# Squamous Cell Carcinoma of the Tonsils: A Molecular Analysis of HPV Associations

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## ABSTRACT

***Background:*** **The role of human papilloma virus (HPV) in the pathogenesis and biological behavior of tonsillar squamous cell carcinoma (TSCC) are areas of intense investigation.**

***Methods:*** **This study used PCR analysis to identify HPV in paraffin-embedded tonsillar and nodal tissue from 52 patients with TSCC and 48 age (+/−5 year)/gender-matched controls with benign tonsillar hyperplasia. Results were correlated with HLA-DRB1 haplotype and clinical outcome.**

***Results:*** **HPV was identified in 46% of patients with TSCC and 6% of controls. DNA sequencing showed the presence of HPV type 16 in 21 patients (40%) with TSCC. There was no statistically significant association between HLA-DRB1 expression and TSCC or HPV infection. Fifteen of 16 patients with HPV-positive TSCC with regional metastases had evidence of HPV in pathologically involved lymph nodes. In eight HPV 16-positive TSCC patients with lymph node metastasis, PCR testing identified HPV 16 in 17 of 23 histologically negative lymph nodes. Patients with HPV-positive TSCC without metastatic disease had no evidence of HPV in their lymphatic tissue. Clinically, HPV-associated carcinoma was present in younger patients in comparison with HPV-negative TSCC patients (mean age, 56.6 *versus* 66 years; $P = 0.001$). The odds for patients with HPV infection to develop TSCC were 18.2 times greater than for patients without HPV infection (95% confidence interval 4.6, 73.1). There was no statistically significant association between presence of HPV and cause-specific survival (hazard ratio = 2.5 for HPV negative *versus* positive; $P = 0.26$), after adjusting for age in a Cox proportional hazards regression analysis.**

***Conclusion:*** **HPV is an independent risk factor for TSCC. Identification of HPV in the histologically positive and negative lymph nodes of patients with HPV-positive TSCC/metastatic disease supports the role of HPV in the oncogenesis of TSCC.**

## INTRODUCTION

Tonsillar cancer affects a diverse patient population. The typical patient has a strong history of tobacco and alcohol exposure. However, young individuals and elderly female patients without exposure to these chemical carcinogens also develop TSCC.[2] Recent data demonstrate the presence of HPV in both primary tumors of the oropharynx and in tonsillar carcinoma's specifically, suggesting that the oropharynx or Waldeyer's tonsillar ring might be a predilection site for HPV-related oncogenesis (1–8). Limited site-specific data exist correlating the presence of HPV with the development of TSCC, and a cause-effect relationship has yet to be defined.

It remains uncertain whether the association of specific HPV subtypes with TSCC represents a true cause-and-effect relationship. One piece of evidence regarding the causal role of HPV in cancers of the uterine cervix was the strict preservation of the HPV DNA in metastatic lesions (9). Similar studies to evaluate lymph node metastases from HPV-associated tonsil cancers for the presence of HPV DNA have not been performed and may provide further evidence that the HPV is essential for maintenance of the transformed state in these cancers. It is also clear in uterine cervical carcinoma that increased risk of cancer is associated with specific HPV subtypes (primarily HPV 16 and 18). The data related to head and neck squamous cell carcinoma are insufficient to establish whether there are low-risk HPV associations.

Genetically determined immune responsiveness may be a critical issue determining regression of dysplastic lesions or progression toward malignancy. It has been suggested that specific HLA-subtype expression may be associated with increased or decreased susceptibility to viral oncogenesis (10–15), *e.g.*, HLA-DRB1 has been reported to be a risk factor associated with cervical squamous cell carcinoma, and an association of cervical cancer and the HLA-DQ3 antigen has been reported for Norwegian (16) and German (17) patients. (12, 18, 19). The HLA-DRB1 haplotype is common in Caucasian Americans (19.8%) and appears to be correlated with both persistent infection by HPV type 16 and associated with carcinoma of the uterine

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[2] The abbreviations used are: TSCC, tonsillar squamous cell carcinoma; HPV, human papillomavirus; OR, odds ratio; CI, confidence interval; HR, hazard ratio; DFS, disease-free survival; OS, overall survival; CSS, cause-specific survival.

cervix (17–19).[3] To the best of our knowledge, HLA-DRB1 has not been identified as a factor in the genetic susceptibility to chemical carcinogens.

Although improved understanding of the role of HPV in the etiology of TSCC may offer strategies for disease prevention, therapeutic interventions are dependent on the correlation with HPV infection and clinical outcome. The purpose of this study was 3-fold: (*a*) to evaluate potential associations between HPV infection, HLA-DRB1 expression, and TSCC in a retrospective case control study; (*b*) to correlate the presence of HPV in TSCC with lymph node involvement; and (*c*) to compare the clinical characteristics and disease outcomes in patients with HPV-positive and -negative TSCC. To the best of our knowledge, this represents the largest study to date correlating HPV with TSCC and better defines the potential oncogenic role and clinical implications of HPV infection in tonsillar tissue.

