNA.

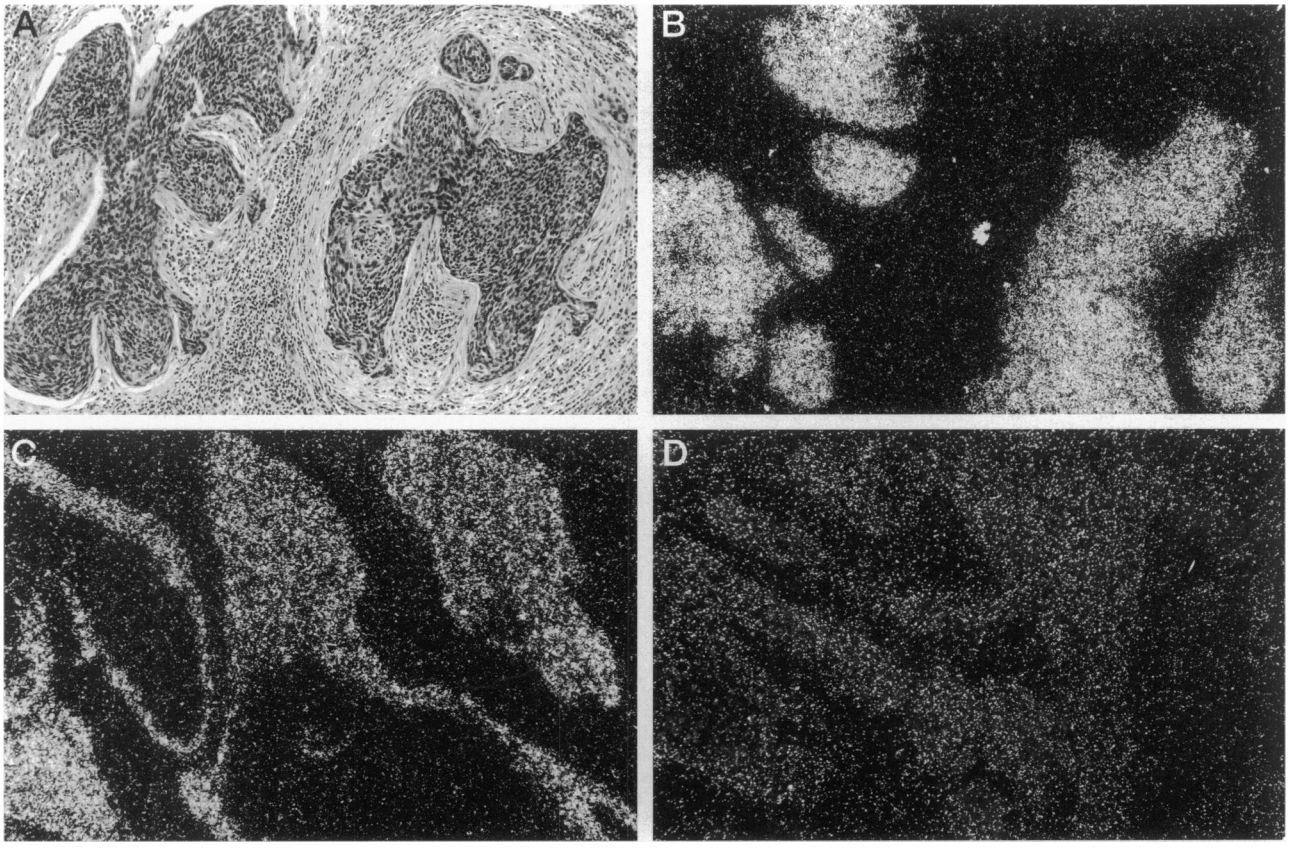

Figure 2. *In situ* hybridization of HPV-positive tumors to HPV-16 ^{35}S-labeled E6/E7 riboprobes. **A:** Photomicrograph of histological section of primary tonsil SCC (case 12) that was positive for HPV-16 E6/E7 transcripts by *in situ* hybridization. H&E; magnification, ×200. **B:** Photomicrograph of dark-field microscopy of *in situ* hybridization of primary tonsil SCC (case 12) with HPV-16 E6/E7 antisense probe. Magnification, ×200. **C:** Photomicrograph of dark-field microscopy of *in situ* hybridization of metastatic tonsil SCC in a cervical lymph node from case 12 with HPV-16 E6/E7 antisense probe. Magnification, ×200. **D:** Photomicrograph of dark-field microscopy of *in situ* hybridization of metastatic tonsil SCC in cervical lymph node with HPV-16 E6/E7 sense probe. The signal is slightly above background due to hybridization to HPV DNA. Magnification, ×200.

deposition of keratinaceous amorphous material, cytoplasmic keratinization, and the formation of keratin pearls. Based on these features, the tumors segregated into two major histological types: well keratinized (K-SCC) and poorly keratinized (P-SCC). Two cases had intermediate features (I-SCC).

