In Situ Hybridization Signal Patterns in Recurrent Laryngeal Squamous Papillomas Indicate that HPV Integration Occurs at an Early Stage

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Abstract Laryngeal papillomas are benign tumors that frequently recur and can compromise airways. We investigated HPV genotype, physical status, and protein expression in juveniles versus adults. Thirty-five laryngeal papilloma specimens were obtained from ten juveniles (1–16 years) and eleven adults (24–67 years). In cases of recurrent papillomatosis (7 juveniles, 7 adults), the first and last papillomas were assayed. HPV type was determined by GP5+/6+ PCR and dot blot hybridization. In situ hybridization (ISH) was performed on 34 specimens; the data were recorded in terms of diffuse (episomal HPV) and punctate (integrated HPV) signal patterns. Immunohistochemistry for the HPV L1 capsid protein, a marker of HPV productive status, was performed on 32 samples. All samples tested HPV positive: HPV 11 in 2/10 (20.0%) juveniles and 5/11 (45.5%) adults; HPV 6 in 7/10 (70%) juveniles and 5/11 (45.5%) adults; and HPV 6/11 double infection was noted in one juvenile and one adult. ISH signals (punctate ± diffuse) were detected among 7/10 (70.0%) juveniles and 7/11 (63.6%) adults. L1 staining was detected in 1/9 (11.1%)

juveniles and 6/10 (60.0%) adults ($P = 0.06$). These data support the idea that integration of low-risk HPV types into the cell genome is an early and common event in the etiology of juvenile and adult recurrent laryngeal papillomas. Productive HPV infections may be more common in adults; accordingly, constant laryngeal re-infection by HPV shed from a productive lesion may contribute to adult recurrent lesions, whereas the mechanism of papilloma recurrence in juveniles may be more attributable to HPV integration.

Keywords Recurrent respiratory papilloma · Laryngeal papilloma · Human papillomavirus · HPV · Larynx

Introduction

Laryngeal papillomas are histologically benign, but can behave in a malignant fashion resulting in airway compromise. Recurrence is frequent, and can result in spread throughout the respiratory tract. The incidence of laryngeal papillomatosis in American children and adults has been estimated at 4.3 and 1.8 per 100,000, respectively [1]. Formerly, laryngeal papillomas were classified based on age at onset and recurrence into four categories: (1) juvenile solitary, (2) adult solitary, (3) juvenile multiple, and (4) adult multiple. These categories have since been condensed into the single disease entity of “recurrent respiratory papillomatosis” (RRP) [2]. Approximately half of RRP cases are first diagnosed in juveniles, and the remainder in adults. The National Registry for Juvenile-Onset Recurrent Respiratory Papillomatosis defines all papillomas diagnosed before the age of 17 years as “juvenile” in nature [3]. Other sources, however, require that juvenile patients to be ≤ 12 years at diagnosis [2, 4].

In both juveniles and adults, the low-risk HPV genotypes 6 and 11 are those most commonly implicated; overall, HPV

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positivity in laryngeal papillomas has been estimated at 76% [2]. Juvenile-onset RRP (JO-RRP) is generally considered to have a more aggressive clinical course than the adult-onset (AO-RRP) [2, 4, 5]. Also, conversion to laryngeal carcinoma occurs more frequently in juveniles [6, 7]. In addition to having a different clinical course, JO-RRP differs in its route of acquisition; while numerous studies have indicated that vertical transmission from maternal genital condylomas is the primary mode of juvenile papillomas, adult papillomas apparently spread secondary to oral-genital contact [8, 9]. The differences in incidence, transmission, and clinical course of JO-RRP versus AO-RRP suggest that the HPV phenotype may also vary between the two. In the current study, we investigated HPV genotype, physical status, and protein expression in juvenile-onset versus adult-onset laryngeal squamous papillomas.

Materials and Methods

Subsequent to institutional review board approval, 35 archival laryngeal papilloma specimens were obtained from 10 juvenile-onset and 11 adult-onset papilloma cases following a computer diagnostic search at Fletcher Allen Health Care in Burlington, Vermont and Baylor College of Medicine in Houston, Texas. Our definition of juvenile-onset papillomatosis was ≤ 17 years old at the time of first papilloma, in accordance with guidelines established by the National Registry for Juvenile-Onset Recurrent Respiratory Papillomatosis [3]. In cases of recurrent respiratory papillomas (RRP), the first and last papillomas were assayed. Papillomas (diagnoses reviewed and confirmed by histopathologists EGB, KC and/or RL) were of endolaryngeal origin; dysplasia, where noted, was defined according to WHO criteria [10, 11].

