Detection of Human Papillomavirus and Relevant Tumor Suppressors and Oncoproteins in Laryngeal Tumors

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Abstract

Purpose: The mechanism of larynx oncogenesis is complex and controlled by various factors, most of them involved in cell proliferation and apoptosis. In this study, we evaluated the levels of two suppressor proteins (pRb and p53) and two oncogenic proteins (c-Myc and Bcl-2), as well as the apoptotic levels and the presence of human papillomavirus (HPV) in both tumor types. Experimental Design: Low- or high-risk HPV viral DNA was determined by PCR and in situ PCR; the level of cellular proteins was examined by immunohistochemistry; the presence of apoptotic cells was evaluated by in situ cell death detection. Results: Most laryngeal papillomatosis samples contained low-risk HPV determined by both techniques. However, 25% of laryngeal carcinoma samples were positive for HPV employing PCR or in situ PCR. In papillomatosis, pRb and p53 levels were higher than in normal larynxes, whereas laryngeal cancer presented the lowest levels. c-Myc oncogene expression was very low in normal and cancer tissues but highly increased in papillomatosis. Bcl-2 expression was low and showed no significant difference between laryngeal papillomatosis and normal larynxes. By contrast, Bcl-2 was clearly up-regulated in cancer. Normal larynx samples and those from laryngeal papillomatosis exhibited similar relatively high numbers of apoptotic cells, whereas in malignant tumors, these cells were scarce. Conclusion: Our results suggest that HPV is an important risk factor in papillomatosis and in some malignant larynx tumors with a strong participation of cellular genes, specifically involved in proliferation and apoptosis. In benign papillomatosis lesions but not in larynx cancer, high p53 activity might preserve the apoptosis process. In larynx cancer, low p53 levels and high bcl-2 expression may be playing an important role to block apoptosis.

Laryngeal carcinoma is a common malignant tumor of the head and neck (1). Besides well-established risk factors like smoking and alcohol abuse (1, 2), the development of laryngeal carcinoma is associated with human papillomavirus (HPV) infection (2, 3). On the other hand, mother-to-child transmission is probably responsible for recurrent laryngeal and pulmonary papillomatosis in infants (4), and actually laryngeal papillomatosis is the most common benign tumor of the larynx in children (5, 6). Laryngeal papillomatosis is induced by low-risk HPV, especially types 6 and 11, and is characterized by recurrence (5, 6). The clinical course is unpredictable but regression may occur following some treatments (7–9). However, in other cases, the disease has a more aggressive course, recurs more frequently, and can extend well into adult life, requiring repeated surgical procedures. In a small number of patients, laryngeal papillomatosis may develop into squamous cell carcinoma (7–9).

HPV-16 is the most common high-risk virus. Its contribution to neoplastic progression is predominantly through the action of the viral oncoproteins E6 and E7 (10–14). Expression of these proteins is sufficient for the immortalization of primary human epithelial cells and induction of histologic abnormalities reminiscent of premalignant HPV-associated squamous intraepithelial lesions (14, 15). It has been well documented that E6 and E7 oncoproteins alter normal cell growth control mechanisms by inactivating two well-characterized tumor suppressor proteins, p53 and retinoblastoma (pRb), respectively (10, 12, 14). pRb plays an important role in the negative regulation of cell proliferation, causing cell cycle arrest in mid to late G1 phase where it is underphosphorylated (16). Wild-type p53 acts as a cell cycle checkpoint after DNA damage and induces G1 arrest or apoptosis, required to maintain genomic stability (17). Oncogenes such as bcl-2 inhibit apoptosis under...
a wide variety of circumstances and predispose for a malignant phenotype (18). Bcl-2 has a potent cooperative effect with c-Myc (19, 20) and it is overexpressed in a variety of cancers (21–23).

Several studies have attempted to identify markers that can predict which patients with laryngeal papillomatosis are at a higher risk for more frequently recurring aggressive disease or malignant transformation. However, results in both benign laryngeal lesions (papillomatosis) and malignant lesions have not been definitive (2, 3, 7). In this study, we approached this issue and we evaluated the expression levels of genes involved in proliferation (c-myc and rb) and in apoptosis (c-myc, bcl-2, and p53), as well as the apoptotic levels both in laryngeal papillomatosis and in laryngeal carcinomas.

