Molecular Identification of 7 Human Papillomavirus Types in Recurrent Respiratory Papillomatosis

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Background: Recurrent respiratory papillomatosis (RRP) is the most frequent benign laryngeal neoplasm, affecting children from the earliest months until adolescence. Its approximate incidence is 4.3 per 100000; 3870 cases are expected in the Mexican infant population, without sex distinction. It is a benign tumor that rarely becomes malignant after radiotherapy. It begins with a mild dysphonia, which may progress to aphonia, but without signs of respiratory insufficiency. Nevertheless, the clinical findings may be complicated by cough with scarce expectoration, which can result in pneumonia with retention of secretion, acute respiratory insufficiency, and asphyxia.

On laryngoscopy, small tumors are observed around the glottic lumen and inner face of the epiglottis, in a cluster formation. The most common inoculate site is the free edge of vocal cords, where their volume can increase until the glottic lumen is obstructed, which requires tracheostomy in some patients. Several different treatments have been proposed; nevertheless, resection is considered the most efficient method. However, even when resection is exhaustive and the obstruction disappears, illness may continue because of viral migration to healthy sites.

The infection process has a torpid course, which may vary widely in time, depending on the patient. While some patients require only a few resections before spontaneous cure, others require many more, leaving a permanent sequela in their voices. This causes patients to become shy and antisocial, with poor psychosocial development.

Two thirds of patients who have RRP in childhood experience spontaneous cure before or at adolescence, but the remaining third continues to be affected for an undefined period in a
MATERIALS AND METHODS

Forty-seven paraffin-embedded papilloma samples were collected from 47 children from the Otorhinolaryngology Service of the Pediatrics Hospital at the Centro Médico Nacional de Occidente (Instituto Mexicano del Seguro Social) in Guadalajara, Jalisco, Mexico. Direct laryngoscopic surgery was carried out in each patient with signs and symptoms of RRP. After clinical diagnosis was confirmed by cytological and histopathological procedures, the tumor tissue was resected to liberate the glottic obstruction.

The resected tissue was treated by means of the Wright-Manos method for DNA extraction. By removal of excess paraffin, the tissue was extracted, cut into small fragments, and placed in a 1.5-mL Eppendorf tube. One milliliter of n-octane (ACROS Organics, a division of Fisher Scientific, Pittsburgh, Pa) was added and the combination was mixed for 30 minutes and centrifuged, after which the octane was discarded. This last step was repeated once. Then, 0.5 mL of 100% ethanol was added; the combination was mixed and subsequently centrifuged at 10,000 rpm for 20 minutes, and the ethanol was discarded. A drop of acetone was added and the tubes were left open at 37°C until dry.

After the deparaffinization process, 100 µL of digestion buffer containing 15 µg of proteinase K per milliliter was added and incubated for 12 hours at 35°C. After incubation, the tubes were centrifuged and left at 95°C for 10 minutes for the proteinase K inactivation. The quantity and purity of the extracted DNA were estimated by spectrophotometry, with 5 µL of it mixed in 1 mL of distilled water, while its integrity was demonstrated by 6% (29:1) polyacrylamide gel electrophoresis with 5 µL of diluted DNA, mixed with 3 µL of loading solution (0.25% bromophenol blue, 0.25% xylene cyanol FF, and 30% glycerol), at 120 V for 90 minutes, for later staining with silver nitrate.

DNA was diluted to a 100-ng/µL concentration and amplification was performed by means of polymerase chain reaction with a 5-µL dilution aliquot during 32 cycles, with the use of the Cpl and CphIG oligonucleotides, which complement in the E1 open reading frame for HPV types 6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 56, and 58. The resulting amplified fragment was 188–base pair (bp) long. Seven microliters of the amplified product was applied with 3 µL of loading solution and subjected to electrophoresis in a 6% polyacrylamide gel; the band of interest was recovered, with 50 µL of recovering buffer (Tris hydrochloride, 10 mmol/L, pH 9.0; potassium chloride, 50 mmol/L; magnesium chloride, 1.5 mmol/L; and 0.1% Triton X-100), and incubated for 20 minutes at 95°C. From this mixture, 3 µL was taken for the reamplification by polymerase chain reaction. To confirm this amplification, 7 µL of the amplified product plus 3 µL of loading solution were applied to a 6% polyacrylamide gel, subjected to electrophoresis, and further stained. Later, 5 µL of the amplified product was mixed with the restriction enzyme Rsal under previously described conditions (Gibco BRL; Life Technologies, Gaithersburg, Md). After incubation, 7 µL of the amplified product plus 3 µL of loading solution underwent electrophoresis in a 2% (1:9:1) polyacrylamide gel to observe the resulting bands from the 188-bp original fragment digestion (Table 1). As markers, a 10-bp ladder as well as pBR 322 digested with HaelIII were used. To confirm the presence of HPV-16, an amplification with specific primers was performed, and the expected fragment of 576 bp was demonstrated in a 6% polyacrylamide gel electrophoresis. Contamination in the different procedures was avoided by the use of suitable controls and procedures.

The results of the 47 patients were analyzed by recovering the following data: sex, age at first resection, number of resections, age at last resection, final stage, persistence of illness, cure or abandonment of treatment, and anatomic resection site. For demographic and epidemiological purposes, the patients’ place of residence was also noted.

Table 1. Expected Band Patterns of the 188-bp Fragment From E1 Open Reading Frame

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<th>Viral Genome</th>
<th>Restriction Sites</th>
<th>Restriction Products, bp</th>
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<td>nt 2015</td>
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<td>HPV-31</td>
<td>nt 1764, 1882</td>
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<td>HPV-39</td>
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* Digested with restriction enzyme Rsal. HPV indicates human papilloma virus; bp, base pair, and nt, nucleotide.