The K-SCCs were characterized by variable sized nests of squamous cells with abundant highly keratinized cytoplasm and numerous central keratin pearls (Figure 3A). The central portions of the tumor nests often contained large concentric keratin whorls. Individual tumor cells had distinct cell borders, abundant homogeneous cytoplasm with a clear, glassy appearance, and numerous easily identified cytoplasmic bridges. The central keratinized cells tended to be sharply demarcated from the more peripheral, often single layer of basal cells (Figure 3B). At the periphery of the tumor, this histological type tended to have an infiltrative pattern with small groups, cords, or bands of tumor advancing into the adjacent stroma (Figure 3C). None of these tumors contained HPV DNA (0/6).

In contrast, the poorly keratinized SCCs (P-SCCs) were composed of a relatively monomorphic population of small ovoid to spindled cells with a high nuclear to cytoplasm ratio arranged in variably sized sheets, nests, and cords (Figure 4A). Often the individual cells had indistinct cell borders, relatively small amounts of cytoplasm with minimal or no squamous maturation, resembling immature basal cells (Figure 4B). In some cases, the cells were more spindled with slightly more abundant cytoplasm, but keratin pearls and cytoplasmic bridges were very rare (Figure 4A). Tumor necrosis tended to be seen in the central portions of the tumor nests (Figure 4C). The advancing front of the tumor into the adjacent stroma tended to be pushing or expansile rather than infiltrating, with well delineated, rounded nests of tumor (Figure 4D). Of the 14 tumors histologically classified as P-SCC, HPV DNA was detected in 13 (13/14, 93%). In one case of P-SCC, the lymphocytic infiltrate was abundant and intermixed with the syncytial tumor cells that had the histological pattern of a lymphoepithelioma. This tumor contained a HPV type that we have not been able to further characterize. To exclude a possible mixed viral infection, *in situ* hybridization for Epstein-Barr virus with EBNA probes was performed (courtesy of Larry Weiss, M.D.) and was negative (data not shown).

Two tumors had intermediate histology (I-SCC) in which the basal cells constituted a higher proportion of the malignant cells than in the K-SCC, but with more individual cell keratinization and more abundant keratin

Table 2. Histological Features of Tonsil Cancers

Case	Tumor type	N/C ratio	Nuclear grade	Cell borders	Tumor borders	Cytoplasm bridges	Keratin pearls	Extra-cellular keratin	Individual cell keratin	Tumor necrosis	Inflam-mation	Apo-ptosis	Peri-nuclear clearing	LVS	Mitotic index
1	K-SCC	L	II	D	M	3	2	3	3	1	2	2	Y	N	1
2	K-SCC	M	III	D	I	3	2	1	3	1	2	3	N	N	3
3	K-SCC	M	II	D	I	2	2	1	2	1	2	1	Y	N	2
4	K-SCC	L	II	D	B	2	3	1	3	1	1	1	Y	N	1
5	K-SCC	M	III	D	I	3	3	1	3	1	2	1	N	N	3
6	K-SCC	M	II	D	I	3	3	1	3	1	1	1	N	N	2
7	I-SCC	H	II	I	B	2	3	3	3	1	1	2	N	N	2
8	I-SCC	L	II	I	M	2	3	2	3	2	1	2	N	N	2
9	P-SCC	M	II	M	B	1	1	1	2	3	1	1	N	N	3
10	P-SCC	M	II	M	B	1	1	1	1	2	1	1	N	N	2
11	P-SCC	M	II	M	B	1	1	1	1	1	1	1	N	N	2
12	P-SCC	M	II	I	B	1	1	1	1	2	2	2	N	N	1
13	P-SCC	M	II	I	B	1	1	1	1	1	1	3	Y	N	1
14	P-SCC	H	II	I	B	1	1	1	1	3	1	1	N	Y	2
15	P-SCC	H	II	I	B	1	1	1	1	2	2	3	N	N	2
16	P-SCC	M	II	I	B	1	1	1	1	1	1	2	N	N	2
17	P-SCC	H	II	I	B	2	1	1	1	2	1	2	N	Y	3
18	P-SCC	H	III	I	B	1	1	3	1	3	2	3	Y	N	3
19	P-SCC	M	III	I	B	1	1	1	1	2	2	3	Y	N	3
20	P-SCC	M	III	I	B	2	1	1	1	1	1	1	N	Y	2
21	P-SCC	H	III	S	B	1	1	1	1	1	3	1	N	N	1
22	P-SCC	M	II	M	M	1	1	1	1	1	1	1	N	Y	2

Nuclear/cytoplasmic ratio (N/C) is recorded as low (L), moderate (M), or high (H). Nuclear grade is recorded as grade I if relatively monomorphic, absent or small nucleoli, and delicate chromatin and grade III if pleomorphic, prominent nucleoli, and coarse chromatin. Grade II is intermediate. Cell borders were recorded as distinct (D), indistinct (I), syncytial (S), or mixed (M). Tumor borders with stroma are described as blunt (B), infiltrative (I), or mixed (M). Cytoplasmic bridges, keratin pearls, extracellular keratin, individual cell keratin, tumor necrosis, inflammation, single-cell apoptosis were recorded by a three point system: 1, none, rare, or minimal; 2, moderate; 3, marked, abundant, or numerous. Presence of lymphovascular space involvement and perinuclear clearing are Y, present; N, absent. The mitotic index is the average of mitotic counts in 10 HPF: 1, <5/10 HPF; 2, 5–20/10 HPF; 3, >20/10 HPF.

