Presence of Human Papillomavirus Type 16 Related Sequences in Verrucous Carcinoma of the Larynx

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ABSTRACT

Verrucous carcinoma of the larynx clinically resembles laryngeal papilloma in that both are wart-like masses on the vocal cords and may be characterized by multifocality and recurrence. Human papillomavirus (HPV) infection is an etiologic factor in laryngeal papilloma, and recent evidence implicates HPV in squamous neoplasias. To determine whether HPV is also associated with verrucous carcinoma of the larynx, we analyzed tissue specimens from six patients with verrucous carcinoma of the larynx by Southern and DNA dot blot hybridization for HPV DNA. From three patients, specimens of normal laryngeal epithelium were also studied. All tissues showed evidence of HPV sequences related but not identical to HPV-16. They were negative for HPV-11. In contrast, four squamous cell carcinomas of the larynx and three normal laryngeal tissues were negative for HPV DNA. Histological sections of the six verrucous lesions were found to contain koilocytosis. Immunoperoxidase staining for HPV capsid antigens was negative in all these cases. The consistent and specific association of HPV with the verrucous carcinomas in this report suggests the possibility of a pathogenic involvement.

INTRODUCTION

Verrucous carcinoma of the larynx is a distinct variant of well-differentiated squamous cell carcinoma characterized by low incidence (1–2% of all carcinomas of the larynx), male preponderance, average age over 60, and local aggressiveness without potential to metastasize. First described by Ackerman (1) in the oropharynx, verrucous carcinomas have been reported in the vulva, vagina, glans penis, esophagus, and tracheobronchial tree as well as in the larynx (2–4). Verrucous carcinomas are generally exophytic with a markedly keratodendritic papillary surface. They bear a number of similarities to laryngeal papilloma. Macroscopically, both appear as warty masses, with notable papillary projections of the epithelial surface. Microscopically, both are composed of cytologically bland thickened squamous epithelium. Clinically, both types of lesions may be characterized by multifocality and have the tendency to recur locally following endoscopic surgical removal. Because HPV infection is known to be an important etiologic factor in laryngeal papilloma (5, 6), and because recent evidence implicates the involvement of HPV in the pathogenesis of squamous neoplasia in a variety of body sites (7, 8), we have examined verrucous carcinoma of the larynx for the presence of HPV. Our findings indicate that HPV-16 related DNA is associated with verrucous carcinoma of the larynx.

MATERIALS AND METHODS

Histology and Immunohistochemistry. Histological sections from formalin-fixed, paraffin-embedded tissues were reviewed and classified according to the method of Batsakis et al. (3). The presence of histological features associated with human papillomavirus infection of the squamous epithelium including koilocytosis, hyperplasia, hyperkeratosis, dyskeratosis, giant cells, and bi- or multinucleate cells (9) was recorded. Tissue sections were stained for papillomavirus group-specific antigen using an antibody produced in rabbits to HPV particles extracted from plantar warts and purified through two cesium chloride gradients. The avidin:biotinylated horseradish peroxidase complex procedure (10) (Vector Laboratories) was used for detection. Sections of skin warts were used as positive controls in each assay.

Molecular Hybridization Studies. Biopsy specimens or excised tissue samples were removed during direct laryngoscopy or following laryngectomy and stored in liquid nitrogen. Tissues were processed without thawing to extract and purify total DNA (11). For Southern blots, 10 μg of cellular DNA or standard reconstructions representing 1, 10, and 100 copies per cell of HPV DNA were digested with PstI using conditions recommended by the supplier (New England Biolabs) [100 μM NaCl:10 μM Tris (pH 7.5):bovine serum albumin (100 μg/ml); electrophoresed through a 1% agarose gel; and transferred to Gene Screen Plus (New England Nuclear Research Products, Boston, MA), according to the procedure of Southern (12). Gene Screen Plus was chosen because of its high strength and binding properties allowing multiple hybridizations without noticeable loss of signal (13).