### Materials and Methods

#### Tissues.

Normal laryngeal samples and pathologic specimens of laryngeal papillomatosis and carcinomas were collected immediately after removal. Samples were obtained from the Department of Pathology, Instituto Nacional de Enfermedades Respiratorias and Hospital Infantil de Mexico. The study was approved by the Ethics Committees. For DNA extraction, they were immediately stored in liquid nitrogen until use. Samples for immunohistochemistry were fixed in metacar buffer (60% methanol, 30% chloroform, and 10% acetic acid) and embedded in paraffin. DNA from laryngeal carcinoma was extracted from paraffin-fixed tissues. The study included tissue specimens of 22 patients with laryngeal papillomatosis (16 males and 6 females; age range, 5–15 years) and 16 samples from patients with laryngeal carcinoma (14 males and 2 females; age range, 46–84 years). Normal laryngeal tissues were obtained in autopsies from 13 individuals who died of nonmalignant causes.

#### DNA extraction.

Frozen tissues from normal larynx and papillomatosis lesions were homogenized in 500 μL of digestion buffer [10 mmol/L Tris-HCl (pH 8), 400 mmol/L NaCl, 25 mmol/L Na₂EDTA (pH 8.0), and 0.5% SDS] and lysed with 0.1 mg/mL Proteinase K (Sigma, St. Louis, MO). DNA was precipitated with 1 volume of 4 mol/L ammonium acetate and 2.5 volumes of absolute ethanol at (Sigma, St. Louis, MO). DNA was precipitated with 1 volume of acetic acid and embedded in paraffin. DNA from laryngeal carcinoma was resuspended and precipitated again with absolute ethanol, washed with 70% ethanol, dried, and dissolved in 20 μL of sterile H₂O.

**DNA extraction from laryngeal carcinoma embedded tissues.** Sections of 5-μm thickness were cut with xylene, ethanol, and acetone. Protein digestion was carried out with Proteinase K (0.2 mg/mL; Sigma) in 50 μL of lysis buffer. DNA was precipitated and washed as indicated above.

#### Detection of HPV by in vitro PCR.

High-risk and low-risk HPV DNA was determined by PCR in purified DNA samples. To increase the accuracy of PCR analysis, consensus primer oligonucleotides L1C1 and L1C2 directed to L1 open reading frame of the HPV genome were used (24). These consensus primers amplify at least nine genital HPV types: 6, 11, 16, 18, 31, 33, 42, 52, and 58. Amplification of the human β-globin gene was done as control of DNA suitability for amplification. The PCR protocol was carried out in a GeneAmp PCR System 2400 Thermal cycler (Perkin-Elmer, Wellesley, MA) and consisted of 35 cycles of denaturation at 94°C for 5 minutes, annealing at 48°C for 1 minute, and extension at 72°C for 1.5 minutes. The reaction mixtures consisted of a total of 50 μL containing 2 μL (40 ng) of genomic DNA, 1 μL (200 μmol/L final concentration) of deoxyribonucleotide triphosphate, 1.5 μL (1.5 mmol/L final concentration) of MgCl₂, 0.5 μL (0.2 mol/L final concentration) of each primer, 3 μL (4 units) of Taq polymerase (Invitrogen, Carlsbad, CA), 5 μL of buffer 10× (Invitrogen), and water. Products (10 μL) were run on 2% agarose gels and stained with ethidium bromide. Images were captured and digitalized using the SnapGene system (Syngene, Cambridge, United Kingdom).

#### HPV DNA typing.

Restriction enzymes were used to typify the viral DNA. The pattern was that described by Yoshikawa et al. (24). In situ PCR. To localize specific HPV DNA sequences, in situ PCR analysis was done as previously described (25). All thermal reactions were carried out using GeneAmp In situ PCR system 1000 (Perkin-Elmer). Molecular reagents and enzymes were purchased from either Invitrogen or Roche Molecular (Roche Diagnostics, Indianapolis, IN). Briefly, sections 5 μm in thickness were taken from the paraffin blocks on silanized slides and were fixed at 42°C and stored until treatment. Samples were permeabilized in 0.2 N HCl for 10 minutes at room temperature, washed in PBS, and then incubated 30 minutes in 0.5 μg/mL Proteinase K at room temperature. Amplification of DNA was accomplished using a hot start method with a PCR solution (final volume: 50 μL) containing 2 mmol/L MgCl₂, 2.5 μL of PCR-DIG mix (2 mmol/L dATP, dCTP, and dGTP each, 0.1 mmol/L digoxigenin-11-dUTP; Roche Diagnostics); two consensus sequence primer pairs within E6 and E7 of high-risk and low-risk HPV (PU primers; ref. 26); and 5 units of Taq DNA polymerase (Invitrogen). Cycling conditions were 2 minutes at 94°C and 18 cycles of 94°C for 1 minute, 60°C (E6/E7) or 58°C (β₂-microglobulin) for 1 minute, and 72°C for 1 minute, using a final extension time of 3 minutes at 72°C. The digoxigenin-11-dUTP–labeled PCR product was detected after washing and incubation with an alkaline phosphatase-antidigoxigenin conjugate (0.75 units/mL in 0.1 mol/L Tris-HCl and 0.15 mol/L NaCl, pH 7.5) for 2 hours at room temperature, and developed in 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate substrate solution (Zymed, San Francisco, CA). Positive test samples showed target DNA signal. Negative controls consisted of omission of Taq DNA polymerase or omission of primers in the PCR. No PCR signal was detectable in all negative controls.

