Human Papillomavirus and Epstein-Barr Virus Infection, p53 Expression, and Cellular Proliferation in Laryngeal Carcinoma

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Key Words: Laryngeal cancer; Human papillomavirus; HPV; Epstein-Barr virus; EBV; p53; Tumor growth fraction

Abstract

Laryngeal carcinomas are aggressive neoplasms with controversial association with the human papillomavirus (HPV) and Epstein-Barr virus (EBV). So far, the impairment of p53 protein function and its impact on cellular proliferation has not been studied adequately in these tumors. In this work, molecular biologic techniques were used to assess the frequency of HPV and EBV in 110 squamous cell carcinomas of the larynx. In addition, accumulation of p53 and Ki-67 cell proliferation antigen expression in malignant cells was assessed by immunohistochemical analysis. High-grade HPV was found in 37.3% of cases, and none had demonstrable EBV infection. Accumulation of p53 was found in 78.2% of the cases, and it was related to a high Ki-67 labeling index and higher histologic grade. The results demonstrate association of HPV with more than one third of laryngeal carcinomas studied, mainly glottic tumors. Tumors with increased cell proliferation were more frequently high grade, with p53 accumulation and lymph node metastasis.

Head and neck cancers, which include tumors from the oral cavity, pharynx, paranasal sinus, and larynx, have a high prevalence and complex pathogenesis. In this setting, laryngeal malignant tumors deserve special attention among all head and neck cancers. According to the International Agency for Research on Cancer and World Health Organization, 161,403 new cases of laryngeal cancer were reported worldwide in 2000. Laryngeal cancer represents 1.5% of all new cases of cancer diagnosed yearly and 30% and 12% of all new cases of head and neck tumors in males and females, respectively. These tumors are responsible for high mortality rates (1.4% of all deaths due to cancer each year) and high morbidity.

Histologically, more than 95% of all laryngeal cancers are squamous cell carcinomas (SCCs). Although it is well known that tobacco and alcohol abuse are major risk factors for the development of laryngeal SCC, infectious agents also may be implicated in some cases. Human papillomavirus (HPV) is the most frequently studied virus that seems to be associated with laryngeal SCC, but some authors claim that Epstein-Barr virus (EBV) also might have a role in the pathogenesis of these tumors. In addition, there are conflicting data in the literature about the importance of some putative prognostic markers in laryngeal SCC, such as cell proliferation markers and expression of the product of the TP53 tumor suppressor gene, the p53 protein.

The aims of the present study were to assess the frequency of HPV infection, EBV infection, p53 accumulation, and Ki-67 cell proliferation antigen in SCC of the larynx and to study a possible association of each with clinicopathologic data.
Materials and Methods

The study was approved by the Research Ethical Committee, Botucatu Medical School, State University of São Paulo, Botucatu, São Paulo, Brazil. We retrospectively evaluated 110 cases of laryngeal SCC from patients treated between April 1980 and July 1999 at Botucatu Medical School. Information retrieved from patient medical files included personal data, tobacco use and alcohol consumption habits, symptoms at diagnosis, primary tumor localization, initial diagnosis, and TNM staging. H&E-stained tissue slides were reviewed for diagnosis and histologic grading. Unstained slides from formalin-fixed, paraffin-embedded tumors (FFPET) were used for immunohistochemical analysis for detection of p53 protein and Ki-67 antigen and for RNA in situ hybridization (RISH) for detection of the EBV-encoded small RNAs 1 (EBER-1). In addition, DNA was extracted from FFPET samples to assess HPV infection by polymerase chain reaction (PCR).

EBV EBER-1 RISH

EBV infection was assessed by RISH with a biotinylated probe for RNA EBER-1, an EBV transcript actively expressed in latently infected cells.5,6 Briefly, tissue sections were placed on poly-D-lysine–treated glass slides and incubated at 70°C for a day. After the slides were routinely deparaffinized, endogenous peroxidase was blocked with a 3% solution of hydrogen peroxide. The enzymatic digestion was performed with Proteinase K (0.02 µg/µL final concentration); the tissue samples subsequently were dehydrated in ethanol and dried at room temperature. The slides were incubated in prehybridization solution (20 mmol/L sodium phosphate/1× Denhardt solution/0.1% dextran sulfate) for 60 minutes at 37°C. After addition of the EBER-1 biotinylated probe (0.25 ng/mL final concentration), tissue samples were incubated overnight at 37°C in a humid chamber. Signal amplification was done with the ABC Elite kit (Vector, Burlingame, CA), and revelation by using a 3,3′-diaminobenzidine (Sigma, St Louis, MO) solution. Cases were evaluated under a conventional light microscope. Positive p53- or Ki-67–immuno-stained cells showed dark brown to black nuclear deposition of chromogen at the antibody-antigen binding site.