DNA Extraction and Polymerase Chain Reaction (PCR)

DNA was extracted from sections of formalin-fixed, paraffin-embedded (FFPE) samples cut into 1.5 ml tubes. Tissues were dewaxed with xylene, washed with ethanol rinses, and after air drying, digested with 0.5 mg/ml proteinase K solution overnight at 55°C. The enzyme was deactivated by heating at 95°C for 10 min. After cooling, the crude DNA extract was purified for PCR via admixture with Chelex-100 resin according to supplied instructions (Bio-Rad Laboratories, Hercules, CA). All DNA extracts were quantified by 'NanoDrop' (Wilmington, DE) spectrophotometric assay. PCR for B-globin sequences [209 base pair (bp) product] was performed to confirm the integrity and quality of the extracted DNA. The HPV status determined by PCR using GP5+/GP6+ primers (~150 bp product) that support the detection of at least 37 different mucosal HPV types [12]. Two GP5+/6+ assays were performed on each sample: a

'touchdown' (TD) protocol [13], and a Slow Ramping (SR) method [14]. HPV type was determined by dot blot hybridization with oligonucleotide probes [12, 13]. Cervical cell line DNA extracts from SiHa [ATCC® number HTB-35™ (HPV16 positive)] and C-33 A [ATCC® number CRM-HTB-31™ (HPV negative)] cells were used as positive and negative controls respectively.

In Situ Hybridization (ISH)

ISH was carried out on 34/35 FFPE sections (papilloma lesion from one specimen was exhausted during sectioning for DNA extraction). A tyramide-based ISH assay (Gen-Point™, Dako North America, Carpinteria, CA) was performed using a Dako biotinylated wide spectrum HPV probe and 3-amino-9-ethylcarbazole (AEC) as the substrate [15]. Cervical lesions were used as ISH positive controls, and ISH was performed minus HPV probe in the hybridization mix as a negative control. The data was recorded in terms of diffuse signal patterns (consistent with episomal DNA), and punctate signals (consistent with HPV integrated into host cell chromosomes) [15, 16]. Non-detection of HPV by ISH was considered consistent with a low-copy number HPV infection below the detection threshold sensitivity of the ISH technique.

L1 Immunohistochemistry (IHC)

Thirty-two HPV positive laryngeal papilloma samples were tested for HPV biological activity by means of IHC using an antibody to detect HPV L1 capsid protein expression the (Clone K1H8, Dako North America, Carpinteria, CA) and the Dako EnVision™ system together with 3,3'-diaminobenzidine tetrahydrochloride (DAB) as secondary detection reagents. Negative control IHC was performed using a mouse monoclonal IgG1 against a protein known to be non-expressed in mammalian tissues (X 0931, Dako North America, Carpinteria, CA). Detection of L1 protein expression by IHC was considered indicative of HPV productive status. Non-detection of L1 protein was considered consistent with loss of L1 expression due to abrogation of the HPV life cycle, possibly as a consequence of integration, or due to a low-level or latent HPV infection. Low-grade cervical lesions were utilized as positive controls.

Results

Patient

At onset of papilloma, juvenile patients ranged from 1 to 16 years in age, while adults ranged from 24 to 67 years.

Table 1 HPV genotype, in situ hybridization signal patterns, and HPV L1 protein expression in adult-onset laryngeal papillomas

Patient	Age (years)	Sex	Epithelial dysplasia	HPV type	ISH pattern	L1 IHC	Number of recurrences
1	64	M	None	11	Punctate & diffuse	Negative	8
1	82	–	Low grade	11	Punctate & diffuse	Extensive	–
2	41	M	None	11	Punctate & diffuse	Focal	9
2	58	–	None	11	Punctate & diffuse	Focal	–
3	67	M	None	11	Punctate & diffuse	Negative	10
3	78	–	None	11	Punctate & diffuse	Focal	–
4	24	M	None	11	Punctate & diffuse	Negative	7
4	30	–	None	11	Punctate & diffuse	Negative	–
5	58	M	None	6	Punctate & diffuse	Negative	2
5	64	–	Low grade	6	Punctate	Negative	–
6	26	M	Low grade	6	Negative	Negative	4
6	31	–	None	6	Negative	Negative	–
7	53	M	None	6	Punctate & diffuse	Focal	5
7	56	–	None	6, 11	Punctate & diffuse	Focal	–
8	34	F	None	6	Diffuse	Focal	1
9	41	F	Moderate grade	11	Punctate & diffuse	Focal	1
10	41	M	Low grade	6	Negative	Negative	1
11	36	M	None	6	Diffuse	Not done	1

The majority of adult-onset patients were male (M:F = 9:2) while the juvenile-onset group was more heterogenous (M:F = 4:6). The number of laryngeal squamous papillomas per patient ranged from 1 to 29 with a slightly higher average in the juvenile than the adult group: 6.5 versus 4.5. The majority of juveniles and adults, respectively, had recurrent respiratory papillomas: 7/10 (70%) versus 7/11 (63.6%) ($P = 1.00$). Furthermore, half of juveniles and adults, respectively, had at least one papilloma with mild to moderate dysplasia: 5/10 (50%) versus 5/11 (45.5%) ($P = 1.00$) (Tables 1, 2).