#### Immunohistochemistry.

Sections 5 μm in thickness were taken from the paraffin blocks on poly-L-lysine (Sigma) and deparaffinized with xylene and ethanol. Endogenous peroxidase activity was quenched by incubation with 30% hydrogen peroxide in methanol. Sections were washed in PBS (pH 7.4) and nonspecific binding was blocked with 10% bovine serum albumin (Sigma) in PBS for 30 minutes. Incubation with monoclonal (c-Myc, Bcl-2, and pRB) or polyclonal (p53) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) was carried out overnight at 4°C. After washing in PBS-Tween solution, the sections were incubated for 1 hour with a biotinylated antirabbit or antimouse immunoglobulin (Vector Laboratories, Burlingame, CA). The sections were then washed and incubated for 1 hour with an AB complex (avidin-biotin-peroxidase; Vector Laboratories). Finally, the specimens were seen with the nucleus or cytoplasm, indicating the presence of the respective proteins. The intensity and pattern of protein immunostaining were evaluated using an image analyzer. The images were digitally processed to obtain the better and homogeneous signal and then selected for analysis of relevant regions. The selected regions were then digitally analyzed using the Image-ProPlus Analysis Software (version 4.0, Media Cybernetics, Inc., Silver Spring, MD). Five hundred cells from each specimen were examined and plotted.

#### Apoptosis assay.

The presence of apoptotic cells was determined using the in situ cell death detection by Fragment End Labeling of DNA (TUNEL). The assay was done according to the instructions of the manufacturer (Oncogene Research Products, Cambridge, MA). Apoptosis was defined according to the presence of condensed chromatin granules in the nucleus.

### Results

Thirty-five nonmalignant laryngeal tissues (13 normal larynx specimens and 22 laryngeal papillomas) and 16 samples from laryngeal carcinoma were studied. Most patients (87%) with...
laryngeal papillomas were children younger than 7 years. By contrast, laryngeal carcinomas were more frequent in adult patients, most of them (72%) of between ages 46 and 65 years.

HPV DNA amplification was achieved using the generic L1C1-L1C2 primers in 20 laryngeal papillomatosis samples (91%; Fig. 1A). By contrast, PCR amplification was only obtained in two (12.5%) laryngeal carcinoma samples, although detection of viral DNA improved with in situ PCR where HPV DNA-positive signals were seen in four (25%) samples using PI primers (Fig. 1B). Restriction enzymes were used to characterize the PCR products for the presence of high-risk (types 16 and 18) or low-risk (types 6 and 11) HPV. Most patients with laryngeal papillomatosis displayed low-risk HPV. Thus, 12 (60%) patients were infected by HPV-6 whereas 6 (30%) patients contained HPV-11. The more severe papillomatosis cases with recurrence were those containing HPV-6. One case had two of the low-risk types and only one patient presented both high-risk and low-risk HPV. On follow-up, this 17-year-old patient with laryngeal papillomatosis containing HPV-6 and HPV-16 types progressed to invasive carcinoma affecting larynx, trachea, and lung, and he died a few months after diagnosis. Interestingly, one papillomatosis patient (HPV-6 positive) suffered invasion to nose and also died.

Regarding the four HPV-positive laryngeal carcinomas detected with primers that reveal both high-risk and low-risk types, two of them had both high-risk and low-risk HPV, one patient had high-risk HPV, and another patient presented low-risk HPV infection. This suggests that in a minority of larynx cancers, HPV could be involved irrespective of their type.