Ki-67 Labeling Index Calculation

Although p53 accumulation was evaluated qualitatively, Ki-67+ cases were analyzed further for growth fraction estimation by quantitative semiautomated image analysis with Kontron KS-300 software (Carl Zeiss Vision, Jena, Germany) Image 1I. Briefly, 5 images of different microscope fields (<500 original magnification) of each Ki-67–stained tissue sample were captured. To select Ki-67+ nuclei (Image 1A), each image is evaluated by the KS-300 software under the instructions of a newly developed automation macro (available by request to the authors). The software creates a mask image for analysis (Image 1B) and shows the automatic count results of Ki-67+ cells (Image 1C). As in some situations, positive nuclei are miscounted by the software (eg, because of weak immunostaining, overlapping cells), image analysis requires operator intervention to achieve optimal results Image 2I. At the end, the estimated growth fraction is defined by the number of Ki-67–labeled nuclei divided by the total area of tissue in the image evaluated.

DNA Extraction From FFPET

For DNA extraction, at least 2 sections of 25 µm were obtained from FFPET samples. Briefly, deparaffinization was performed with xylene treatment (10 minutes at 64°C, 10 minutes at room temperature with agitation, and another 10 minutes at 64°C). Later, the samples were centrifuged for 5 minutes (at 15,800g), and xylene was removed. This sequence was repeated twice; afterwards, tissues were dehydrated with ethanol and centrifuged for 10 minutes (at 15,800g). Ethanol was removed, and the steps were repeated once more. Tissue sections were incubated overnight with Proteinase K (400 ng/µL in 10 mmol/L of tris(hydroxymethyl)aminomethane (Tris)/1 mMol/L of EDTA/0.5% polysorbate-20 buffer) at 56°C. After enzyme inactivation by heat (94°C for 10 minutes), DNA purification was performed by adding 100 µL of 5-mmol/L concentration of sodium chloride plus 100 µL of cetyl trimethyl ammonium bromide/sodium chloride solution (0.87 mol/L of sodium
chloride and 0.34 mol/L of cetyl trimethyl ammonium bromide) to the samples. After 10 minutes of incubation at 65°C, the samples were treated with chloroform/isoamylic alcohol 24:1 and centrifuged for 5 minutes (at 15,800 g). The upper phase was transferred to a new tube with ethanol and kept at –20°C for 10 minutes. After another centrifugation (15,800 g), the precipitated DNA was treated with 70% ethanol at room temperature, and the pellet was resuspended in 50 µL of TE solution (10 mmol/L of Tris-HCl, pH 7.5, and 1 mmol/L of EDTA). DNA quantity and quality were verified, respectively, by UV spectrophotometry and agarose-gel electrophoresis. Samples in which the DNA concentration was higher than 0.4 µg/µL were diluted to 0.1 µg/µL to avoid PCR inhibition.

Assessment of HPV Infection by PCR

HPV infection was first evaluated by PCR with consensus primers GP5+ and GP6+, which give an amplicon of 138 to 150 base pairs (bp) and allows detection of a broad spectrum of distinct HPV genotypes. To assure the results of HPV infection, HPV+ cases were evaluated once more for GP5+/GP6+ amplification with a more stringent PCR protocol (see conditions in Table 1). Cases that remained positive for HPV amplification in the second GP5+/GP6+ PCR were evaluated with a multiplex PCR with specific primers directed to HPV-6 or HPV-11, HPV-16, and HPV-18. Positive control samples used were DNA extracted from HeLa cells (used in GP5+/GP6+ PCR and as the HPV-18-positive control samples in the multiplex PCR), DNA from CaSki cells (HPV-16 positive control sample in the multiplex PCR), and 1 case of anal condyloma acuminatum (HPV-6/HPV-11 positive control sample in multiplex PCR). All negative DNA samples were evaluated for PCR suitability by amplifying a 123-bp segment of the β-chain globin gene with the primers PCO3+ and PCO4+. PCR reactions were monitored for