For DNA dot blots, 1 μg of undigested cellular DNA was applied to a Gene Screen Plus membrane using a manifold (Scliecher and Schuell). For standards, HPV inserts were separated from appropriately digested plasmid DNAs (7, 8, 14) by agarose gel electrophoresis and recovered by electroelution. Probes were cloned HPV types 18, 16, and 11 (7, 8, 14) nick-translated with all four nucleotide [α-32P]triphosphates (15) to specific activities of 0.2–0.9 × 10^6 cpm/μg. Southern and dot blots were hybridized sequentially with the probes of choice in 5X SSC (1X SSC = 0.15 M NaCl:0.015 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 1% sodium dodecyl sulfate, 30% formamide, and 10% dextran sulfate at 42°C overnight (Tm —34°C). Blots were first washed in 2X SSC:C% sodium dodecyl sulfate at 50°C (Tm —41°C, “nonstringent”) and sometimes rewashed in 0.1X SSC at 60°C (Tm —9°C, “stringent”). Between hybridizations, the probe was eluted as described (16). The effectiveness of the elution was verified autoradiographically. For autoradiography, wet filters were wrapped in Saran Wrap and exposed to Kodak XAR5 film with DuPont Lightening Plus intensifying screens for varying lengths of time (see Legends to Figs. 1–3).

RESULTS

Clinical Presentation. Six patients, males aged 51–59, presented with a common history of smoking and persistent hoarseness. At direct laryngoscopy all the lesions were diagnosed as verrucous carcinoma based on their clinical appearance. Each was circumscribed, papillary, and leukoplakic and involved one or both vocal cords.

Histology and Immunohistochemistry. Table 1 summarizes the major histological findings of the six patients. Four were diagnosed as verrucous carcinoma, having thickened processes of cytologically bland squamous epithelium extending into the underlying connective tissue and broad invading pushing margins (17). Two (Patients 1 and 6) showed no penetration of the basement membrane in the sections examined but were otherwise consistent with verrucous carcinoma. Koilocytosis, characteristic of HPV infection, was identified in all cases.

Adjacent tissues in four of the six patients showed focal
Table 1  The presence of koilocytosis, papillomavirus group-specific antigen, and human papillomavirus DNA in verrucous carcinoma of the larynx

Koilocytosis (14) was graded to indicate the relative abundance of koilocytic cells. The presence of HPV capsid antigen was determined by use of a rabbit antibody to human plantar wart virus (see “Materials and Methods”). The presence of HPV DNA was assessed under nonstringent conditions.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Koilocytosis</th>
<th>HPV capsid antigen</th>
<th>HPV DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2+</td>
<td>—</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>1-2+</td>
<td>—</td>
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<td>3</td>
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<td>1+</td>
<td>—</td>
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</tr>
<tr>
<td>5</td>
<td>1+</td>
<td>—</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>1+</td>
<td>—</td>
<td>+</td>
</tr>
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</table>

Molecular Hybridization Studies. Moderately nonstringent hybridization conditions were used to permit the detection of related as well as homologous types of HPV (18). The Southern blot in Figs. 1 and 2 were hybridized sequentially in the following order to probes containing HPV-16, HPV-11, HPV-18, HPV-16, and HPV-11. Results obtained with the HPV-16 and HPV-11 probes were reproducible on rehybridization.

Hybridization of tissue DNAs to HPV-16 (Fig. 1) revealed definite evidence of the presence of related sequences in at least four (Patients 2–5) and probably all five of the verrucous lesions (Fig. 1). Differences in the restriction pattern of the DNAs relative to the cloned HPV-16 are noted. Very faint hybridization to HPV-18 was also detected (data not shown). The hybridization patterns were similar with both probes, but stronger with HPV-16. In addition, the DNAs from visually normal sites adjacent to the tumors of three patients (Fig. 1) were also positive for HPV DNA. It is especially important to note that the adjacent site from Patient 1 is definitely positive. Despite this patient’s lesion being borderline, his larynx does harbor HPV-16 related sequences. It is of interest to note that the amount of detectable HPV-16 related DNA in the lesion did not correlate with the extent of koilocytosis (Table 1). Stringent washing of this blot reduced but did not totally remove signals from the verrucous tissue DNAs, whereas it made hybridization to the inverted nasal papilloma DNA no longer detectable.