## MATERIALS AND METHODS

**Patients.** Archival tissue from 108 patients with primary TSCC diagnosed during 1987–1995 was randomly selected from the Mayo Clinic Tissue Registry. Tissue sections from all patients were reviewed by a head and neck pathologist (J. L.), and the diagnosis was reconfirmed histologically in all specimens. DNA of sufficient quality for PCR amplification could be detected by β-Globin assay in 50% of the specimens. In 2 patients, the tonsils could not be clearly identified as the primary tumor site. These were excluded, and the remaining 52 TSCC patients were included in the study. Forty-eight gender and age- (±5 years) matched (1:1) patients diagnosed with benign hyperplasia of the tonsils during the same time period served as controls, after detecting DNA of sufficient quality by β-Globin assay.

The charts of all patients were reviewed to extract data on patients' demographics, diagnosis, stage at presentation, history of other diseases, carcinogenic risk factors, (alcohol and/or tobacco), and the type of therapeutic intervention. If past or present tobacco use was reported, smoking was graded positive. In this series, alcohol consumption was graded as either none, mild if less than one drink per week, moderate if several or more drinks per week, and severe if daily use was reported by the patient.

**DNA Extraction from Paraffin Sections.** Tonsillar tissue and lymph node histology were reviewed. Paraffin-embedded tonsillar tissue was cut in 25-μm sections using disposable microtome blades. Two 25-μm sections were cut from a maximum of three paraffin-embedded metastatically involved nodes and up to four uninvolved nodes from each of the cases. Lymph nodes from patients without metastases were prepared in an identical fashion. To indicate potential sample-to-sample contamination (if present), paraffin-embedded mouse tissue was cut and used for amplification after every two tonsil samples. All tissues were digested in 500 μl of buffer containing 500 μg/ml Proteinase K, 0.45% Tween 20, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.001% gelatin (16). Tissues were incubated at 55°C for 18–24 h with intermittent mixing followed by boiling for 10 min to inactivate proteinase K.

**PCR-quality Control.** To avoid contamination leading to false positive results, all PCR-related work was carried out in specialized zones within a PCR laboratory that undergoes UV purification at least once every 24 h. To detect crossover contamination, nonhuman, viral-free DNA and negative controls (PCR reagents containing no DNA) were included in each PCR amplification. To eliminate crossover contamination if present, dUTP was used in place of 50% of dTTP. All negative controls and nonhuman, viral-free DNA samples were negative for β-Globin, HPV, and HLA-DRB1 assay. Positive controls containing the MJ and Caski cell lines (20) always amplified β-Globin and HPV DNA, respectively.

**β-Globin Amplification.** To verify integrity of the DNA, each sample was amplified using the human β-Globinprimers GH20 and PC04 (product length, 268 bases). An additional 83 mouse control specimens were amplified with the same human β-Globin primers to screen for sample contamination.

**HPV-DNA Detection by PCR.** The L1 consensus primers MY09 and MY11 (14) were used as the primary method for detecting HPV. The amplification reaction was performed in 50 μl containing 10 mM Tris (pH 8.3), 50 mM KCl, 2.5 μM $MgCl_2$, 200 μM each deoxynucleotide triphosphate (100 μM dUTP and dTTP), 2.5 units of Amplitag gold (5 units/μl, Perkin-Elmer), 0.1% BSA, 19.5 μl of RNase-free water, 0.5 μm of each primer, and 5 μl of the sample. PCR cycling conditions were 95°C for 10 min followed by 40 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by 10 min at 72°C. Samples negative for HPV in the L1 region were nested using the consensus HPV L1 primers 6582–23D (5′-GCMCAGGGWCATAAYAATGGYAT-3′) and 7033–22U (5′-CGTCCMAARGGAWACTGATCTA-3′) using the above-described PCR solution, with 1 μl instead of 5 μl of sample and 35 instead of 40 cycles.

Samples were also amplified using specific primers to the E6 region of HPV 16. PCR conditions were the same as for the L1 region, except for 2 mM $MgCl_2$. The HPV 16 E6 primers are as follows:

5′CCACAGTTATGCACAGAGCTGCAAACAACTATACAT (HPV16-E6–140-36D)

5′TTGTCCAGATGTCTTTGCTTTTCTTCAGGACACAGT (HPV16-E6–465-36U)

DNA from the Caski cell line was used as a positive PCR control to assess the success of the amplification. PCR reagents lacking DNA (no sample added) served in each PCR amplification as a negative control. The amplicon size for the HPV-16-E6 PCR product was 325 bp.