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**Image 1** Semi-automated image analysis for growth fraction estimation in laryngeal squamous cell carcinomas. Immunohistochemical reaction for Ki-67 cell proliferation antigen was performed, and immunostained tissues were evaluated quantitatively by software (see text for details). A, Ki-67 immunostained laryngeal squamous cell carcinoma. B, Automated software selection of Ki-67–labeled nuclei of neoplastic cells. C, Image result after automatic software count of Ki-67–labeled nuclei of neoplastic cells (A-C, methyl green counterstain, original magnification ×500).
crossover contamination with amplification of samples with no DNA; in addition, DNA extracted from a known HPV–tissue sample was used. Table 1 and **Table 2** show primers and PCR assay conditions, respectively.

**Data Analysis**

Descriptive statistics were used for evaluation of clinicopathologic data. Analysis of variance was used to verify all possible correlations among categorical variables (eg, tumor localization, TNM staging, clinical stage, and histologic grade) and continuous variables (eg, age at diagnosis, Ki-67 labeling index). The analysis of categorical variables was performed with contingency 2 × 2 tables and the $\chi^2$ test when possible. Results were considered statistically significant when $\alpha$ was less than .05; a trend was considered when $\alpha$ ranged between .05 and .1.

**Results**

**General Data**

Among 110 patients with laryngeal carcinomas, 89 (80.9%) were men and 21 (19.1%) were women. Age at diagnosis ranged from 25 to 86 years (mean, 59.4 years). Symptoms that induced medical consultation were dysphonia (79.1%), odynophagia (33.6%), dysphagia (25.5%), and dyspnea (21.8%); lymphadenopathy (mainly cervical) and otalgia were found less frequently (7.3% and 2.3%, respectively).

Almost all patients were tobacco users (100/110 [90.9%]). A mean of 49.1 pack-years (py, expressed as number of packs of cigarettes smoked per day multiplied by the number of smoking years) was verified. Considering smoking habits, patients with laryngeal carcinoma could be grouped as nonsmokers (py, 0; 9.1%), light smokers (py, <10, 4.5%), moderate smokers (py, ≤10 to <20, 2.7%), and heavy smokers (py, ≥20, 53.6%). Owing to insufficient data about tobacco use, 30 patients (27.3%) could not be further classified. In addition, 59 tobacco users also reported alcohol consumption (37.3% distilled drinks, 5.1% fermented drinks, and 3.4% both types), in volumes of less than 500 mL (25.9%), 500 mL to 1 L (20.3%), or more than 1 L (10.2%), and frequency that ranged from occasional (18.6%) to daily (59.3%).

**Table 1**

**PCR Conditions for HPV Detection and Genotyping and β-Globin Amplification**

<table>
<thead>
<tr>
<th>PCR Purpose</th>
<th>Primers Used</th>
<th>PCR Reaction</th>
<th>Thermocycling</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV screening</td>
<td>GP5+/GP6+</td>
<td>PCR buffer (28 mmol/L of Tris-HCl, pH 8.4; 70 mmol/L of KCl; 3 mmol/L of MgCl₂, 0.2 mmol/L of each dNTP; 1 U Taq DNA polymerase, 0.5 µmol/L of each primer, and glycerol 10%)</td>
<td>1 cycle: 94°C, 5 min; 45 cycles: 94°C, 30 s; 45°C, 45 s; 72°C, and 1 min; and 1 cycle: 72°C, 7 s</td>
</tr>
<tr>
<td>HPV detection validation</td>
<td>GP5+/GP6+</td>
<td>Same as above</td>
<td></td>
</tr>
<tr>
<td>HPV genotyping (HPV-6/HPV-11, HPV-16, and HPV-18; multiplex PCR reaction)</td>
<td>SL6/11 F&amp;R, VdB16 F&amp;R, VdB18 F&amp;R</td>
<td>PCR buffer (28 mmol/L of Tris-HCl, pH 8.4; 70 mmol/L of KCl; 3 mmol/L of MgCl₂, 0.2 mmol/L of each dNTP; 1.25 U Taq DNA polymerase, and 0.5 µmol/L of each primer)</td>
<td>1 cycle: 94°C, 5 min; 40 cycles: 94°C, 1 min; 55°C, 1 min, and 72°C, 1 min; 1 cycle: 72°C, 7 min</td>
</tr>
<tr>
<td>Assessment of DNA suitability for PCR amplification</td>
<td>PCO3+/PCO4+</td>
<td>PCR buffer (28 mmol/L of Tris-HCl, pH 8.4; 70 mmol/L of KCl; 3 mmol/L of MgCl₂, 0.2 mmol/L of each dNTP; 1.25 U Taq, 0.5 µmol/L of each primer)</td>
<td>1 cycle: 94°C, 5 min; 40 cycles: 94°C, 30 s; 55°C, 45 s; and 72°C, 1 min; 1 cycle: 72°C, 7 s</td>
</tr>
</tbody>
</table>