Cellular gene expression. The expression of two suppressor proteins (pRb and p53) and two oncogenic proteins (c-Myc and Bcl-2) was examined by immunohistochemistry, and relative levels were evaluated by digital procedures. In laryngeal papillomatosis, pRb expression was higher than in normal larynx, whereas laryngeal cancer presented the lowest level, suggesting that the absence of this important tumor suppressor protein is implicated in laryngeal cancer development \((P = 0.0001; \text{Fig. 2})\). Similarly, p53 expression was also higher in papillomatosis and normal larynx compared with cancer \((P = 0.00001; \text{Fig. 3})\), supporting an important role for this protein in cancer suppression. c-Myc oncogene expression was very low in normal cells and in cancer tissues and highly increased in papillomatosis \((P = 0.00001; \text{Fig. 4})\). Bcl-2 expression was low and showed no significant difference between laryngeal papillomatosis and normal larynx. By contrast, Bcl-2 was clearly up-regulated in cancer samples, suggesting that this oncoprotein may be blocking apoptosis in larynx cancer \((P = 0.005; \text{Fig. 5})\). To further explore the percentage of apoptotic cells in the different tissues, a DNA fragmentation assay was used. As exemplified in Fig. 6, normal samples and those from laryngeal papillomatosis exhibited similar relatively high numbers of apoptotic cells, whereas in malignant tumors, they were very low \((P = 0.0001; \text{Fig. 6})\).

Discussion

Laryngeal papillomatosis are solitary or multiple benign tumors of the respiratory tract \((8)\). Because of the recurrent clinical course, respiratory papillomas have an important adverse significance to affected patients and may even provoke severe obstruction of the airways \((5, 7)\). The disease is associated with HPV and likely results from infection with HPV-6 and HPV-11 \((27–30)\). In contrast, the role of HPV in laryngeal carcinomas is unclear and studies thus far have shown contradictory results \((31, 32)\). In this context, we aimed to
investigate the frequency and the type of HPV present in benign and malignant laryngeal tumors using in vitro and in situ PCR. Our results in papillomatosis were similar with both techniques, and showed that most (91%) of them contained HPV-DNA detected by in situ PCR and in vitro PCR. Low-risk HPV was present in all papillomatosis patients whereas one case contained both low-risk HPV and high-risk HPV types. HPV-6 and HPV-11 were the predominant types, HPV-6 being the most prevalent including in severe and recurrent cases. This finding agrees with other reports (6, 33), supporting that HPV-6 and HPV-11 play an important role in papillomatosis. We found only one patient with malignant degeneration and, interestingly, this case presented infection by HPV-6 and also by HPV-16. These rare cases have previously been reported (7–9).

In laryngeal cancer, we found that in situ PCR was more sensitive than in vitro PCR, detecting HPV-DNA in 25% of the samples as compared with 12.5% when in vitro PCR was employed. The large difference in the percentages reported by other authors can be explained partly by the different detection methods used. In situ PCR combines the sensitivity of solution PCR with the histologic localization provided by the traditional in situ hybridization technique (2), resulting in locally increased readily detectable copy numbers. Two cases with laryngeal cancer showed high-risk and low-risk HPV whereas the other two contained either low-risk HPV or high-risk HPV, suggesting that both low-risk and high-risk HPV might be involved in the development of laryngeal malignant tumors in a subset of patients, and that these viruses may be a synergistic risk factor for malignant development.

Fig. 3. Immunohistochemical analysis of p53 gene expression. A, papillomatosis; B, laryngeal cancer; C, negative control (papillomatosis sample without primary antibody); D, normal larynx. Magnification, 40× (A and B); 10× (C). Immunohistochemical analysis and hematoxylin staining were done as indicated in Fig. 2. Columns, percentile average of normal larynx (n = 13), papillomatosis (n = 22), and laryngeal cancer (n = 16).

The transforming processes induced by HPVs depend on E6 and E7 proteins (10–14, 27, 28). These viral proteins support a particularly large number of functions and interactions with cellular proteins, some of which are specific for the carcinogenic HPVs, whereas others are shared by low-risk and high-risk HPVs (10, 28). High-risk HPVs can frequently persist in infected host cells at a low copy number for decades, often without causing clinically overt lesions that turned over very rapidly. Low-risk HPVs effectively induce epithelial hyperplasia and produce copious amounts of progeny virus. Low-risk HPV E6 and E7 proteins critically contribute to viral life cycle, but they have a substantially lower transforming activity and do not induce genomic instability (28, 33). Low-risk HPV E7 protein binds to pRb at a decreased efficiency (27) and does not induce pRb destabilization (11, 27). Low-risk HPV E6 proteins do not efficiently interact with and are incompetent to degrade p53 (10, 34, 35); in addition, E6 from low-risk HPV do not induce telomerase activity (35). It is possible that under certain conditions, low-risk HPVs can contribute to cellular transformation. For example, E7 from HPV-6 and HPV-11 can cooperate with ras to transform primary BRK cells, but at a lower level than HPV-16 E7 (27).