After amplification, 15 μl of each sample were run on a 2% agarose gel (Seakem GTG; FMC Bioproducts, Rockland, ME) containing 20 μg of ethidium bromide (Sigma Chemical Co.; 10 mg/ml) in 100-ml gel to visualize products. Each HPV L1-positive specimen was sequenced (PE ABI 377 DNA Sequencer; PE Applied Biosystems, Foster City, CA) after DNA purification using Wizard Preps PCR DNA Purification system (Promega, Madison, WI). The product sequence was matched with published sequences (Gene Bank) using commercial software (Wisconsin Package Version 9.1; Genetics Computer

[3] Gostout, TAP1, TAP2, and HLA-DR2 alleles are predictors of cervical cancer risk. Submitted for publication.

*Table 1* HPV presence and type predominance in cancer and control patients

HPV was detected using both L1 consensus primers and specific primers to the E6 region. Samples negative for HPV in the L1 region were nested using the HPV L1 consensus primers 6582-23D and 7033-22U. Samples negative for the HPV 16 E6 region were analyzed by Southern blot (*left column*). The numbers of HPV-positive specimens, including the detected type (determined by sequencing analysis), are listed.

| | Cancer specimen | | | | | Benign hyperplastic specimen | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | HPV presence | | HPV types | | | HPV presence | | HPV types | | |
| | Yes | No | 16 | 12 | 59 | Yes | No | 16 | 12 | 59 |
| HPV L1 | 13 | 39 | 13 | 0 | 0 | 0 | 48 | 0 | 0 | 0 |
| HPV L1 including nested | 24 | 28 | 21 | 2 | 1 | 0 | 48 | 0 | 0 | 0 |
| HPV E6 | 21 | 31 | 21 | 0 | 0 | 0 | 48 | 0 | 0 | 0 |
| Southern blot[a] | N/A | 31 | | | | 3 | 45 | 3 | | |

[a] Performed on all gel-negative E6 samples.

Group, Madison, WI) to determine the HPV type. Samples negative for HPV 16 E6 by ethidium bromide gel were further analyzed by performing Southern blotting of the PCR product. A 210-bp chemiluminescent probe was applied to the membrane-bound PCR product to detect low-level amplification product that was not evident by visual inspection alone (ECL direct nucleic acid-labeling and detection systems; Amersham Life Science, Little Chalfont, Buckinghamshire, England).

**HPV and Lymph Node Involvement.** Regional lymphadenectomy had been performed in 47 of the 52 TSCC patients, including all 24 of the HPV-positive cases. From the 24 HPV-positive tonsil tumors 4 were identified with no metastatic involvement of regional nodes. The remaining 20 patients had from 1 to 30 lymph nodes involved with metastatic squamous cell cancer. Extracapsular involvement was reported in 9 patients. No tissue was available for this study for 4 of the patients with metastatically involved lymph nodes, leaving a final sample size of 16 for analysis of positive lymph nodes in patients with HPV-associated tumors. Up to three metastatically involved nodes (depending on availability of tissue) were selected from each of the 16 patients for PCR evaluation for HPV DNA. Benign lymph nodes were also sampled from 8 patients with HPV 16-associated tumor and histological evidence of nodal metastases. One to four representative positive and negative lymph nodes were sampled from non-HPV-associated tumors.

**HLA Detection.** Using a commercially available HLA-DRB detection kit (Dynal) with the HLA-DRB Pattern Matching program, the HLA-DRB-status in each patient was determined according to manufacturer's instructions.

**Statistics.** Among the TSCC patients, associations between HPV infection and HLA-DRB1 expression, chemical risk factors, and histopathological features were evaluated based on Fisher's exact test, the two-sample *t* test, or the Wilcoxon rank-sum test, as appropriate.

The association between HPV, HLA-DRB1 expression, chemical risk factors, and the presence (*versus* absence) of TSCC was summarized using ORs. Corresponding 95% CIs were estimated from two separate analyses, unmatched and matched, because gender and age- (+/−5 years) matched controls with DNA of sufficient quality could not be identified for all TSCC patients. In the unmatched analysis, each risk factor was evaluated in a separate logistic regression model, adjusted for age and gender. The matched analysis was performed on just the 48 TSCC patients and their matched controls by fitting conditional logistic regression models, adjusted for age. An OR summarizes the odds of TSCC in patients with a risk factor relative to the odds of TSCC in patients without a risk factor and indicates how much more likely TSCC is to occur in a patient with a risk factor present.

Follow-up was calculated from the date of the resection to the date of last follow-up or death. Estimates of OS, CSS (death because of TSCC), and DFS were calculated using the Kaplan-Meier method. DFS was defined as survival free of having a local or regional recurrence or distant metastasis. Multivariable Cox regression models were fit to assess the association between HPV and survival after adjusting for age. The association between HPV and survival was summarized with a HR and corresponding 95% CI. The calculated HRs indicate how much more likely a TSCC patient will not survive if they are HPV negative *versus* HPV positive. All calculated *P*s were two sided, and $P$s $< 0.05$ were considered statistically significant.