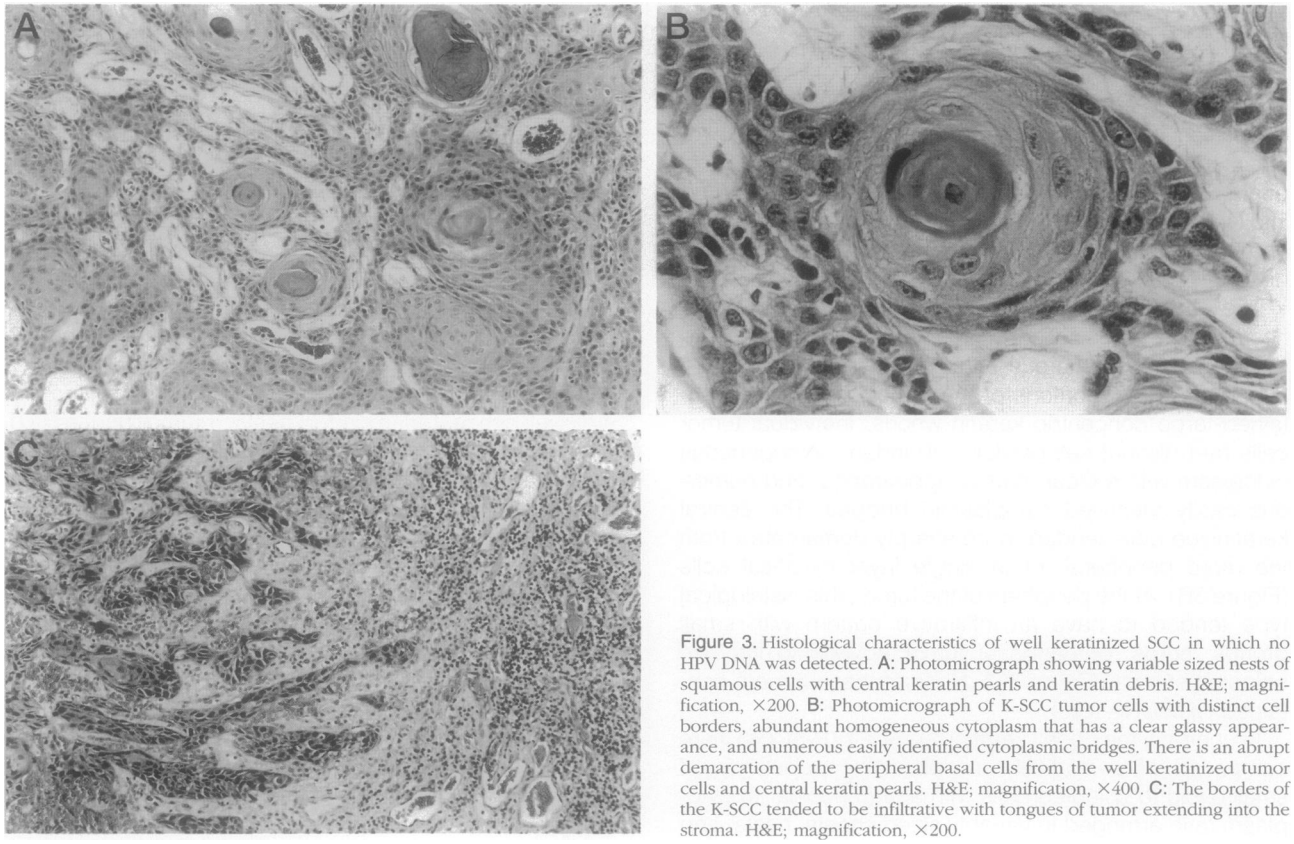

Figure 3. Histological characteristics of well keratinized SCC in which no HPV DNA was detected. A: Photomicrograph showing variable sized nests of squamous cells with central keratin pearls and keratin debris. H&E; magnification, ×200. B: Photomicrograph of K-SCC tumor cells with distinct cell borders, abundant homogeneous cytoplasm that has a clear glassy appearance, and numerous easily identified cytoplasmic bridges. There is an abrupt demarcation of the peripheral basal cells from the well keratinized tumor cells and central keratin pearls. H&E; magnification, ×400. C: The borders of the K-SCC tended to be infiltrative with tongues of tumor extending into the stroma. H&E; magnification, ×200.