Following moderately nonstringent hybridization to the HPV-11 probe, no hybridization to the DNA of the verrucous tissues was seen (Fig. 2). HPV-11 and HPV-16 are only distantly related, and cross-hybridization is not detectable even under nonstringent conditions, except in the case of extremely high HPV copy numbers. The two bands seen in the standards in Fig. 2 are pBR DNA. These bands had equal intensity to those in Fig. 1 when exposed for equivalent time. Longer exposures revealed increased nonspecific background binding, but, again, no bands were visualized in any of the nine lanes containing the verrucous DNAs. The inverted nasal papilloma, however, was strongly positive, and little or no probe washed off in the subsequent stringent wash.

DNAs from several other specimens (Fig. 3A) were then extracted and tested by DNA dot blot hybridization. Histological review of these specimens was not done. Of all the tissues studied on this blot, the only one positive for HPV-16 related sequences (Fig. 3, B and C) was the sixth verrucous carcinoma
Fig. 3. Detection of HPV-16 related DNA in verrucous and other tissues by dot blot hybridization. A shows a diagram of the tissues analyzed: verrucous carcinoma (V); normal epithelium (N); squamous cell carcinoma (C); and papilloma (P). All tissues are laryngeal except the left-hand V, which is a verrucous carcinoma of the mandible, and the lower P, which is an inverted papilloma of the nose (Patient 7 in Fig. 1). B and C show the signals obtained following hybridization to HPV-16 probe: nonstringent wash (B) and stringent rewash (C). D and E show the signals obtained following hybridization to HPV-11 probe: nonstringent wash (D) and stringent rewash (E). Autoradiograms (B) and (D) were exposed for 3 days; C and E were exposed for 1 wk.

Table 2: The presence of HPV DNA in various diseases of the head and neck

<table>
<thead>
<tr>
<th>Pathology</th>
<th>HPV-16 Presence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Larynx</td>
</tr>
<tr>
<td>Verrucous</td>
<td>0/6</td>
</tr>
<tr>
<td>Squamous</td>
<td>0/6</td>
</tr>
<tr>
<td>carcinom</td>
<td>0/7*</td>
</tr>
<tr>
<td>Papilloma</td>
<td>12/14*</td>
</tr>
<tr>
<td>Polyp</td>
<td>0/3</td>
</tr>
<tr>
<td>Normal</td>
<td>0/3</td>
</tr>
</tbody>
</table>

* Three squamous cell carcinomas and 14 laryngeal papillomas were previously analyzed under stringent conditions to HPV-6/HPV-11 DNA (18).

of the larynx. The laryngeal papilloma, which was positive under nonstringent conditions, did not contain an HPV-16 related virus, because the probe eluted under stringent conditions. All other tissues were negative, including the four squamous cell carcinomas of the larynx and the verrucous carcinoma of the mandible.

When this blot was rehybridized with the HPV-11 probe (Fig. 3, D and E), both of the papillomas showed continued strong hybridization following stringent washing. In contrast, the verrucous carcinoma did not contain HPV-11, because the initial weak hybridization was nearly totally abrogated following the stringent wash.

We also screened additional samples to examine the prevalence of HPV-16 related sequences in other lesions. Table 2 summarizes the composite of our findings. In the larynx, verrucous carcinomas were consistently found to harbor HPV-16 related sequences. In contrast, no HPV DNA was identified in squamous cell carcinomas. This laboratory has previously reported testing 20 papillomas for HPV-6 or HPV-11 DNA. All 20 hybridized to HPV DNA under nonstringent conditions, and 12 of 14 tested under stringent conditions were positive for HPV-6 or HPV-11 (6). In the present study, one papilloma of five tested with HPV-16 appeared to hybridize better to HPV-16 than to HPV-11. In addition, one vocal cord polyp proved positive for HPV-16 related sequences. Among the other head and neck tissues examined, two additional positives were identified. One of these was a recurrent inverted nasal papilloma in a 54-yr-old man. The other was a particularly aggressive papilloma of the conjunctiva in a 12-yr-old boy.