## RESULTS

**Prevalence of Risk Factors.** Among the 52 patients with TSCC, the average age was 61.7 years (range, 40–85 years), and 83% were male. A history of tobacco and alcohol use was more common among the study patients with TSCC than among the controls. Of the patients with TSCC, 44.2% had a history of smoking and consumption of a moderate to severe amount (several or more drinks per week) of alcohol; an additional 42.3% had a history of smoking and alcohol consumption of one drink or less per week. The remaining 13.5% of the TSCC patients did not have a history of smoking and reported alcohol consumption of one drink or less per week. In comparison, these rates were 18.8, 35.4, and 45.8%, respectively, among the control patients.

**HPV and HLA-DRB1.** The amplification pattern of the tumor DNA with the HPV L1, nested HPV L1, and specific HPV 16 E6 primer pairs showed the presence of HPV DNA in a total of 24 (46.2%) of the 52 TSCC specimens (Table 1). HPV types were determined in all positive specimens (HPV 16: $n = 21$, HPV 12: $n = 2$, and HPV 59: $n = 1$). The presence of the high-risk HPV virus type 16 was detected in all of the L1 HPV 16-positive specimens using HPV 16-specific E6 primer pair. Southern blot performed on the PCR product for remaining HPV-negative tumor samples did not detect any additional HPV 16-positive specimens. Three samples were positive for HPV in

*Table 2* Correlation of HLA-DRB1 with the presence of HPV[a]

| Risk factor | TSCC ($n$ = 52) $n$ (%) | Controls ($n$ = 48) $n$ (%) |
|---|---|---|
| Expression of HLA-DRB1 | | |
| Present | 20 (38.5%) | 14 (29.2%) |
| Absent | 25 (48.1%) | 33 (68.7%) |
| Unknown | 7 (13.5%) | 1 (2.1%) |
| Presence of HPV and HLA-DRB1 | | |
| Both present | 11 (21.2%) | 2 (4.2%) |
| HPV only | 11 (21.2%) | 1 (2.1%) |
| HLA-DRB1 only | 9 (17.3%) | 12 (25.0%) |
| HPV present/HLA status unknown | 2 (3.8%) | 0 |
| HPV absent/HLA status unknown | 5 (9.6%) | 1 (2.1%) |
| Neither | 14 (26.9%) | 32 (66.7%) |

[a] One control patient had no chart documentation on smoking history.

the L1 region but did not amplify with the HPV 16-specific primers (Table 1). By sequence analysis, HPV 12 was detected in two (8.3%) and HPV 59 in one (4.2%) of the 24 HPV-positive cancers (Table 1). The matching rates of the sequencing products to the published sequences of the HPV types were always >96%. In the 48 control samples, DNA amplification was negative with all primers used for the cancer specimens. However, HPV 16 could be detected by Southern blot in a total of 3 (6.3%) samples (Table 1), indicating very small quantities of viral DNA.

In 20 (38.5%) of the TSCC patients, HLA-DRB1 expression was detected. In comparison, HLA-DRB1 expression was detected in 14 patients (29.2%) of the control group (Table 2).

**Associations between HPV and Other Risk Factors among TSCC Patients.** Among the TSCC patients, those with HPV DNA were less likely to have a history of smoking and were younger. Nearly all (27 of 28; 96.4%) of the TSCC patients without HPV had a history of smoking, compared with 75% of the TSCC patients with HPV ($P$ = 0.04). One patient had neither HPV infection nor exposure to chemical risk factors but was of advanced age (83 years). The mean age of the TSCC patients with HPV DNA was significantly lower than the mean age for the TSCC patients without HPV infection (mean, 56.6 *versus* 66 years; $P$ = 0.001). There was no statistically significant association between HPV and gender, alcohol history, or HLA-DRB1 expression among the patients with TSCC. Of the 24 TSCC patients positive for HPV, 45.8% had HLA-DRB1 expression compared with 32.1% of the TSCC patients without HPV infection. Because only three of the control patients had HPV, the association between HPV and the other risk factors could not be assessed in the control group.