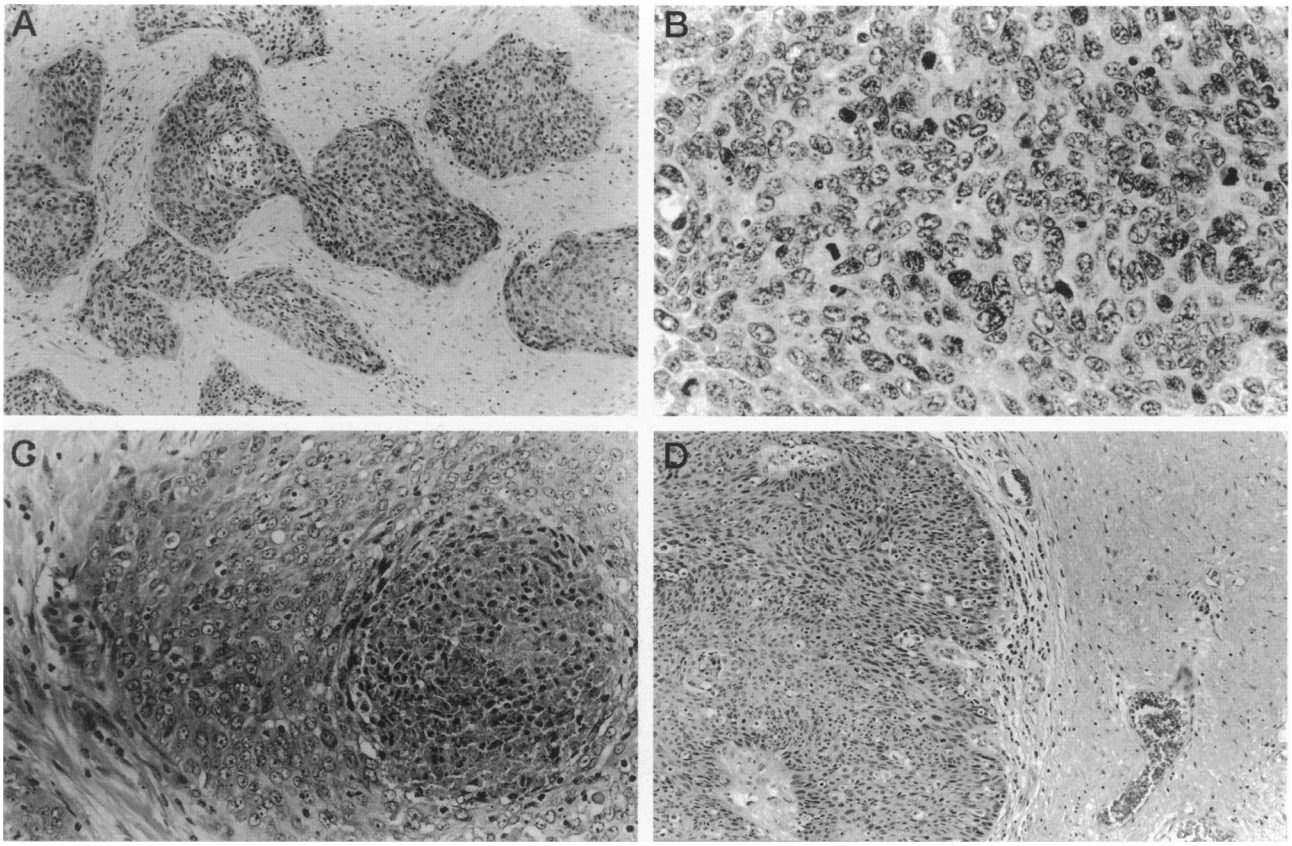

Figure 4. Histological characteristics of poorly keratinized SCC in which HPV DNA was detected. **A:** Tumor tended to form variably sized sheets and nests with blunt contours and rare if any keratin pearls. H&E; magnification, ×200. **B:** Individual tumor cells have a high nuclear/cytoplasmic ratio with indistinct cell border and relatively small amount of cytoplasm with minimal or no squamous maturation. H&E; magnification, ×400. **C:** The central portions of the P-SCC tumor nests frequently had necrotic debris. H&E; magnification, ×200. **D:** The P-SCC tended to have expansile borders, invading the stroma in a broad front. H&E; magnification, ×200.

pearls than in the P-SCC. One of these contained HPV DNA, and the other did not.