HPV Genotyping

All 35 samples were HPV positive by both TD and SR PCR assays: 7/10 (70%) juvenile-onset and 5/11 (45.5%) adult-onset patients were positive for HPV 6 ($P = 0.38$) (Fig. 1a, b). HPV 11 was detected in 2/10 (20%) juveniles and 5/11 (45.5%) adults ($P = 0.36$). One juvenile-onset and one adult-onset patient were positive for both HPV 6 & 11. HPV 11 was not specifically associated with recurrence or dysplasia in juveniles: 2/7 (28.6%) juveniles with recurrent papillomas had HPV 11 and 2/5 (40%) juveniles with dysplasia had HPV 11 ($P = 1.00$). HPV 11 was more common in adults with papilloma recurrence: 5/7 (71.4%) of adult-onset patients with recurrent respiratory papillomas were positive for HPV 11 (adults vs. juveniles, $P = 0.29$). There appeared to be no correlation between HPV 11 and dysplasia in adults: 2/5 (40%) adults with dysplasia had HPV 11 ($P = 1.00$).

In Situ Hybridization

Among PCR positive samples, 7/10 (70%) juvenile-onset patients had at least one ISH positive papilloma compared with 9/11 (81.8%) adult-onset patients ($P = 0.63$) (Fig. 1a, b). Punctate signals ($\pm$ diffuse) were detected in ≥ 1 papilloma in 7/10 (70%) juvenile-onset patients. The majority of juveniles with recurrent papillomas had punctate signals in 1 or more papillomas, as did the majority of juveniles with dysplasia: 5/7 (71.4%) and 4/5 (80%), respectively. Punctate signals ($\pm$ diffuse) were detected in ≥ 1 papilloma in 7/11 (64%) adult-onset patients. In 2 adults, the signal was diffuse only. The majority of adults with recurrent respiratory papillomas had punctate signals in ≥ 1 papilloma as did the majority of adults with dysplasia: 6/7 (85.7%) and 3/5 (60%), respectively.

L1 Immunohistochemistry

L1 staining was detected in ≥ 1 papilloma in 1/9 (11.1%) juvenile-onset and 6/10 (60%) adult-onset patients ($P = 0.06$) (Fig. 1c). In the case of one juvenile and one adult, immunohistochemistry could not be performed due to insufficient sample. L1 positivity in ≥ 1 papilloma was seen in 4/7 (57%) adults with recurrent respiratory papillomas and 2/5 (40%) adults with dysplasia. L1 staining was also seen in 4/5 (80%) adults with HPV 11 and one adult with both HPV 6 & 11. A correlation between HPV pattern/L1 IHC results and recurrences and/or dysplasia was not established.

Table 2 HPV genotype, in situ hybridization signal patterns, and HPV L1 protein expression in juvenile-onset laryngeal papillomas

Patient	Age (years)	Sex	Epithelial dysplasia	HPV type	ISH pattern	L1 IHC	Number of recurrences
1	6	M	None	6	Punctate & diffuse	Negative	4
1	7	–	None	6	Negative	Negative	–
2	3	F	None	6	Negative	Negative	7
2	13	–	Moderate grade	6	Punctate & diffuse	Negative	–
3	1	F	None	6	Punctate & diffuse	Negative	10
3	3	–	None	6	Punctate	Negative	–
4	10	F	None	6	Negative	Negative	5
4	14	–	Moderate grade	6	Negative	Negative	–
5	1	F	None	6	Negative	Negative	4
5	4	–	None	6	Negative	Negative	–
6	1	M	Moderate grade	11	Punctate & diffuse	Focal	29
6	6	–	Low grade	11	Negative	Negative	–
7	11	M	Low grade	11	Punctate & diffuse	Negative	1
8	16	M	None	6	Negative	Negative	1
9	4	F	Low grade	6	Punctate & diffuse	Negative	1
10	16	F	None	6, 11	Not done	Not done	3
10	19	–	None	6	Punctate	Not done	–