**Evaluation of Risk Factors for TSCC.** The odds for patients with HPV DNA to develop TSCC were 18.2 times greater (95% CI for OR, 4.6 and 73.1) than the odds for patients without HPV DNA, after adjusting for age and gender. In addition, the odds were significantly increased for patients with a history of smoking or alcohol usage, respectively (Table 3). Because HPV was associated with smoking history among the patients with TSCC, it was of interest to evaluate the OR for HPV after adjusting for age, gender, and smoking history. After controlling this potential confounder, the OR increased to 42.6 (95% CI 6.6 and 273.4). The odds for patients with HLA-DRB1 expression to develop TSCC was 1.8 (95% CI 0.7 and 4.3) times greater than the odds for patients without HLA-DRB1 expression. However, because the 95% CI for this association bounds 1, the odds of developing TSCC was not significantly different among patients with *versus* without HLA-DRB1 expression. Furthermore, having both HPV DNA and HLA-DRB1 expression did not further increase the risk of TSCC compared with patients with HPV DNA only, after adjusting for age and gender (OR 0.3; 95% CI 0.03 and 4.3). Eleven of the 13 patients with HPV and HLA-DRB1 expression developed TSCC compared with 13 of the 14 patients with HPV DNA only. The results were similar based on a matched analysis of just the 48 of the 52 TSCC patients with matched controls and are reported in Table 3.

**Histopathological Correlation of HPV with TSCC.** Of the HPV-positive patients, 2 (8.3%) presented as stage I, 1 (4.2%) as stage II, 1 (4.2%) as stage III, and 20 (83.3%) as stage IV. Of the HPV-negative patients, 2 (7.1%) presented as stage I, 3 (10.7%) as stage III, 21 (75%) as stage IV, and 2 (7.1%) had unknown stage (Table 4). There was no statistically significant association between stage at presentation and presence of HPV ($P$ = 0.95). Regional lymphadenectomy was performed in 47 of the 52 TSCC patients and all HPV-positive TSCC patients. Seventeen (70.8%) of the HPV-positive patients received radiation therapy as opposed to 18 (64.3%) of the HPV-negative patients. Four HPV-positive patients (16.7%) required a partial mandibulectomy as opposed to 11 of the HPV-negative patients (39.3%). Postoperative radiation therapy was used for patients with close surgical margins, extracapsular spread, and N2 or N3 neck disease.

Pathologically, the median size of the tumor specimen was 2.2 cm for the HPV-positive group *versus* 3.5 cm for the HPV-negative group ($P$ = 0.015). Patients in the HPV-positive group tended to have lower T stages compared with patients in the HPV-negative group ($P$ = 0.038). In terms of degree of atypia, there were no patients in either group with grade one squamous cell carcinoma. Of the HPV-positive specimens, 3 (12.5%) had grade 2 squamous cell carcinoma, 21 (87.5%) had grade 3, and no patients had grade 4 malignancies. Of the HPV-negative specimens, the degree of patients with grade 2, 3, and 4 squamous cell carcinoma was 3 (10.7%), 21 (75%), and 4 (14.3%), respectively. There was no statistically significant association between presence of HPV and grade ($P$ = 0.22). A portion (43%; 10 of 23) of HPV-positive specimens exhibited keratinization *versus* 66.7% (18 of 27) of the HPV-negative specimens ($P$ = 0.10).

**Lymph Node Analysis.** Results of the lymph node analysis are presented in Table 5. Fourteen patients were identified with HPV 16-associated tumors and lymph node metastases; 2 had other HPV types. A total of 27 positive nodes from the 14 HPV 16 patients were processed for HPV detection as described above. HPV 16 DNA was identified in all of the metastatically involved nodes. HPV 16 was not identified in lymph nodes from the 2 patients with node-positive tumors that were associated with HPV types other than HPV 16. Twenty-three histologically negative lymph nodes were selected from 8 of the HPV 16-associated, node-positive tumors. HPV DNA was detected in 17 of these 23 nodes. The only patient with metastatic disease

*Table 3* Summary of the association of risk factors with TSCC

The ORs for tonsillar squamous cell car were evaluated based on a matched and an unmatched analysis. ORs 95% CIs, and *P* are given.

| Risk factor | Unmatched analysis[a] | | Matched analysis[b] | |
|---|---|---|---|---|
| | OR (95% CI)[c] | *P* | OR (95% CI) | *P* |
| Presence of HPV: | | | | |
| Yes *vs.* no | 18.2 (4.6, 73.1) | <0.001 | 26.3 (3.2, 213.9) | 0.002 |
| Presence of HLA DRB1: | | | | |
| Yes *vs.* no | 1.8 (0.7, 4.3) | 0.19 | 1.6 (0.5, 5.0) | 0.38 |
| Smoking history: | | | | |
| Past/current *vs.* never | 8.0 (2.4, 26.2) | <0.001 | 17.7 (2.3, 138.9) | 0.006 |
| Alcohol history: | | | | |
| Several or more per week-*vs.* one or less drinks per week | 4.0 (1.5, 10.5) | 0.005 | 3.4 (1.3, 9.0) | 0.015 |

[a] Results based on an unmatched analysis of 52 TSCC patients and 48 controls, after adjusting for the matched factors, age, and gender.
[b] Results based on a matched analysis of the 48 TSCC patients and their matched controls.
[c] An OR summarizes the odds of TSCC in patients with the risk factor relative to the odds of TSCC in patients without the risk factor.

