Human Papillomavirus and Host Variables as Predictors of Clinical Course in Patients with Juvenile-Onset Recurrent Respiratory Papillomatosis

M. GABBOTT,1 Y. E. COSSART,1 A. KAN,2 M. KONOPKA,2 R. CHAN,3 AND B. R. ROSE1*

Department of Infectious Diseases, The University of Sydney,1 Department of Histopathology, The New Children’s Hospital Sydney,2 and Microbiology Department, Liverpool Hospital,3 Sydney, New South Wales, Australia

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This study provides the first systematic evaluation of papillomavirus type and viral mutation occurring during the course of juvenile-onset recurrent respiratory papillomatosis. One hundred ninety-nine consecutive papillomas excised from 47 children between 1981 and 1996 at The New Children’s Hospital in Sydney, Australia, were tested for human papillomavirus (HPV) DNA by PCR. PCR products from the viral upstream regulatory region (URR) enhancer were sequenced, and variation was related to clinical variables. Forty-four of the 47 children had HPV-induced papillomas, with type 11 accounting for 24 (55%) and type 6 accounting for 19 (43%); one (2%) was positive for either type 6 or 11. Overall, 183 (98%) of the 186 samples with amplifiable DNA were HPV positive. There was no change in HPV type over time and no statistically significant association between HPV type and disease aggressiveness. One novel, large-scale URR duplication was identified in an HPV type 11 isolate in the last of a series of six papillomas examined and the first from the bronchus. However, the duplication was not found in HPV type 11 isolates from the associated pulmonary carcinoma and its metastases in other organs. Three of 14 URR point mutations coincided with transcription factor binding sites, but there were no obvious associations with clinical course. Chi-square and multiple linear regression analyses of clinico pathological variables revealed early age at diagnosis (less than 4 years) as an independent predictor of aggressive disease ($P < 0.001$). A bimodal distribution of the age at diagnosis was noted, with peaks at 2 and 11 years of age.

Clinical variables such as frequency of excisions and sites of involvement provide indications of the aggressiveness of juvenile-onset recurrent respiratory papillomatosis (RRP), but there are no reliable predictors of the clinical course in individual cases. Human papillomavirus (HPV) type 6 (HPV6) and HPV11 are accepted etiologic agents of RRP (22, 29), and there is some evidence that HPV11 is associated with a more protracted clinical course requiring a greater number of treatments than HPV6 (14). This claim remains to be fully validated.

Other viral markers of biological importance may also be significant in determining the behavior of this disease. There is evidence in HPV16- and HPV18-associated genital malignancies that sequence variation in the viral upstream regulatory region (URR) affects the binding of cellular factors which help regulate E6/E7 expression (20, 26), and large-scale URR duplications have been a feature of both HPV6- and HPV11-positive malignancies (3, 8, 18). It has also been reported that some point mutations in the URR of HPV11 clinical isolates (some from RRP) adversely affect the level of E6 promoter activity in vitro (21), providing a hint that there may be a subset of variants with reduced biologic potential, but it is not known if changes of this nature influence the clinical course.

Identification of HPV markers associated with the progression or regression of disease could be of great benefit in the management of individual RRP cases and, in the long term, may lead to development of effective gene therapies, as well as therapeutic and preventive vaccines. This retrospective observational study investigated the relationship between HPV and the associated clinical course of juvenile-onset RRP by analyzing 199 serial biopsies collected from 47 children during the course of their disease. All samples were tested for HPV type by PCR, and sequence analysis was performed on the URRs of isolates from individual patients to detect variation from the prototype and to identify any change occurring over the course of disease. Associations between molecular findings and clinical variables were sought.

MATERIALS AND METHODS

Study population. Investigations were carried out on formalin-fixed, paraffin-embedded sections from the most recent 199 histologically diagnosed respiratory tract papillomas excised from patients treated at The New Children’s Hospital (formerly the Royal Alexandra Hospital for Children), Sydney, New South Wales, Australia. They were derived from 47 patients receiving treatment between 1981 and 1996. The clinical records were reviewed, and data related to sex, age at diagnosis, surgical episodes, and site of involvement were extracted. Of the 47 patients in the study, 23 (49%) were male. The median age at diagnosis was 4 years (range, 0.5 to 21 years). The median number of samples available for analysis from each patient was two (range, 1 to 23), although the total number of excisions per patient ranged from 1 to 168 with a median of 19. Median treatment time was 3 years (range, 0 to 18 years), with a median frequency of 5 excisions per year (range, 2 to 17).