*dNTP, deoxynucleoside triphosphate; F&R, forward and reverse primers; HPV, human papillomavirus; KCl, potassium chloride; MgCl₂, magnesium chloride; PCR, polymerase chain reaction; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.*

*Invitrogen, Carlsbad, CA.*
**Tumor Site, TNM Staging, and Histopathologic Findings**

In tumor localization, laryngeal carcinoma cases were classified as follows: 52 supraglottic, 53 glottic, and 5 infraglottic or transglottic **Table 3**. Despite a male predominance in cases at all tumor sites, we found an important difference in the male/female ratio among patients with glottic tumors in relation to those with supraglottic tumors ($P = .0046$). Also, our data show the importance of tumor localization on symptoms at diagnosis: patients with glottic tumors frequently showed dysphonia (49/53 cases [92%]; $P = .0007$), whereas patients with supraglottic tumors had odynophagia (23/52 cases [44%]; $P = .0102$) and dysphagia (19/52 [37%]; $P = .0120$).

Most patients had advanced disease, as indicated by the high frequency of T3 and T4 cases (31.8% and 20%, respectively).

There were 24 patients (21.8%) with disease in clinical stage 3 and 40 (36.4%) with clinical stage 4. There was a trend for patients with glottic tumors to be at clinical stage 1 or 2 (26/53 cases [49%]), whereas patients with supraglottic tumors frequently had clinical stage 3 or 4 tumors (33/52 cases [63%]; $P = .0718$).

As shown in **Table 4**, tumors were graded as follows: well-differentiated SCC (grade I), 37 (33.6%); moderately differentiated SCC (grade II), 45 (40.9%); and poorly differentiated to anaplastic SCC (grade III), 28 (25.5%). The presence of lymph node metastasis correlated positively with tumor size and grade ($P = .0048$ and $P = .0041$, respectively), and grade III SCCs had significantly more lymph node metastasis than grade I SCCs ($P = .0010$; odds ratio [OR], 7.35). There was an

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**Table 2**

Oligonucleotides Used as Primers for HPV and Genotyping and β-Globin Amplification by Polymerase Chain Reaction

<table>
<thead>
<tr>
<th>Primers</th>
<th>Target</th>
<th>Oligonucleotide Sequence (5'-3')</th>
<th>Amplicon (bp)</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP5+</td>
<td>HPV L1 gene (consensus primers)</td>
<td>TTT GTT ACT GTG GTA GAT ACT</td>
<td>138-150*</td>
<td>de Roda Husman et al8</td>
</tr>
<tr>
<td>GP6+</td>
<td>HPV-6/HPV-11</td>
<td>GAA AAA TAA ACT GTA AAT ATT C</td>
<td>302</td>
<td>Soler et al10</td>
</tr>
<tr>
<td>SL6/11.U</td>
<td>HPV-16</td>
<td>TAC ACT GCT GGA CAA CAT GC</td>
<td>152</td>
<td>van den Brule et al11</td>
</tr>
<tr>
<td>SL6/11.D</td>
<td>HPV-18</td>
<td>GTG CGC AGA TGG GAC ACA C</td>
<td>216</td>
<td>van den Brule et al11</td>
</tr>
<tr>
<td>VdB16.U</td>
<td>HPV-18</td>
<td>TGC TAG TGC TTA GTC AGC AA</td>
<td>123</td>
<td>Saiki et al9</td>
</tr>
<tr>
<td>VdB16.D</td>
<td>HPV-18</td>
<td>ATT TAC TGC AAC ATG ACT</td>
<td>123</td>
<td>Saiki et al9</td>
</tr>
<tr>
<td>VdB18.U</td>
<td>HPV-18</td>
<td>AAG GAT GCT GCA CCG GCT GA</td>
<td>123</td>
<td>Saiki et al9</td>
</tr>
<tr>
<td>VdB18.D</td>
<td>β-globin gene</td>
<td>CAC GCA CAC GCT TGG CAG GT</td>
<td>123</td>
<td>Saiki et al9</td>
</tr>
<tr>
<td>PCO3+</td>
<td>β-globin gene</td>
<td>CTT CTG ACA CAA CTG TGT TCA CTA GC</td>
<td>123</td>
<td>Saiki et al9</td>
</tr>
<tr>
<td>PCO4+</td>
<td>β-globin gene</td>
<td>TCA CCA CCA ACT TCA TCC ACC</td>
<td>123</td>
<td>Saiki et al9</td>
</tr>
</tbody>
</table>