The current concept of oncogenesis is based on the interaction between a variety of factors. These factors could modulate cellular proliferation, apoptosis, and differentiation, involving particular cellular oncocenes, tumor suppressor genes, and in certain cases viral oncogenes (10, 36–38). The linkage of cell cycle and apoptosis has been recognized for c-Myc, p53, pRb, Ras, and Bcl-2. However, different variables,
including cell type, cellular environment, and genetic background, make it difficult to predict the outcome after activity modulation. In this work, we compared the expression of two suppressor genes and two oncogenes (p53, rb and c-myc, bcl-2, respectively) and we measured apoptosis levels in laryngeal papillomatosis and laryngeal cancer. It has been shown that pRb is involved in down-regulation of S-phase specific genes by binding to and sequestering the positive transcription factor E2F (16, 39). We observed that ~50% of the papillomatosis samples presented higher than normal levels of pRb, suggesting that E2F is sequestered and that other mechanisms could favor cell cycle progression in these benign lesions (i.e., c-myc). The unique papillomatosis case with HPV-16 had low levels of pRb expression, suggesting that high-risk HPV E7 is promoting pRb degradation, as previously reported (40, 41). These results indicate that pRb expression has an important role for growth suppression in laryngeal papillomatosis. Consistent with this finding, in laryngeal cancer, pRb level was lower than in normal larynx irrespective of the HPV type. These results indicate that low pRb expression is a frequent finding in laryngeal carcinomas and suggest that free E2F might participate in larynx tumor growth.

In cervical cancer, the p53 protein is rapidly degraded by high-risk HPV E6 oncoproteins (10, 14, 41), thus inhibiting p53-dependent apoptosis. In the present study, 90% of the papillomatosis lesions showed higher than normal levels of p53. The only papillomatosis case infected with HPV-16 displayed normal levels of p53 protein, suggesting that p53 is partially degraded by E6 oncoprotein encoded by this high-risk HPV. In all cases, we observed nuclear staining, suggesting that p53 is active. By contrast, the levels of p53 in laryngeal cancer were lower than in normal larynx irrespective of the HPV type. The results of the current study indicate that low p53 expression is a frequent finding in laryngeal carcinomas and could explain, at least partially, the low apoptosis level observed in this neoplasia. The decreased levels of p53 and pRb observed in larynx cancer may lead to deregulation of the cell cycle and to chromosomal instability and aneuploidy as regularly observed in human cancer.

Deregulated expression of some oncoproteins, such as Myc, Ras, or E2F, may result in stabilization, accumulation, and activation of p53 (36, 41–44). Thus, a possible explanation for the p53 elevation observed in papillomatosis is that deregulation of oncoprotein expression in these lesions is leading to unscheduled cellular proliferation. This predicts a simultaneous increase in the rate of both proliferation and apoptosis. In this context, the very high c-Myc signal observed in papillomatosis compared with normal tissue or larynx cancer samples could be associated with uncontrolled proliferation in these benign lesions. c-Myc has been implicated in both the activation and repression of transcription of genes controlling cellular proliferation, programmed cell death, and differentiation (44–46). Thus, it is possible that in papillomatosis, c-Myc leads to both hyperproliferation and apoptosis, particularly in the presence of active p53. On the contrary, a remarkable decrease in c-Myc levels was noticed in malignant tumors. In this case, proliferation could be driven by other oncogenes such as ras or by E2F family members (very low pRb levels in larynx cancer). In our study, bcl-2 was faintly expressed in
papillomavirus, which was no different from normal larynx. In contrast, strong up-regulation of Bcl-2 was observed in malignant tumors, supporting important differences in apoptotic processes between benign and neoplastic lesions of the larynx. In effect, we observed that in malignant tumors, the percentage of apoptotic cells was very low compared with those detected in papillomatosis and normal larynx.

In conclusion, our results suggest that HPV is an important risk factor in papillomatosis and in some malignant larynx tumors with a strong proliferation of cellular genes, specifically involved in proliferation and apoptosis. In benign papillomatosis lesions, but not in larynx cancer, p53 activity might preserve the apoptosis process. In larynx cancer, low p53 levels and high bcl-2 expression may be playing a major role to block apoptosis. Lowering antiapoptotic bcl-2 gene expression by antisense oligonucleotides or, alternatively, by functionally antagonizing Bcl-2 protein with ligands of the mitochondrial benzodiazepine receptor may be used to explore novel approaches directed at reestablishing sensitivity to apoptosis-based larynx cancer chemotherapy (47).

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