Regardless of age at onset, all children underwent a resection. The age at onset in 8 of the 47 children was younger than 12 months (the earliest at 2 weeks of age), and resections were done after 1 year of age. Table 2 shows the number of resections per case, the age at first and last resection, evolution time, virus types found, incidence, and cases that resulted in total remission. The data regarding patients who showed adult-onset RRP were not available. A chest radiographic study was not performed in any of the cases because it is not included in the current outpatient protocol.

The DNA was positive for virus in all 47 biopsy specimens from the children with RRP, as shown by the band corresponding to 188 bp (Figure 1). From the the band patterns that appeared in 12% (19:1) polyacrylamide gel electrophoresis after digestion with the restriction en-
zyme Rsal in the 47 samples studied (Figure 2), we concluded that 35 samples (74%) were coinfected with 2 or more viral types and the remaining 12 (26%) showed only 1 virus. The relative frequency of the virus types was as follows: 15 samples (32%) were positive for HPV type 6; 18 (38%) for type 11; 39 (83%) for type 16; 2 (4%) for type 31; 26 (55%) for type 33; 13 (28%) for type 35; and 7 (15%) for type 39. Of the 35 coinfected samples, 13 (37%) showed 2 virus types; 9 (26%), 3 types; 10 (29%), 4 types; 2 (6%), 5 types; and 1 (2%), 6 types.

The most frequently found HPV type was 16 (39 cases [83%]), followed by type 33 with 26 (55%), type 11 with 18 (38%), type 6 with 15 (32%), type 35 with 13 (28%), type 39 with 7 (15%), and type 31 with 2 (4%). In this study, the frequencies of types 6 and 11 were not statistically significantly different (P>.05) from frequencies in previous studies.

Of the 266 surgeries performed, 187 (70.3%) were planned according to the clinical course of the patients' disease; the remainder were necessitated by respiratory insufficiency. One hundred seventy-one (64.3%) of the surgeries were performed by the staff surgeons and the remaining cases were performed by trainees, all of them under supervision. Complete excision of the papillomas

Table 2. Patient Data, Clinical Variables, Human Papilloma Virus (HPV) Types, and Outcome

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<th>Patient No./Sex</th>
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* Or age at the end of the study period disease.
† Between the age at first excision and the age at last excision.
The association of HPV with RRP has been described by different authors in 100% of cases. Only HPV types 6 and 11 have been described previously, the first is associated with the greatest frequency and the latter with the most severe clinical symptoms. Coinfection between these 2 types with other viruses, such as herpes simplex, cytomegalovirus, and Epstein-Barr virus, has been described in 80%. In some previous reports, HPV-16 was identified in tissue from patients with RRP, the majority belonging to adults.

By means of polymerase chain reaction, our study allowed the detection of HPV DNA in 100% of the analyzed samples. With the use of the restriction enzyme RsaI, HPV types 6, 11, 16, 31, 33, 35, and 39 were identified in tissue from patients with RRP. The most frequent sole causal agent (8 cases [17%]), HPV-31 was the least frequent, being present in only 2 cases (4%) and always associated with other HPV types. Regarding the high frequency of type 16, this is the second report that describes this virus type in children with RRP. Previously, HPV-16 had always been associated with the genital epithelia and considered oncogenic. However, in 1994, Doyle et al described 1 case of RRP that began at 4 years old, with death at 13 years from epidermoid carcinoma; in that case, HPV-16 was identified in a biopsy specimen from the right bronchi.

In our study, the presence of HPV-16 was confirmed by polymerase chain reaction with the use of specific primers that showed a positive 576-bp band in a polyacrylamide gel (Figure 3). On the other hand, on the basis of the clinical data, the patients were divided into mild and aggressive categories. Patients with more than 10 total resections, more than 5 years of evolution, and more than 3 resections per year, as well as nonlaryngeal respiratory papillomatous lesions, were considered to have dysplastic changes, inflammatory atypia without dysplasia, nondysplastic atypia, mild dysplastic foci, juvenile papilloma with and without mild dysplasia, and viral atypia without dysplastic or anaplastic data in all the cases, without showing an association with particular types of HPV by statistical tests.
aggressive cases; cases without these criteria were considered mild. Eleven cases (23%) were considered severe or aggressive. Moreover, 21 cases (45%) had 3 or more HPV types. Five cases both were severe and involved 3 or more HPV types; in these, HPV-33 was the preponderant type. A $\chi^2$ test used to compare the clinical severity with the viral types present found no statistical significance. The logistic regression analysis that relates the clinical severity and the single viral types, in combination or all simultaneously, was negative; nevertheless, type 16 showed a relationship near statistical significance ($P=0.05$), in contrast with the other viral types. The high incidence of HPV types 16, 33, 35, and 39, plus those traditionally associated with RRP, identified in this study indicates that it is necessary to carry out a similar study in a clinically healthy control group to obtain the frequency and distribution of the viral types.

CONCLUSIONS

In the RRP samples studied, HPV was present in 100% of the cases. The viral types and frequencies found were different from those described by other authors (except for the frequencies of types 6 and 11). In addition, the presence of types 16, 33, 35, and 39 was observed in papillomatosis from laryngeal tissue. The most and least frequent types were 16 and 31, respectively. Coinfection was the most frequent event in this population. There was no relationship between any HPV type and the severity of illness, nor with its clinical aggressiveness.

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Manuel Peñaloza-Plascencia, MD, died on December 26, 1998. Certainly it is a great loss for the Molecular Medicine Division, for the Otorhinolaryngology Service of Pediatrics Hospital, and for his family, friends, and science. May he rest in peace.

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REFERENCES