bp, base pairs; HPV, human papillomavirus.
* Amplicon size depends on the HPV genotype.

**Table 3**

Tumor Site, Sex Ratio, and Patient Age at Diagnosis of 110 Laryngeal Squamous Cell Carcinoma Cases Studied

<table>
<thead>
<tr>
<th>Tumor Site</th>
<th>No. (%) of cases</th>
<th>M/F ratio</th>
<th>Age (y)</th>
<th>Mean</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supraglottic</td>
<td>52 (47.3)</td>
<td>2.3:1*</td>
<td>58.5 ± 9.7</td>
<td>25-86</td>
<td></td>
</tr>
<tr>
<td>Glottic</td>
<td>53 (48.2)</td>
<td>12.3:1*</td>
<td>60.1 ± 11.8</td>
<td>41-83</td>
<td></td>
</tr>
<tr>
<td>Transglottic</td>
<td>5 (4.5)</td>
<td>1.5:1</td>
<td>62.0 ± 11.4</td>
<td>47-77</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>10 (100.0)</td>
<td>4.2:1</td>
<td>59.4 ± 10.8</td>
<td>25-86</td>
<td></td>
</tr>
</tbody>
</table>

* $P = .0046$ (odds ratio, 4.97; confidence interval, 1.38-19.47).

**Table 4**

Distribution of 110 Laryngeal Squamous Cell Carcinomas Studied by Tumor Site, Histologic Grade, and Presence of Lymph Node Metastasis*

<table>
<thead>
<tr>
<th>Tumor Site</th>
<th>Supraglottic (n = 52)</th>
<th>Glottic (n = 53)</th>
<th>Transglottic (n = 5)</th>
<th>Total (N = 110)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade I</td>
<td>9 (17)</td>
<td>27 (51)†‡</td>
<td>1 (20)</td>
<td>37 (33.6)</td>
</tr>
<tr>
<td>II</td>
<td>27 (52)†</td>
<td>15 (28)</td>
<td>3 (60)</td>
<td>45 (40.9)</td>
</tr>
<tr>
<td>III</td>
<td>16 (31)†</td>
<td>11 (21)</td>
<td>1 (20)</td>
<td>28 (25.5)</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td>22 (42.3)</td>
<td>13 (24.5)</td>
<td>2 (40)</td>
<td>37 (33.6)</td>
</tr>
</tbody>
</table>

CI, confidence interval; OR, odds ratio.
* Data are given as number (percentage).
† $P < .0003$ (OR, 0.23; CI, 0.07-0.76).
‡ $P = .0046$ (OR, 0.23; CI, 0.07-0.76).
§ $P = .0046$ (OR, 0.23; CI, 0.07-0.76).
association between tumor grade and site: glottic tumors frequently were well-differentiated, whereas supraglottic tumors were mostly of higher grade ($P = .0013$). In addition, supraglottic tumors had almost twice the number of lymph node metastases as glottic tumors ($P = .0232$; OR, 2.82).

**EBV and HPV Infection**

None of the cases had detectable EBV infection in neoplastic cells, as verified by EBER-1 RISH [Image 3]. On the other hand, HPV infection was detected by GP5+ and GP6+ consensus primers in PCR in 41 cases (37.3%). Among all 41 HPV+ cases, PCR with specific primers showed HPV-16 and HPV-18 amplification in 15 (37%) and 18 (44%), respectively [Image 4]. None of the cases had detectable HPV-6/HPV-11 infection, and 8 (20%) of 41 cases could not be amplified with primers directed to HPV genotypes 6/11, 16, and 18. A 123-bp segment of the β-globin gene was amplified successfully in all evaluated cases (data not shown).