*Table 4* Characteristics of squamous cell carcinoma of the tonsil by presence or absence of HPV[a]

| Characteristic | Human papillomavirus[b] | | |
|---|---|---|---|
| | Present ($n$ = 24) | Absent ($n$ = 28) | *P* |
| Tumor, *n* (%) | | | 0.038 |
| T1 | 9 (37.5) | 4 (14.3) | |
| T2 | 7 (29.2) | 9 (32.1) | |
| T3 | 4 (16.7) | 3 (10.7) | |
| T4 | 3 (12.5) | 10 (35.7) | |
| Unknown | 1 (4.2) | 2 (7.1) | |
| Nodal involvement, *n* (%) | | | 0.88 |
| NX | 0 (0) | 4 (14.3) | |
| N0 | 4 (16.7) | 3 (10.7) | |
| N1 | 2 (8.3) | 2 (7.1) | |
| N2 | 17 (70.8) | 16 (57.1) | |
| N3 | 1 (4.2) | 1 (3.6) | |
| Unknown | 0 (0) | 2 (7.1) | |
| Stage, *n* (%) | | | 0.95 |
| I | 2 (8.3) | 2 (7.1) | |
| II | 1 (4.2) | 0 (0) | |
| III | 1 (4.2) | 3 (10.7) | |
| IV | 20 (83.3) | 21 (75.0) | |
| Unknown | 0 (0) | 2 (7.1) | |
| Tumor size, cm | | | 0.015 |
| Median | 2.2 | 3.5 | |
| Range | 1.0–5.5 | 1.0–8.3 | |
| Histologic differentiation, *n* (%) | | | 0.22 |
| Grade 1 | 0 (0) | 0 (0) | |
| Grade 2 | 3 (12.5) | 3 (10.7) | |
| Grade 3 | 21 (87.5) | 21 (75.0) | |
| Grade 4 | 0 (0) | 4 (14.3) | |
| Keratinization, *n* (%) | | | 0.10 |
| Yes | 10 (41.7) | 18 (64.3) | |
| No | 13 (54.2) | 9 (32.1) | |
| Unknown | 1 (4.2) | 1 (3.6) | |

[a] Patients with unknown characteristics were ignored in the assessment of statistical significance.
[b] Values are number (percentage) unless otherwise indicated.

without evidence of HPV 16 in the pathologically negative nodes had a nodal stage of N1, whereas all of the other patients were N2.

One tumor was associated with HPV59 and one with HPV 12 had metastatically involved lymph nodes. The patient with the HPV 59-associated tumor had three metastatically involved lymph nodes. Nested L1 PCR produced an appropriate size amplification signal from two of the three the nodes tested. DNA sequencing confirmed HPV 59. The patient with the HPV 12-associated tumor had two metastatically involved lymph nodes. L1 consensus primer amplification failed to show a characteristic HPV amplification signal from the two nodes. Nested PCR was again used, but no band was detected. Additional attempts to amplify HPV DNA using type-specific HPV 12 E6 primers or alternative consensus primers GP5+/6+ (21) and ME/MH[4] also failed to produce a band.

All other nodes tested did not contain detectable HPV DNA, including three noninvolved lymph nodes from the HPV 59-associated tumor, as well as involved and uninvolved nodes from tumors that were not associated with HPV. All of the mouse DNA-negative controls were negative for HPV 16, whereas all of the positive controls were positive for HPV 16.

**Survival Analysis among TSCC Patients.** Among the 31 patients alive at last follow-up, the median duration of follow-up was 3.7 years with a range of 0.3 to 12.3 years. Fourteen of the 52 patients have experienced a local or regional recurrence or distant metastasis, including 3 with local recurrences only; 2 local and regional recurrences; 6 regional recurrences; 1 local, regional and breast metastasis; 1 regional recurrence and lung metastasis; and 1 lung metastasis. Four of these occurred in HPV patients (1 local and 3 regional). Overall, the DFS at 3 years was 74.6%. Presence of HPV was not significantly associated with DFS (at 3 years; HPV positive, 85.2%; HPV negative, 66.1%; $P = 0.19$).

Of the 21 deaths, 14 were related to disease. At 3 years, the OS and CSS were 64.1 and 73.3%, respectively. The absence of HPV was significantly associated with poorer OS (at 3 years; HPV positive, 80.8%; HPV negative, 52%; HR = 2.8; $P = 0.033$) and CSS (CSS at 3 years; HPV positive, 95.2%; HPV negative, 57.3%; HR = 5.1; $P = 0.018$; Fig. 1). However, after

[4] B. S. Gostout, S. E. Strome, A. C. Clayton, R. M. McGovern, K. D. Olsen, and M. J. Webb. Two cases of coincident carcinomas of the head and neck and the uterine cervix. Accepted for publication in Gyn. Oncology.