There was no significant difference between K-SCC and P-SCC in the following features: nuclear grade, mitotic index, number of apoptotic bodies, lymphovascular space involvement, or inflammation. Tumor necrosis was present in both histological types, although in the P-SCC it tended to be localized to the central portions of the tumor nests whereas in the K-SCC, there were large sheets of necrotic tumor. Six of the tumors had cytological features that resembled koilocytes characterized by marked pleomorphism with irregular nuclear contours, multinucleation, and perinuclear clearing. The chromatin was irregularly clumped, and in many cells there were prominent nucleoli but no nuclear smudging. These changes were seen, often very focally, in tumors that otherwise had typical K-SCC or P-SCC features and did not correlate with the presence of HPV DNA (Figure 5). Alterations in adjacent epithelium, including the presence of dysplasia, could not be consistently evaluated in this population due to erosion by tumor, denudation, or treatment effects.

The correlation of HPV status with histological features was determined. HPV DNA was detected in 13 of the 14 cases of P-SCC (93%) and in none of the cases of K-SCC (0/6, 0%; $P < 0.0001$).

Immunohistochemical Studies

Twenty-one of the cases had sufficient tumor tissue remaining in paraffin blocks for immunohistochemical studies. Table 3 compares the immunohistochemistry results with histology and HPV status. Tumors with more than 30% of nuclei staining were interpreted as positive for cyclin D1 overexpression. Seven cases showed positive cyclin D1 staining, including all six tumors classified as

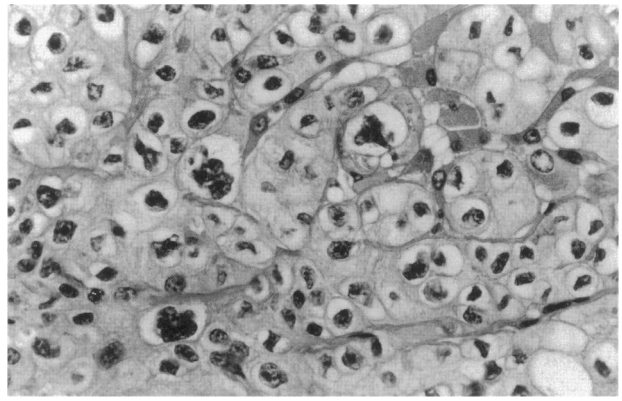

Figure 5. Perinuclear clearing in a K-SCC in which no HPV DNA was detected. H&E; magnification, ×600.

Table 3. Summary of HPV Status, Histology, and Oncoprotein Expression

Case	Histology	HPV status	bcl-1*	p53*	pRB*
1	K-SCC	–	+	–	+
2	K-SCC	–	+	–	+
3	K-SCC	–	+	+	+
4	K-SCC	–	+	–	+
5	K-SCC	–	+	+	+
6	K-SCC	–	+	+	+
7	I-SCC	–	–	–	+
8	I-SCC	16	–	–	+/–
9	P-SCC	16	–	–	ND
10	P-SCC	16	–	–	+/–
11	P-SCC	16	–	+/–	ND
12	P-SCC	16	–	–	+
13	P-SCC	16	–	–	ND
14	P-SCC	16	–	–	–
15	P-SCC	16	–	–	+/–
16	P-SCC	16	–	–	–
17	P-SCC	59	–	–	–
18	P-SCC	16	–	–	+/–
19	P-SCC	16	–	–	+
20	P-SCC	33	ND	ND	ND
21	P-SCC	X	–	+/–	+
22	P-SCC	–	+	–	+

For case 20, there was insufficient tumor in biopsy specimen for evaluation. HPV-X could not be typed by restriction fragment length polymorphism analysis. ND, not done.

*Epicritic value: +, >30% of the cells positive; +/–, 10 to 29% positive; –, <10% positive.

K-SCC (Figure 6A) and the case of P-SCC that lacked detectable HPV DNA. Neither of the tumors with intermediate histology, nor the P-SCC with HPV DNA, showed more than minimal staining, although scattered lymphocytes were positive (Figure 6C). The pattern of cyclin D1 staining tended to be diffuse with variable intensity of nuclear staining (Figure 6B). Only three tumors had more than 30% of their nuclei positive for p53, and all were K-SCCs (Figure 7). Two P-SCCs, including the tumor with lymphoepitheliomatous histology, had scattered p53-positive nuclei (between 10 and 30% of the cells) and were considered marginally positive. None of these five patients with p53 expression by immunohistochemistry had radiotherapy before the biopsy or surgical specimen was stained. In all cases, at least a few tumor cells stained with the pRB antibody; however, the tumors that contained HPV DNA tended to have fewer nuclei positive and with lower intensity than the tumors in which HPV was not detected (Figure 8, A and B). The tumor staining was evaluated in areas of the slide with positive internal controls, and nuclear pRB staining was not seen in sections of a retinoblastoma.