HPV infection was more frequent in glottic tumors (24/53 [45%]) than in supraglottic tumors (14/52 [27%]), and this difference was statistically significant ($P = .0274$; OR, 0.39; confidence interval, 0.16-0.98). However, there was no association of HPV infection with tumor size, tumor grade, lymph node metastasis, tobacco use, or clinical stage of the disease. In addition, there was no significant difference between HPV-16 and HPV-18 infection by tumor site.

**p53 Expression and Ki-67 Labeling Index**

As verified by immunohistochemical analysis, most of the SCCs (86/110 cases [78.2%]) had p53 accumulation [Image 5]. However, there was no association of p53 accumulation with tumor size, tumor grade, lymph node metastasis, tobacco use, or clinical stage of the disease.

The Ki-67 labeling index could be calculated by semiautomated image analysis in 104 (94.5%) of the cases. There was a significant correlation between p53 accumulation and a higher Ki-67 labeling index ($P = .0375$), and the Ki-67 labeling index also correlated with tumor grade: grade I SCCs had lower Ki-67 expression than grade II SCCs ($P = .0237$). In addition, there was a trend toward HPV-16+ SCC of the larynx to manifest a higher Ki-67 labeling index than HPV-18+ cases ($P = .0866$).

**Discussion**

The development of epithelial malignant neoplasms in the upper aerodigestive tract is a complex phenomenon because a multitude of carcinogenic agents collaborate for cell transformation at this site. Smoking is the most important independent risk factor for development of laryngeal carcinomas, and it acts in a synergistic way with alcohol consumption. It is noteworthy that nonsmokers with laryngeal carcinoma have a better clinical course than smokers. In 1997, Agudelo and coworkers showed that in patients with no history of tobacco or alcohol use, lesions developed later than in patients with a history of smoking and alcohol consumption. The former group had predominantly glottic tumors, which are easier to diagnose and have a more favorable prognosis. In fact, it was proposed that glottic and supraglottic carcinomas
behave as distinct clinicobiologic entities. In the present study, glottic tumors had a lower histologic grade and a lower rate of lymph node metastasis than supraglottic tumors, features often associated with more favorable clinical behavior.

HPV infection also is implicated as an important risk factor for the development of head and neck carcinomas. HPV DNA was detected successfully in blood serum from patients with head and neck cancer, including laryngeal tumors. In laryngeal SCCs, the viral DNA is detected in frequencies that range from 15% to 50%. This marked variation could be explained partially by the different methods used for HPV detection (including conventional PCR, nested-PCR, PCR–restriction fragment length polymorphism, PCR–enzyme-linked immunosorbent assay, in situ hybridization, Southern blot, and dot blotting). However, a distinct epidemiologic setting for each study also must be considered because there is significant variation in factors that may influence the prevalence of HPV infection in different human populations worldwide.

In normal mucosa of the larynx, HPV can be found in up to 25% of samples, and vocal cord lesions harbor DNA from HPV-6 or HPV-11 in 23% of cases. Pou and coworkers suggested that the presence of HPV in laryngeal papillomas causes an increased risk for cancer development. They also stated that concomitant infection by herpes simplex virus, EBV, or cytomegalovirus is associated with more aggressive disease. Laryngeal papillomas often are associated with infection by low-risk HPV genotypes, and malignant transformation accounts for only 3% to 7% of cases. Fouret and coworkers reported HPV infection in 14% of cases of laryngeal hyperplasia, keratosis, and dysplasia, lesions that may represent premalignant conditions in the larynx. Furthermore, in 1999, Nishioka and coworkers reported a significant increase in the frequency of HPV-16 and HPV-18 infections in cancers from different head and neck sites, including the larynx, nasal sinus, pharynx, and oral cavity.
In the present study, HPV was detected overall in 37.3% of the cases of laryngeal carcinomas. Considering only glottic tumors, the rate increased to 45%, which is reasonable considering the normal histologic features of the larynx and some known viral features. HPV requires terminal differentiation of squamous epithelial cells to complete its biologic cycle. Therefore, it could be speculated that glottic squamous cell epithelium is more prone to HPV dissemination and may be more susceptible to viral oncogenic properties in comparison with normal respiratory epithelium in laryngeal supraglottic and infraglottic sites. This is a plausible mechanism of synergetic association between smoking and HPV infection in laryngeal carcinogenesis because, under persistent chemical injury plus viral infection, squamous cell metaplasia in occurring in nonsquamous laryngeal tissues of smokers is prone to evolve with dysplasia followed by malignant transformation.