At diagnosis, 44 (94%) of the 47 patients had laryngeal lesions; among these, there was concomitant involvement of the pharynx in 2, of the esophagus in 2, and of the trachea in 1. Of the three cases without laryngeal lesions at diagnosis, two had tonsillar and one had tracheal papillomas. Subsequent extralaryngeal spread of disease was observed in 16 (36%) of 44 patients. In total, 12 (26%) of the 47 patients had tracheal and/or bronchial and/or lung (TBL) involvement at some stage of their clinical course. To provide an overall assessment of the severity of disease, patients were categorized as having either aggressive (10 or more total excisions with a frequency greater than or equal to 3 excisions per year and/or TBL involvement) or nonaggressive disease, in accordance with the method used by Doyle et al. (9). Twenty-eight (60%) of the 47 patients were classified as having aggressive disease. At the conclusion of the study, 26 (55%) of the patients were believed to be in remission, 10 (21%) were still being treated, and 10 (21%) had been lost to follow-up. One patient (no. 27) developed
Selected clinical variables of the individual patients are shown in Table 1. HPV typing. Ninety-two percent (183 of 199) of the samples from the 47 patients were HPV DNA positive. Thirteen (81%) of the 16 HPV-negative samples, all from a single patient (no. 42), contained no amplifiable DNA as judged by negative results of the β-globin PCR assay. Other samples from this patient were HPV DNA positive, and the negative results were attributed to complete loss of the tiny biopsies during processing rather than to the presence of PCR inhibitors. Three other patients, all with single, nonrecurring lesions, had β-globin-positive, HPV-negative lesions.

The HPV type was always the same in serial samples from the same patient, and no sample was positive for more than a single HPV type. HPV11 was present in 24 (55%) of 44 HPV-positive cases, and HPV6 was present in 19 (43%) (10 subtype 6a, 7 subtype 6b, and 2 not subtyped due to lack of material); a single lesion from one patient contained either type 6 or 11, but the sample was insufficient for delineation of the type. In this study, HPV6a sequences are defined by the presence of a 20-nucleotide (nt) insert following position 7718, compared to the HPV6b prototype, where this insert is not present, in accordance with the criteria of a previous study (16).

**URR sequence variation.** Sequencing with primer TS1 or TS2 allowed analysis of a 244-bp (7620-to-7863) region of the HPV 11 enhancer, a 285-bp (7664-to-7948) segment of the HPV 16 enhancer, and a 265-bp (7576-to-7840) region of the HPV6b enhancer. Isolates from 21 of the 24 patients with HPV11-positive lesions and 17 of 19 patients with HPV6-positive lesions were sequenced. First and last samples were available from 11 patients with HPV11-positive lesions and 6 with HPV6-positive lesions. Of the remaining 10 HPV11- and
eleven HPV6-positive cases, 6 (4 and 2, respectively) had single lesions. In other cases, there was insufficient material for sequencing.

**HPV11.** None of the 21 isolates analyzed was identical in sequence to the prototype. A GC insert following position 7716 in the HPV11 URR was present in all of the isolates. In addition, one large-scale change and four point mutations were identified.

(i) **Large-scale mutation.** A novel 51-nt duplication was present only in the last of the six HPV11-positive benign samples from patient 27. The first five samples collected from the larynx and trachea all produced bands with the predicted sizes on electrophoresis, but the sixth, from the bronchus, showed the additional presence of a larger band (Fig. 1). Sequence analysis confirmed the presence of a duplication of nts 7707 to 7755, introducing extra AP1 and Oct-1 transcription factor binding sites. No antemortem malignant tissues were available for analysis. All 11 postmortem samples subsequently analyzed (papillomas from the epiglottis and uvula, primary SCC from the lung, and secondary deposits in the heart, liver, and spleen) tested positive for HPV11. However, there was no evidence of the duplication in any of these samples.

(ii) **Small-scale mutations.** Isolates from two patients showed point mutations in the sequenced region (Table 4).

**HPV6.** One of the 17 HPV6 isolates sequenced was identical to the HPV6b prototype in the segment analyzed. In total, there were 10 point mutations and the numbers in individual isolates ranged from 3 to 5. Samples from 10 patients contained the HPV6a subtype (Table 5).