*Table 5* PCR detection of HPV DNA in lymph nodes of patients with tonsil carcinoma

| Tumor HPV status | No. tumors tested | Lymph node metastases identified? | Histology of nodes for PCR | No. nodes tested | PCR Results | |
|---|---|---|---|---|---|---|
| | | | | | HPV 16 primers | Consensus primers |
| HPV 16 | 14 | Yes | Positive | 27 | Positive | |
| HPV 12 | 1 | Yes | Positive | 2 | Negative | Negative |
| HPV 59 | 1 | Yes | Positive | 3 | Negative | 2/3 Positive |
| HPV 16 | 8 | Yes | Negative | 23 | 17/23 Positive | |
| HPV 59 | 1 | Yes | Negative | 3 | Negative | Negative |
| HPV 16 | 3 | No | Negative | 9 | Negative | |
| HPV 12 | 1 | No | Negative | 3 | Negative | Negative |
| HPV negative | 5 | Yes | Positive | 10 | Negative | Negative |
| HPV negative | 5 | Yes | Negative | 14 | Negative | Negative |
| HPV negative | 5 | No | Negative | 15 | Negative | Negative |

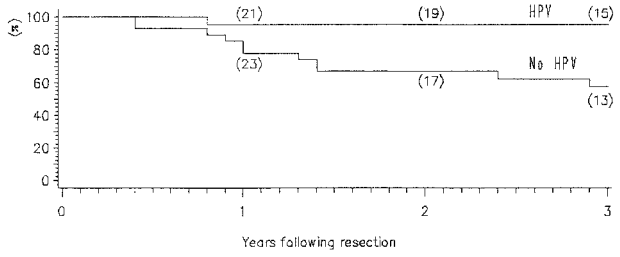


*Fig. 1* CSS after resection in TSCC patients with and without HPV. The numbers in parentheses indicate the number of patients still at risk at each time point. The absence of HPV was significantly associated with poorer CSS (at 3 years; HPV positive, 95.2%; HPV negative, 57.3%; HR = 5.1, $P = 0.018$). However, after adjusting for age in a Cox regression model, there was no statistically significant association between the presence of HPV and CSS [HR = 2.5, 95% CI (0.5, 12.5), $P = 0.26$]. Given that age at resection was associated with presence of HPV (*i.e.*, patients with HPV were more likely to be diagnosed at a younger age than those without HPV), HPV presence was not identified as an independent factor in a multivariable model, including both age and presence of HPV as covariates.

adjusting for age in a Cox regression model, there was no statistically significant association between the presence of HPV and OS [HR = 1.3 for HPV negative *versus* positive, 95% CI (0.4 and 3.9; $P = 0.65$)] or CSS [HR = 2.5 for HPV negative *versus* positive, 95% CI (0.5 and 12.5; $P = 0.26$)]. Given that age at resection was associated with presence of HPV (*i.e.*, patients with HPV were more likely to be diagnosed at a younger age than those without HPV), HPV presence was not identified as an independent factor in a multivariable model, including both age and presence of HPV as covariates.

## DISCUSSION

This investigation was designed as a retrospective case control study to define the prevalence of HPV in TSCC and evaluate the possible coexistence of HPV DNA and HLA-DRB1 as independent risk factors in TSCC. To further evaluate the role of HPV in oncogenesis, molecular analysis was extended to determine the presence of HPV DNA in the lymph nodes of patients with and without metastatic disease. Results from primary site analysis were correlated with patient exposure to known chemical carcinogens to characterize the relative risk of malignancy in tonsillar tissue containing HPV DNA and the impact of the presence of HPV on OS. The critical findings of this study are that HPV is an independent risk factor for the development of TSCC. We were not able to show a statistically significant association with HPV-related tonsil cancer and the presence of the HLA DRB1 haplotype, although there is a trend toward increased frequency of DRB1 in cases compared with controls. In cervix cancer patients, a DRB1 association has been demonstrated in United States southwestern Hispanic women (18, 19) and recently in a cohort of Midwestern Caucasian women.[5]

Our results establish a strong association between HPV 16 and TSCC. In fact, HPV was identified in only 3 patients with benign tonsillar hyperplasia (Table 2), and the limited amount of viral DNA in this control group required Southern blot of PCR product for identification. Less sensitive tests gave positive results in all patients from the cancer group who were positive for HPV, indicating greater HPV DNA in the cancer patients. We found HPV 16 to be the predominant type in tonsillar cancers. However, we must acknowledge a potential bias toward detecting type 16 in that type-specific E6 primers are used for HPV type 16 only. If the type-specific primers have greater sensitivity, or if the viral integration results in L1 loss in some samples, our results would be skewed toward detection of HPV 16. This has not been a major problem with genital tract neoplasms.