In the present study, lack of EBV infection was verified in all cases of laryngeal carcinomas. EBV is highly associated with nasopharyngeal undifferentiated carcinomas, but the association with other head and neck neoplasms is still under debate. In 1985, it was suggested that the biologic reservoir of EBV maps to the respiratory tract, but Liavaag and coworkers did not find the viral genome in virtually all 231 samples evaluated of normal head and neck tissues, including 7 from the larynx. In 1993, Tyan and coworkers reported EBV infection in 6 of 10 cases of laryngeal carcinoma, and only 1 of these showed HPV infection. These results, however, must be considered with caution because the PCR approach used by Tyan and coworkers did not find the viral genome in virtually all 231 samples evaluated of normal head and neck tissues, including 7 from the larynx. In 1997, Logullo and coworkers reported accumulation of LAT in head and neck carcinomas, mainly laryngeal cancers. Because it has been estimated that EBV latently infected cells, including lymphocytes and nonneoplastic epithelial cells. Because it has been estimated that EBV latently infected B cells may be found in approximately 1 of 10^6 normal circulating cells in healthy individuals. EBV false-positive results must be considered when PCR-based methods are used for EBV detection.

Dysfunction of the TP53 tumor suppressor gene seems to be an early event in head and neck cancer, and it is associated with disease progression; p53 protein accumulation often is found in preneoplastic and neoplastic lesions of the larynx. In 1997, Logullo and coworkers reported accumulation of p53 in head and neck carcinomas, mainly laryngeal cancers. Because it is well known that some HPV oncoproteins interact with p53, some authors have studied the association of p53 accumulation, TP53 genetic alterations, and HPV infection in head and neck cancer. Although no conclusive data about this issue are available, it seems that there is no positive association between HPV infection and TP53 alterations in SCCs from the upper aerodigestive tract.

In 2002, Jacob et al reported a correlation between HPV infection and increased p53 accumulation in their series of 44 cases of invasive laryngeal carcinomas. Such a correlation was not found in the present study, and possible explanations for these discrepancies are differences in the number of HPV+ cases evaluated and methodological issues. Actually, HPV-induced oncogenesis and TP53 malfunction due to genetic abnormalities may be independent events during laryngeal carcinogenesis. It recently was reported that a subset of head and neck cancers that harbor active HPV-16 have a distinct pattern of genetic abnormalities and lack TP53 mutations. Suzuki and coworkers reported that HPV-16 E7 expression alone was able to immortalize laryngeal epithelial cells in vitro, and these cells showed TP53 spontaneous mutations later in culture, which may mimic the genetic instability that occurs during tumor progression in vivo.

To date, some putative prognostic factors studied in head and neck cancer have limited value because of subjective evaluation and technical issues, which may explain conflicting results in the literature. For example, Kreckik and coworkers reported better survival for patients with laryngeal SCCs with a low Ki-67 labeling index, but their results did not achieve statistical significance. Later, the authors observed differences in Ki-67 labeling between malignant and nonmalignant laryngeal lesions, and there was a trend in the Ki-67 labeling index for malignant lesions to be associated with tumor grade. In the present study, Ki-67–labeled laryngeal SCCs were evaluated quantitatively by semiautomated image analysis to achieve results that could precisely reflect the tumor growth fraction. It was verified that the Ki-67 labeling index was associated positively with p53 accumulation and tumor grade. Not only was this association statistically significant, it also was biologically reasonable. Furthermore, we found a trend toward higher Ki-67 labeling in laryngeal carcinomas that harbor HPV-16 compared with HPV-18+ cases. Whether this difference is consistent remains to be studied in a larger series of HPV+ laryngeal carcinomas.

In the present study HPV infection (only high-grade genotypes) was found in more than one third of 110 laryngeal SCCs from Brazilian patients, notably glottic tumors, and EBV infection was consistently absent. Accumulation of p53 protein was frequent in the laryngeal carcinomas evaluated, and it was associated significantly with higher malignant cell proliferation and a higher histologic grade of tumors. These results strengthen the hypothesis that HPV infection, but not EBV infection, has a role in the pathogenesis of a subset of laryngeal carcinomas.

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