**Clinical associations.** All 38 patients with recurring lesions had HPV-related disease, compared with 6 of the 9 with single, nonrecurring lesions. Twenty-two (58%) of the patients with

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**TABLE 2.** PCR primers and thermocycling profiles used in the study

<table>
<thead>
<tr>
<th>PCR no. (primer[s])</th>
<th>Target(s)</th>
<th>Genome position(s)</th>
<th>Product size (bp)</th>
<th>Thermocycler profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (TS1, TS2)</td>
<td>HPV11</td>
<td>7583–7602, 7889–7870</td>
<td>307</td>
<td>94°C for 4 min (1 cycle); 94°C for 30 s, 52°C for 1 min, 72°C for 1 min (38 cycles)</td>
</tr>
<tr>
<td>1 (TS1, TS2)</td>
<td>HPV6</td>
<td>7539–7558, 7860–7841</td>
<td>322</td>
<td>94°C for 4 min (1 cycle); 94°C for 30 s, 52°C for 1 min, 72°C for 1 min (38 cycles)</td>
</tr>
<tr>
<td>2 (TS3, TS4)</td>
<td>HPV11</td>
<td>7687–7886, 7791–7772</td>
<td>105</td>
<td>94°C for 4 min (1 cycle); 94°C for 30 s, 52°C for 1 min, 72°C for 1 min (38 cycles)</td>
</tr>
<tr>
<td>3 (pU-2R, pU-31B)</td>
<td>HPV6/11</td>
<td>400–419, 627–608</td>
<td>228</td>
<td>94°C for 4 min (1 cycle); 94°C for 30 s, 52°C for 1 min, 72°C for 1 min (38 cycles)</td>
</tr>
<tr>
<td>4 (HPV16 specific)</td>
<td>HPV16</td>
<td>320–339, 429–410</td>
<td>110</td>
<td>94°C for 4 min (1 cycle); 94°C for 30 s, 52°C for 1 min, 72°C for 1 min (38 cycles)</td>
</tr>
<tr>
<td>5 (HPV18 specific)</td>
<td>HPV18</td>
<td>46–66, 130–111</td>
<td>85</td>
<td>94°C for 4 min (1 cycle); 94°C for 30 s, 52°C for 1 min, 72°C for 1 min (38 cycles)</td>
</tr>
<tr>
<td>6 (GH2O, PCO4)</td>
<td>β-Globin gene</td>
<td>Unknown</td>
<td>268</td>
<td>94°C for 4 min (1 cycle); 94°C for 30 s, 52°C for 1 min, 72°C for 1 min (38 cycles)</td>
</tr>
<tr>
<td>7 (GP5, GP6)</td>
<td>HPV6, HPV11, HPV16, HPV18, HPV31, HPV33, HPV35, HPV52, HPV58</td>
<td>L1 ORF</td>
<td>140–150</td>
<td>94°C for 4 min (1 cycle); 94°C for 30 s, 52°C for 1 min, 72°C for 1 min (38 cycles)</td>
</tr>
<tr>
<td>8 (CP-I, CP-IIG)</td>
<td>HPV6, HPV11, HPV16, HPV18, HPV31, HPV33, HPV35, HPV52, HPV58</td>
<td>E1 ORF</td>
<td>188</td>
<td>94°C for 4 min (1 cycle); 94°C for 30 s, 55°C for 1 min, 72°C for 1 min (38 cycles)</td>
</tr>
</tbody>
</table>

* Primer sequences are the HPV11 sequence.
* Genome position given is for the HPV6b prototype.
* Described by Fujinaga et al. (11).
* Described by Shibata et al. (28).
* Developed previously by our group.
* Developed by Rosnick et al. (25).
* Described by de Roda Husman et al. (6).
* Described by Tieben et al. (31).

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**TABLE 3.** PCR protocols

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value (PCR no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction volume (μl)</td>
<td>25 (3, 4, 6, 7, 8), 50 (1, 2, 5)</td>
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<tr>
<td>MgCl₂ concn (mM)</td>
<td>2.0 (1, 2, 3, 4, 5, 6), 3.5 (7), 3.6 (8)</td>
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<tr>
<td>Deoxynucleoside triphosphate concn (μM)</td>
<td>250 (1, 2, 3, 4, 5, 6, 7, 8)</td>
</tr>
<tr>
<td>Primer concn (μM)</td>
<td>0.2 (6), 0.6 (4), 1.1 (8), 1.2 (1, 2, 3, 7), 1.5 (5)</td>
</tr>
<tr>
<td>No. of U of Tth plus DNA polymerase</td>
<td>0.5 (3, 4, 6, 7, 8), 1.0 (1, 2, 5)</td>
</tr>
</tbody>
</table>

* Biotech.
* The PCR numbers correspond to those in Table 2.