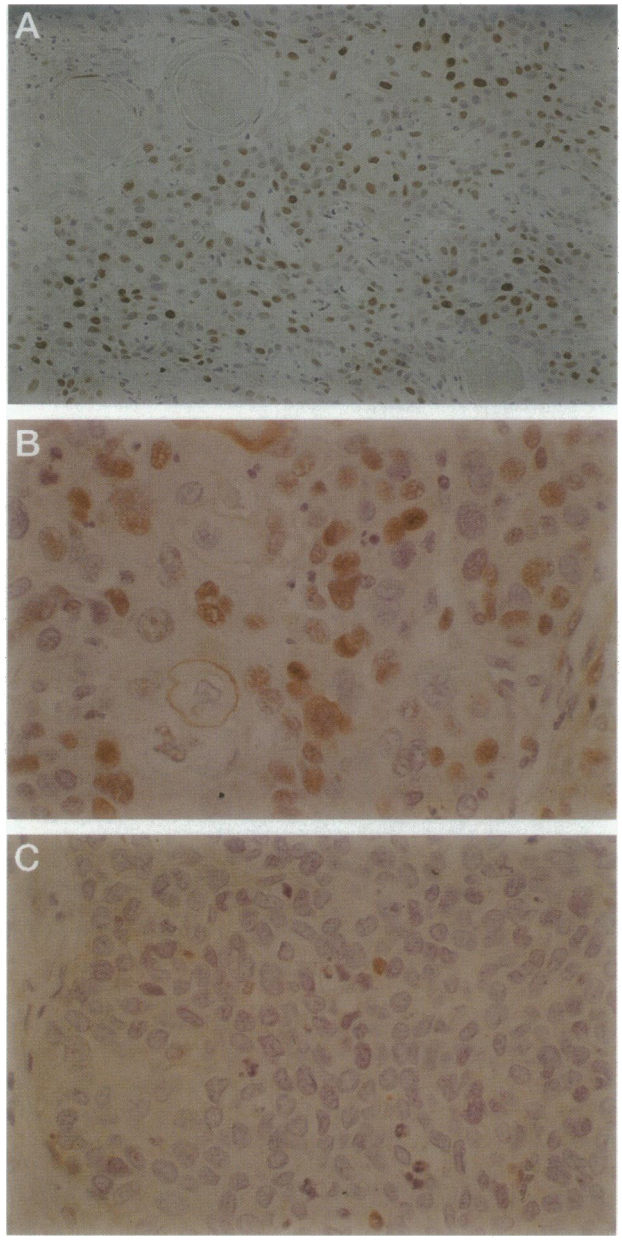

Figure 6. Immunohistochemical staining for bcl-1 (cyclin D1). **A:** Low-power photomicrograph of positive immunohistochemical staining for bcl-1 (cyclin D1) in a K-SCC that lacked detectable HPV DNA. Magnification, ×200. **B:** High-power photomicrograph of positive immunohistochemical staining for bcl-1 in a K-SCC. Note the variable nuclear staining intensity. Magnification, ×400. **C:** Negative immunohistochemical staining for bcl-1 (cyclin D1) of a P-SCC that had HPV-16 DNA. Note scattered positive lymphoid cells. Magnification, ×400.

Discussion

In the head and neck region, HPV appears to have a particular predilection for SCC of the tonsil.[10,11,16–18] In our previous study,[18] we found HPV DNA in 15% of 167 tumors of the head and neck, but 60% (9/15) of tonsil tumors had viral DNA by PCR analysis. Using restriction enzyme digestion and Southern blot hybridization of the tumor DNA, the levels of HPV DNA in the tonsil cancers were shown to be comparable to that found in cervical cancers. In this study, we added 7 cases of SCC of the tonsil, and 14 of the 22 tumors were positive for HPV DNA (63.6%) by PCR. As in genital cancers, HPV-16 is the most common genotype, being present in 11 of the 14 HPV-positive tumors. All seven HPV-16 positive tumors and a metastasis contained viral transcripts, further supporting HPV as an etiological agent in these tumors.

Our findings that tonsil carcinomas with and without detectable HPV can be distinguished by histological fea-