Fig. 1 **a** Diffuse in situ hybridization (ISH) signals (black arrows) in a laryngeal papilloma indicating episomal virus. **b** Punctate (red arrows) and diffuse ISH signals (black arrows) in a laryngeal

papilloma indicating viral integration and episomal HPV, respectively. **c** HPV L1 immunohistochemical staining in a laryngeal papilloma showing viral capsid protein expression

Discussion

In the current study, we investigated HPV in juvenile-onset versus adult-onset respiratory papillomas. To our knowledge, this study is the first to correlate HPV genotype, ISH signal patterns, and L1 capsid expression in laryngeal papillomas. The finding that punctate ISH signals are common

in both juvenile-onset (7/10) and adult-onset (7/11) patients infected with low-risk HPV genotypes, suggests that HPV integration into the cell genome occurs at an early stage and may be an important factor contributing to the recurrent nature of these lesions. Possibly, if HPV integration occurs in a basal stem cell, HPV is then locked into the cell genome at the site of laryngeal infection and thereafter may support

the recurrent papilloma phenotype and eventually the conversion of laryngeal papillomas to carcinomas, especially in juveniles [6, 7]. In cervical tumors, high-risk HPV integration is associated with the loss of the HPV *E2* gene that regulates the *E6* and *E7* oncogenes that promote abrogation of cell cycle control and cell division; low-risk HPV integration may support a parallel situation in recurrent papillomas. It can be speculated that the tendency for HPV integration and/or aberrant cell growth and lesion recurrence in neonates and children may be exacerbated by the changes associated with the developing larynx. In newborns, the vocal fold length is ~3.0 mm increasing up to 21.0 mm in adulthood. Accompanying this growth, are transitional changes involving cuboidal, columnar, brush cell, ciliated and non-ciliated cell types, as well as alterations in cell layer thickness and degree of differentiation. The larynx also undergoes modifications in the proportions of mucinous, cartilaginous, ligamentous and collagen structures, and changes its overall shape from a conical to a cylindrical form while descending from the first to third cervical vertebrae in childhood to the sixth to seventh vertebrae in adulthood [17]. Animal studies also show that the process of laryngeal growth and epithelial maturation incurs considerable metaplastic transformation [18]; perhaps the cell processes that otherwise prevent HPV integration are more susceptible to aberrant functioning in tissues undergoing (chronic) transformation. Additionally, infants (and immunocompromised adults) may be especially susceptible to chronic HPV infection and integration through failure to clear the virus from the respiratory tract. Episomal HPV perpetuation (diffuse signal patterns) may also be explained as the consequence of a stem cell infection. Diffuse-only ISH signal patterns were absent from juvenile papillomas but were detected in 2/11 (18.2%) adult-onset cases. Additionally, 60% of adult-onset cases had ≥ 1 papilloma positive by immunohistochemistry for viral L1 capsid protein expression, whereas only 11% of juvenile cases showed L1 expression ($P = 0.06$). Taken together, the diffuse signal patterns and L1 expression data suggest that the mechanism of papilloma recurrence in juveniles may be more likely due to HPV integration rather than constant laryngeal re-infection by HPV shed from a productive lesion. In this respect, our results agree with those of Sharma et al. [19] who found that 65% of ISH positive juvenile laryngeal papillomas had a low viral load. Conversely, late (adult) onset RRP may be more likely than juvenile RRP to retain features of the productive HPV phenotype, which may also contribute to adult lesion recurrence.

Previous studies have shown HPV 11 to be associated with a more aggressive clinical course in children, including development of tracheal disease, pulmonary disease, and greater frequency of surgical procedures including tracheotomies [20–22]. However, HPV 11 was

not demonstrated to be a significant biomarker of recurrence or dysplasia in juveniles in this study: only 28.6% of juveniles with recurrent papillomas, and only 40% of those with ≥ 1 dysplastic papilloma were positive for HPV 11. HPV11 was found to be more prevalent in adult-onset than juvenile-onset papillomas (45.5% vs. 20%), but due to our relatively small sample size, this difference was not statistically significant ($P = 0.36$). Further longitudinal studies of HPV with a larger sample set are required to better determine the association of the HPV 11 genotype with disease character.

In conclusion, we found evidence that integration of low-risk HPV types into the cell genome is an early event (as evidenced by the detection of punctate signals in incident recurrent papillomas and in juveniles as young as 1 year) and a common event in the etiology of both juvenile and adult recurrent laryngeal papillomas. Productive HPV infections may be more common in adults; accordingly, constant laryngeal re-infection by HPV shed from a productive lesion may also contribute to adult recurrent lesions. The mechanism of papilloma recurrence in juveniles, conversely, may be more attributable to HPV integration. Larger studies are warranted to examine this trend and shed more light on the biological evolution of these curious lesions.

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