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**FIG. 1.** Polyacrylamide gel electrophoresis of URR (TS1/TS2) PCR products from patient 27. Lanes: 1, size marker (HpaII digest of pUC-19); 2 to 6, samples 27/1 to 27/5, respectively, showing a band of the predicted size (307 bp); 7, sample 27/6, showing an additional band of 358 bp; 8, HPV11-positive control.
TABLE 4. Point mutations identified in HPV11 enhancer

<table>
<thead>
<tr>
<th>Nucleotide position</th>
<th>Nucleotide in HPV11 prototype</th>
<th>Mutation</th>
<th>Presence of mutationa in patient no.:</th>
<th>7, 9, 10, 18, 20, 25, 36, 37, 40</th>
<th>15</th>
<th>534</th>
<th>45</th>
<th>6</th>
<th>17</th>
<th>16</th>
<th>38</th>
</tr>
</thead>
<tbody>
<tr>
<td>7625</td>
<td>C</td>
<td>T</td>
<td>+</td>
<td>3, 11, 14, 19, 21, 23, 24, 26, 27, 39-33, 35, 39, 42-44</td>
<td>28</td>
<td>41</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7626</td>
<td>A</td>
<td>C</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7682</td>
<td>T</td>
<td>C</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7819</td>
<td>A</td>
<td>C</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

a A plus sign indicates that the mutation was present.

Table 4. Point mutations identified in HPV11 enhancer

recurrent disease had lesions containing HPV11, 15 (39%) had HPV6-positive samples, and the papilloma of 1 patient had either HPV6 or HPV11. Among the six with HPV-positive lesions in the nonrecurring group, HPV6 was present in the lesions of four (67%) of the patients, while two (33%) typed as HPV11.

Four patients were excluded from the statistical analysis, owing to either the absence of detectable HPV DNA in their samples (patients 1, 2, and 46) or inability to determine the exact HPV type (patient 13). Univariate analysis showed that age at diagnosis and disease aggressiveness were significantly related (chi square, 8.748; P = 0.003), with an age of less than 4 years associated with a greater likelihood of aggressive disease. Neither sex nor HPV type was significantly related to disease aggressiveness (chi square, 1.132 [P = 0.287] and 0.995 [P = 0.319], respectively). In the multiple linear regression model with all three independent variables included, an age at diagnosis of less than 4 years remained a significant predictor of a greater likelihood of being classified as having aggressive disease (βage = 0.472, t = 3.660, P < 0.001) but neither sex (βsex = 0.221, t = 1.650, P = 0.107) nor HPV type (βtype = −0.133, t = −1.022, P = 0.313) was a significant predictor of disease aggressiveness. To account for potential misclassification among the 10 (21%) patients lost to follow-up and the 10 still being treated, the analysis was repeated with all of the patients in these groups assigned to the aggressive disease category (4 of the 20 patients involved). The sole significance of a young age at diagnosis was sustained on univariate (chi square = 9.958, P = 0.002) and multivariate (βage = 0.488, t = 4.149, P < 0.001) analyses. The relationship between URR mutations and clinical variables could not be assessed statistically due to low frequency.

DISCUSSION

This study surveyed the prevalence and persistence of different HPV types in consecutive histologically diagnosed respiratory papillomas. It is unique in its evaluation of sequence variation in the HPV URR in relation to the natural history of RRP. The findings also provide new insight into clinical variables which influence the aggressiveness of RRP.

The results of the study support a previous large survey showing that RRP is evenly distributed between the sexes (19). The median age at diagnosis of 4 years in our group is also in accordance with previous studies (17, 30), but it is interesting that there was some evidence of a bimodal distribution of age at diagnosis for HPV-positive cases (Fig. 2). Such a feature has been noted previously (30) and could be accounted for by relatively recent acquisition of infection by adolescents, for instance, via sexual activity, or by hormonal activation following a very lengthy incubation. The proportion of patients with extralaryngeal involvement (36%) was similar to that of a previous study (5).

The study reaffirms HPV as the major etiologic agent of respiratory papillomatosis. The use of optimized PCRs amplifying different parts of the genome undoubtedly increased the power of the study to detect HPV DNA in archival specimens, bringing positivity rates close to 100%. Two of the three β-globin-positive, HPV-negative samples were single, nonrecurring tonsillar lesions, pointing to an etiology unrelated to HPV. The exclusive identification of HPV6 and HPV11 in our samples was predictable from previous surveys, although worldwide variation in the distribution of types of genital lesions (2) might lead to an expectation of different types in RRP patients with different geographical and ethnic origins. The predominance of HPV11 over HPV6 is also in accordance with previously published reports of RRP in children (7, 14, 23). Evidence is accumulating that the distribution of HPV6 and HPV11 in RRP is age dependent, with HPV6 reportedly more common in children (7, 14, 23). Evidently, the exclusive identification of HPV6 and HPV11 in our samples was predictable from previous surveys, although worldwide variation in the distribution of types of genital lesions (2) might lead to an expectation of different types in RRP patients with different geographical and ethnic origins. The predominance of HPV11 over HPV6 is also in accordance with previously published reports of RRP in children (7, 14, 23). Evidence is accumulating that the distribution of HPV6 and HPV11 in RRP is age dependent, with HPV6 reportedly more common than HPV11 in adult-onset disease (4, 24). Although HPV6a was slightly more common than HPV6b overall in our isolates, there was no convincing evidence to support the findings of previous, smaller studies that this subtype prevails among HPV6 isolates in juvenile-onset RRP (16, 18).