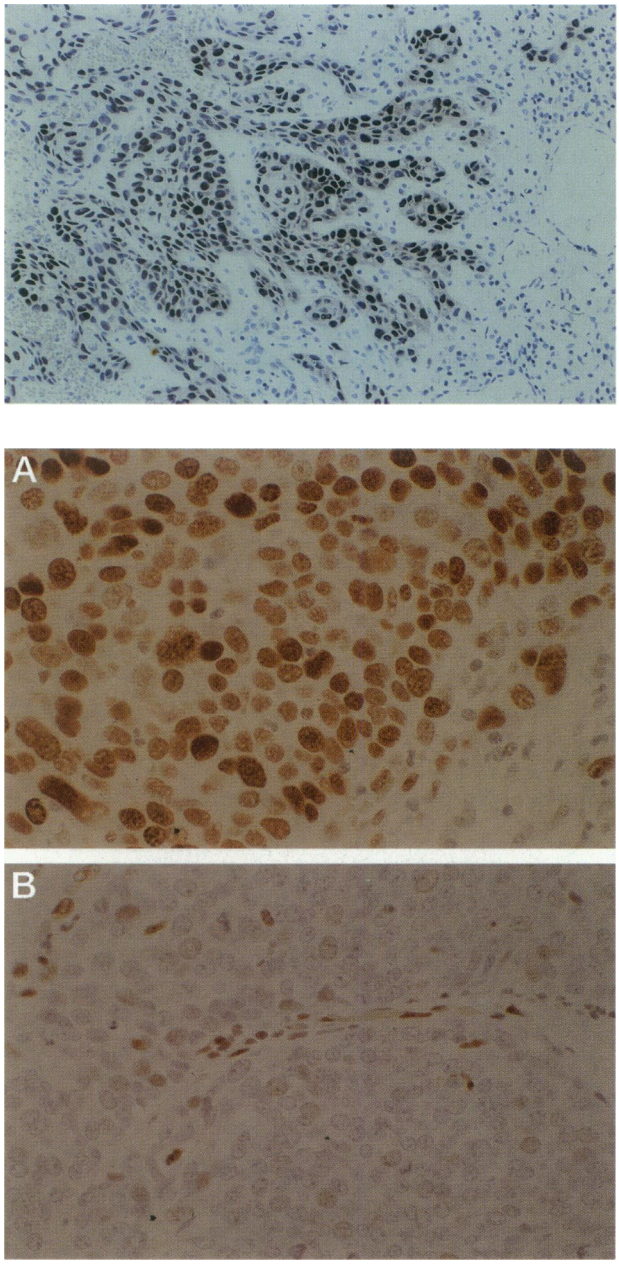

Figure 7. (top) Strong positive overexpression of nuclear p53 by immunohistochemical staining of a K-SCC that lacked detectable HPV DNA. Magnification, ×200.
Figure 8. (bottom) Immunohistochemical staining for pRb. **A:** Strong positive staining for pRb in most tumor cells of a K-SCC that lacked detectable HPV DNA. Magnification, ×400. **B:** Only a rare pRb-positive tumor cell in a P-SCC with HPV-16 DNA. Note the strong staining of endothelial nuclei. Magnification, ×400.

tures, and differences in oncogene expression by immunohistochemistry, support at least two distinct histopathological entities for SCC of the tonsil. In SCC without detectable HPV DNA, the tumors tended to be composed of cells with well keratinized cytoplasm, numerous keratin pearls, and often an abrupt demarcation of basaloid cells at the periphery of the tumor nests from the more central keratinizing cells. These tumors overexpressed cyclin D1 by immunohistochemistry, and several showed marked overexpression of p53 as well. In contrast, the HPV-positive tumors consisted primarily of a relatively monomorphic population of small ovoid to spindled cells with scanty cytoplasm and minimal cytoplasmic keratinization. These tumors did not overexpress cyclin D1 and only occasionally showed marginal immunoreactivity with the p53 antibody.

The histological features of the tonsil cancers with and without HPV DNA are similar to the distinctive types of SCC of the vulva, described as basaloid carcinomas and well differentiated keratinizing SCC, respectively.[35] However, in the vulva, an additional histological tumor type, the warty SCC, has been described with prominent condylomatous cytological features,[35] and HPV DNA is detected in most. In several of the K-SCCs of the tonsil we found prominent perinuclear clearing and marked nuclear pleomorphism with wrinkling of the nuclear membrane, but DNA from these tumors did not amplify with consensus primers for either the L1 or E1 HPV genes. It is possible that these K-SCCs in the tonsil may contain an undetected HPV type. The primers used for this study detect over 25 HPV types, but these are predominantly the genital types that infect mucosal surfaces. Many HPV types do not amplify with these primers, including those that belong to the EV group as well as many of the cutaneous types. Additional studies are needed to determine whether known or unknown HPV types are present in these cases with histological changes resembling koilocytosis or whether these are degenerative changes and unrelated to viral infection.

The results of the immunohistochemical studies for p53, pRB, and cyclin D1 in HPV-positive and -negative tonsil cancers are consistent with our current understanding of how HPV oncoproteins interact with cellular proteins involved in cell cycle regulation and support a viral etiology for the majority of tonsil SCCs. The level of p53 protein increases after damage to cellular DNA, and the wild-type p53 protein is involved in arresting the cell cycle until that damage can be repaired. It is postulated that loss of p53 function can be achieved by at least two different pathways: deletion or mutation of the p53 gene or binding of p53 to viral oncoproteins (HPV E6), which increases its degradation through ubiquitin-dependent proteolysis.[26] Somatic mutations of the p53 gene are infrequent in HPV-positive cervical[36,37] or vulvar carcinomas, although they are common in HPV-negative vulvar cancers.[38] P53 gene mutations and deletions can be detected in 25 to 50% of head and neck cancers.[19–21] In studies that have examined both p53 by mutational analysis and HPV detection in head and neck cancers, most of the HPV-positive tumors had normal p53.[39,40] Mutations in the p53 gene lead to stabilization of an abnormal protein that can often, but not always, be detected by intense immunohistochemical staining of most tumor nuclei in formalin-fixed and paraffin-embedded tissue.[41] Although a negative immunostain does not exclude alterations in p53, there is a tendency in this study to find p53 overexpression and, by inference, mutations in the gene in HPV-negative K-SCCs (3/6, 50%).