DISCUSSION

This study demonstrates a strong correlation between the presence of HPV-16 related sequences and verrucous carcinoma of the larynx in our six patients. Indeed, analysis of the DNA hybridization results indicates the presence of HPV-16 related sequences not only in all six verrucous tumors but also in the four tissues outside the margins of these tumors that were studied. In addition, the laryngeal specimens of all six verrucous patients were reviewed and found to contain koilocytosis, a well-established marker of HPV infection (9). The absence of immunohistochemically detectable HPV antigens is not surprising, since peroxidase positivity for HPV antigens has been shown to be extremely low in squamous intraepithelial neoplasia and virtually absent in squamous cell carcinoma (10, 19). Immunohistochemistry is limited to the detection of productive viral infections. DNA hybridization, on the other hand, has the advantage of being able to detect latent and transforming as well as productive infections, which renders it potentially more sensitive.

The Southern and dot blot analyses presented here clearly demonstrate that HPV-16 related sequences are present in verrucous carcinoma of the larynx. HPV-16 and -18 have been implicated in human genital squamous carcinomas and its precursors, and HPV-11 is known to be involved in both laryngeal papilloma and cervical condyloma (5, 6, 20, 21). The one blot presented here does not permit definitive conclusions to be drawn regarding the type of HPV DNA present in the verrucous tissues. However, among the three types of HPV studied, the strongest signals were detected using HPV-16 DNA, indicating that the sequence in the verrucous tissues are more closely related to HPV-16 than to either of the other two types tested. This finding was obtained twice during the series of sequential hybridizations performed in this study.

The hybridization signals detected in the verrucous DNAs are specific to HPV-16 and not the result of cross-hybridization to cellular sequences. Under the moderately nonstringent hybridization conditions used, sequences which are only distantly related do not cross-hybridize, as evidenced by the fact that the HPV-16 viral-specific DNA fragments on the Southern blot in Fig. 2 are not appreciably detected by the HPV-11 probe. (The two fragments which are detected are the plasmid fragments.) Note, too, that the pattern observed in the verrucous DNAs is not found in the lane containing DNA from a human oropharyngeal papilloma (Fig. 1, Patient 6) which was negative for all the HPV tests. Moreover, HPV-negative human DNAs hybridized in our laboratory on other Southern blots under conditions identical to those used in this study have never produced hybridization patterns such as those detected here in the verrucous tissue DNAs.

The differences in restriction patterns between the verrucous tissue and cloned HPV-16 DNAs could be explained either by the presence of a different but related type of HPV or by incomplete enzyme digestion. We suspect the presence of a new
type, because the hybridization was weak and because some of the probe eluted during the stringent wash (data not shown); also, the DNA from the inverted nasal papilloma (Fig. 1, Patient 7) was cleaved to completion.

In addition to the findings of HPV-16 related sequences in the verrucous lesions, we also found similarly related sequences in four “normal” laryngeal tissues of three of these patients taken from sites well outside the margins of the tumor. As in laryngeal papilloma (6), the presence of a latent viral infection in adjacent tissues of verrucous carcinoma could certainly be important in explaining the recurrent and multifocal nature of the disease.

No specific hybridization to DNAs from the verrucous tissues was observed to the HPV-11 probe. The latter finding is particularly important for two reasons. (a) It indicates that the virus associated with the verrucous lesions is distinct from the HPV's found in laryngeal papillomas. (b) The absence of hybridization to the HPV-11 probe indicates that the hybridization to the HPV-16 probe is not due to cross-hybridization to bacterial plasmids contaminating the human tissues. (All probes contained pBR DNA.) Unfortunately, further characterization of the DNAs from these six patients is precluded by the lack of sufficient material for further analysis. Whether HPV-16 related DNA will be found in all cases of verrucous carcinoma of the larynx is not known. As additional verrucous specimens are made available to us, attempts will be made to identify, clone, and characterize the HPV sequences associated with verrucous carcinoma of the larynx. Potentially, HPV typing may be useful in predicting the biological behavior of laryngeal squamous lesions, as has been shown in the female genital tract, and may serve as a marker for patients at high risk for the development of carcinoma (20, 21).

What role, if any, the HPV-16 related virus plays in the generation of verrucous carcinoma of the larynx is not clear. Although the possibility of a passenger virus cannot be ruled out at this time, the consistent and specific association with the verrucous lesions in this paper clearly suggests the possibility of a pathogenic involvement.

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