There was no evidence of the concurrent infection with different HPV types which has been reported in some studies
While it is difficult to exclude coinfection with HPV6 and HPV11 due to extensive sequence homology, the high quality of our sequence data and the consistency of the types detected in serial biopsies from individual patients indicate that multiple infection involving these types is uncommon, at least in our cohort.

We were unable to draw a conclusion about any firm relationship between HPV type and clinical disease aggressiveness. The predominance of HPV6 over HPV11 in single, nonrecurring lesions indirectly supports the aggressiveness of HPV11, but large, multicenter studies are needed to clarify the clinical significance of HPV type.

This study confirms the remarkable stability of the HPV11 URR enhancer in comparison with that of HPV6 (and even HPV16 and HPV18). Nineteen of the 21 HPV11 isolates examined were identical in sequence to the prototype (apart from a GC insert following position 7716, which was also a feature of all of the isolates in previous studies [16, 21]). In contrast, only 1 of 17 HPV6 isolates was identical in sequence with the HPV6b prototype. The tendency of HPV6 mutations to bridge the gap between HPV6a and HPV6b (16) was confirmed.

The significance of URR sequence diversity in terms of the aggressiveness and/or progression of respiratory papillomatosis was a major focus of this study. We hypothesized that those with particularly aggressive disease may have been infected with variants containing mutations mediating upregulation of the E6/E7 promoter or, alternatively, that such mutations might occur during the course of disease in response to various forms of physical or chemical treatment or to the unstable environment created by rapidly cycling cells. We were also interested in establishing whether less aggressive (particularly nonrecurring) lesions were associated with isolates containing mutations mediating down-regulation of the E6 promoter such as those identified in the study of Heinzel et al. (16). The findings of that study support our hypotheses to a limited extent. The 51-nt duplication identified during the course of disease occurred in an isolate from patient 27, who required the greatest number of excisions (n = 168) over the longest period of time (18 years), was one of three with dissemination of disease to the lung, and was the only one with progression to malignancy, which ultimately claimed his life. The fact that the mutation was identified in only the last of six benign papillomas and the first from the bronchus supported a role for this mutation in the spread of the disease, particularly as radiological evidence of pulmonary malignancy was first noted at this time.

It was therefore surprising that none of the HPV11 isolates from this study that any single mutation or pattern of point mutations predisposed to either a favorable or an unfavorable outcome. Nonetheless, we were unable to exclude the possibility that the variants identified by Heinzel and colleagues (16) were associated with a reduced biologic potential since none of our isolates showed corresponding patterns of mutation—a reflection of the geographic clustering of variants.

This is the first report of a relationship between age at diagnosis and aggressive disease in juvenile-onset RRP. The link between young age at disease onset and persistence and aggressiveness of disease has a parallel in hepatitis B and may be explained by immaturity of the immune response mechanisms.

It is acknowledged that incomplete follow-up of 10 (21%) of the patients may have resulted in misclassification of aggressiveness of disease for some patients, and the failure to observe all of the patients over the entire juvenile period is a further potential limitation of this essentially descriptive study. However, in the first instance, only four of the patients who did not reach remission were classified as having nonaggressive disease and the effect of young age was sustained in a reanalysis of the worst-case scenario in which all of these were aggressive. With regard to the second issue, current understanding of the natural history of childhood disease suggests that recurrence following remission in childhood is uncommon.

We conclude that further studies of both viral and host factors are needed to confirm the results of this study. The critical steps in virus-host interactions accounting for the emergence of this rare clinical manifestation of a common viral infection remain open to question. There is some indication of a relationship between the HLA DR3 phenotype and susceptibility to RRP (1), but this has not been shown for other HPV-induced lesions. It seems probable that regression of lesions is brought about by an HPV type-specific, cell-mediated immune response, but there has been no association between RRP and immune deficiency status. In this study, we have shown that mutation in the URR can be ruled out as the cause of either aggressive cell proliferation or long duration of RRP, although characterization of other viral genes such as the E6/E7 oncogenes and L1 seems worthwhile.

ACKNOWLEDGMENTS

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REFERENCES