The retinoblastoma gene product is constitutively expressed in the nucleus of every normal cell. Alterations in

the gene that result in loss of the pRB protein or in production of an abnormal protein product have been found in a wide variety of epithelial tumors, including breast carcinoma, bladder cancer, and non-small-cell lung cancer.[42–44] Expression of pRB may also be related to the differentiation state of tumors, as immunoreactivity is increased in differentiated cells of teratocarcinoma compared with the undifferentiated malignant cells.[45] One study examined pRB immunoreactivity in head and neck cancers and found the complete absence of nuclear staining in two of seven cases and also that staining was increased in well differentiated tumors compared with poorly differentiated tumors.[46] We found that well keratinized SCCs showed enhanced nuclear pRB immunoreactivity compared with more poorly differentiated tumor cells containing HPV DNA. It has also recently been reported that pRB levels in HPV E7-expressing cells is low due to degradation through the ubiquitin-proteasome pathway.[47]

Cyclin D1 promotes transition of the cell cycle from G1 to S by interacting with cyclin-dependent kinases CDK4 and CDK6. The active complex phosphorylates pRB, which leads to the release of a transcription factor (E2F) that stimulates expression of genes required for DNA synthesis.[24] Cyclin D1 is overexpressed in a variety of human epithelial tumors.[24] In SCC of the head and neck, bcl-1 gene amplification, as well as protein overexpression, is frequently observed and correlates with high stage and poor prognosis.[19,22] The antibody used in this study, DCS-6, does not cross-react with cyclin D2 or cyclin D3[48] and has been shown to reproducibly detect overexpression of cyclin D1 in head and neck cancers.[49] In this study we report that cyclin D1 expression correlates inversely with HPV status. Seven of eight SCCs of the tonsil with no amplifiable HPV DNA showed immunoreactivity for cyclin D1, whereas all of the HPV-positive tumors had less than 10% of the nuclei staining. Similar results have been found in other HPV-positive tumor systems. In cultured cells expressing HPV oncoproteins E6 and E7, cyclin D1 protein levels are low or undetectable, whereas cyclin B and cyclin A complexes remain intact.[50,51] *In vivo,* cyclin D1 protein levels are undetectable by immunohistochemistry in tissue sections from cervical cancers and high-grade dysplasia.[52]

HPV E7 protein binds to the same site on pRB protein as cyclin D1, effecting release of E2F and essentially eliminating the requirement for a functional cyclin D1 for cell cycle progression.[27] The pRB complex regulates cyclin D1 transcription so that inactivation of pRB by interaction with HPV E7 may set up a negative feedback loop for cyclin D1 synthesis.[24,50,51] The *in vivo* regulation of cyclin D1 may be more complex, as increased cyclin D1 mRNA levels were shown in cervical cancer and high-grade dysplasia by *in situ* hybridization, although the cyclin D1 protein was not expressed.[52] The levels of cyclin D1 mRNA in head and neck cancers expressing HPV viral oncoproteins is not known. Alternative mechanisms for down-regulation of cyclin D1, such as increased degradation of cyclin D1 protein released from pRB by E7 binding, may also contribute. In HPV-positive tumors, the viral oncoprotein E7, by binding to pRB, may be responsible for altering cyclin-D1-mediated growth control or, alternatively, cell turnover may be increased by E7 interactions with other cell cycle regulators, such as p27 or cyclins A and E.[53,54] In the HPV-negative tumors, overexpression of cyclin D1 by a viral-independent mechanism, such as amplification of the cyclin D1 gene (bcl-1) or alterations in p16 (a cyclin-dependent kinase inhibitor), may promote cell proliferation.[24,55]

It is not clear why the tonsil is more susceptible to HPV transformation than adjacent sites in the aerodigestive tract such as pharynx, hypopharynx, or pyriform sinus.[18] Similar to the uterine cervix, the tonsil is anatomically located at a portal between the exterior environment and internal organs, and the site is potentially subjected to both immunogenic and carcinogenic stimulation. In addition, the squamous epithelium at both sites is embryonically derived from endoderm[56] and may undergo metaplastic processes. The tonsil has two types of squamous epithelium: a stratified squamous mucosa that forms the free surface, histologically similar to that seen in the oral cavity, and a reticulated epithelium lining the crypts, in which the cells above the germinal layers are cytologically uniform and infiltrated with lymphoid elements.[57] The histological differences we see in SCC of the tonsil with and without detectable HPV DNA may be a reflection of the epithelium transformed, either reticulated squamous epithelium of the crypts or the native stratified squamous epithelium from the surface.

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