Interestingly, HPV was identified in both the pathologically positive and negative nodes of patients with HPV-positive TSCC who developed regional metastases. HPV was not identified in the lymph nodes of patients with HPV-positive TSCC without metastatic disease. In cancer of the uterine cervix, the strict preservation of HPV DNA in metastatic lesions reflects viral integration into the host genome in most cases and is consistent with the critical role of the E6 and E7 oncogenes in supporting the transformed state. We have demonstrated conservation of HPV DNA in cervical lymph node metastasis from 14 HPV 16-associated tonsil cancers. The identification of HPV 16 DNA in histologically negative nodes may represent tumor metastases that are too small to be reliably detected histologi-

[5] B. S. Gostout, unpublished data.

cally. Alternatively, the detected HPV DNA in histologically negative nodes may represent debris from tumor cell necrosis or debris from a positive lymph node transferred during processing. Regardless, there is extensive spread of viral DNA from tumors with involved nodes compared with the absence of spread from tumors with uninvolved nodes.

Similar to previous reports, the present trial identified an association between the presence of HPV and decreased tobacco usage and identified a survival advantage in patients with HPV-positive TSCC; however, the association between HPV and survival was not statistically significant after adjusting for age (7). The potential role of the tonsils in HPV-mediated cancer is not well documented. Only two authors discriminate accurately enough between different anatomical sites of the oropharynx to allow conclusions on differing susceptibility between anatomical sites (1, 5). Both come to the conclusion that Waldeyer's tonsillar ring and the oropharynx, respectively, show an especially high incidence of HPV-associated cancer. Our study, to the best of our knowledge, investigates for the first time a sufficiently large number of TSCC patients to allow conclusions on the specific risk of viral oncogenesis, for this tissue type and lends considerable support to the hypothesis that HPV 16 plays a causal role in certain cancers of the head and neck region.

The 1 patient with HPV 12 detected in her tumor, but not in metastatically involved lymph nodes, may be an important demonstration of the converse. When HPV is associated with a tumor in a noncausal role, the viral DNA is not strictly preserved in metastases. HPV 12 is generally regarded as a nononcogenic, group B HPV. This group of papillomaviruses has oncogenic capacity, but immune competent individuals appear to be resistant to cancers caused by this HPV group. The patient with the HPV 12-associated tumor was not immunosuppressed. Thus, the HPV 12 detected may simply represent mucosal carriage of a virus not causally related to the malignancy. Because of the limited number of HPV subtypes other than HPV 16 identified in this and other studies of head and neck squamous cell cancer, it remains uncertain whether the association of specific HPV subtypes with TSCC promotes consideration of high- or low-risk grouping of HPV subtypes.

Correlation of HPV status with known chemical risk factors and disease-specific outcomes provides an important link between infection and potential clinical intervention. Among patients with cancer, those with HPV infection were less likely to have a history of smoking and were younger. More importantly, whereas both 3-year OS and CSS were significantly improved in patients with HPV infection, this effect was lost after age adjustment using a Cox regression model (Fig. 1). These findings are not the result of differences in tumor stage or histopathological type as these parameters did not differ significantly among the two groups. Our findings differ from those reported recently by Gillison *et al.*, which included 60 patients with primary tumors from all oropharyngeal sites and identified a significant survival advantage in patients with HPV-positive squamous cell cancer. Potential explanations for these diverse findings include a lack of site specificity in the Gillison trial, because the present study was strictly limited to a single subsite of the oropharynx. Additionally, the historical treatment bias for patients with tonsillar carcinoma at the Mayo Clinic has been surgical with radiotherapy used in an adjuvant setting depending on defined prognostic indicators. In the trial by Gillison *et al.* (7), 25% of patients were treated with definitive radiotherapy. Thus, observed differences in patient outcomes may result from institutional preferences regarding treatment.

Preventive strategies can reduce HPV infection and might decrease the prevalence of TSCC. Screening methods for early diagnosis of tonsillar cancer should be developed to screen for oncogenic viruses. Additional studies to investigate the behavior of HPV-induced oropharyngeal squamous cell carcinoma could offer new treatment strategies with improved results. The preservation of HPV DNA in the metastases of HPV-associated tumors provides a rational basis for immune therapy directed toward viral antigens. HPV vaccination trials are under way for prevention and treatment of HPV-related cancers. This report and other recent evidence supporting a role for HPV in a subset of tonsil cancers emphasize the need for vaccine therapies and other novel antiviral therapies for the prevention and treatment of HPV-related cancers of the head and neck.

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# Squamous Cell Carcinoma of the Tonsils: A Molecular Analysis of HPV Associations

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