Longitudinal Measures of Human Papillomavirus 6 and 11 Viral Loads and Antibody Response in Children With Recurrent Respiratory Papillomatosis

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Objectives: To measure human papillomavirus (HPV) 6 and 11 viral load and antibody response in longitudinal specimens obtained from children with recurrent respiratory papillomatosis and to examine the association of type-specific viral load with clinical severity of disease.

Design: Longitudinal pilot study with a median follow-up of 5.4 months.

Subjects: The study included 15 children undergoing therapy for recurrent respiratory papillomatosis at the Egleston Children’s Hospital, Atlanta, Ga, between January 22, 1999, and June 13, 2000.

Main Outcome Measures: The kinetics of HPV-6 and HPV-11 viral load and antibody level were examined over time. Longitudinal HPV-6 and HPV-11 viral loads were analyzed for associations with clinical indicators of disease severity.

Results: Four children were infected with HPV-11, 4 were infected with HPV-6, and 7 had mixed infections. The HPV-6 and HPV-11 viral loads were stable over time in most of the children. Among children with mixed infections, HPV-6 viral loads were inversely correlated with those of HPV-11 ($r = -0.80$, $P < .001$). The HPV-11 infection was significantly associated with more annual surgical procedures ($P = .02$). Neither HPV-6 nor HPV-11 viral loads were associated with demographic factors or markers of clinical severity. None of the children had detectable antibodies against HPV-6, and only 3 had detectable antibodies against HPV-11 virallike particles.

Conclusions: Our data support the association of HPV-11 infection with clinical severity. Measures of HPV-6 and HPV-11 viral loads are relatively stable over time in most children with recurrent respiratory papillomatosis, suggesting that multiple samples may not be necessary. Cytobrush samples may substitute for tissue biopsy specimens in HPV detection and typing, but not for absolute viral load determination.


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anatomical sites, increased frequency of surgery, spread to the lower respiratory tract, and extended duration of disease.\textsuperscript{3,6,7} Although several studies have reported an association between infection with HPV-11 and disease severity, we are not aware of longitudinal studies that evaluated type-specific kinetics of viral markers. We performed this pilot study to determine if viral load varied over time and if the method of sampling (biopsy vs cytobrush) affected results. In addition, we examined the correlation of viral load and host antibody response to clinical aspects of the disease.

**METHODS**

**STUDY POPULATION AND DESIGN**

All children who presented for care of JORRP at the otolaryngology clinic of Emory University, Atlanta, between January 22, 1999, and June 13, 2000, were eligible. We observed enrolled children for a median of 5.4 months (range, 0–16.1 months) on a schedule of clinic visits determined by their need for treatment. All children had been enrolled in the national registry of JORRP database,\textsuperscript{2} from which we abstracted demographic data (date of birth, sex, race, and ethnicity) and clinical data (date of diagnosis and number of previous surgical treatments). The duration of disease at enrollment and the average number of annual surgical procedures during the illness were computed based on these data. Indicators of clinical severity included the age of the child at diagnosis, the duration of illness, the average number of months between treatments, the average number of annual surgical procedures, the number of anatomical sites where lesions were detected, and the detection of lesions on the trachea. The JORRP-affected anatomical sites were grouped as oral cavity, nasopharynx, oropharynx, hypopharynx, larynx, trachea, and bronchi/lungs. The main outcomes were the number of HPV-6 and HPV-11 viral copies and the presence of type-specific antibody obtained in specimens collected at each operating room visit.

The human subjects committees of the Centers for Disease Control and Prevention and Emory University approved the study protocol. Human experimentation guidelines of the US Department of Health and Human Services were followed in the conduct of this study.

**SPECIMEN COLLECTION AND PROCESSING**

Endoscopy or, at a minimum, microlaryngoscopy was performed at all operative visits. During endoscopy, a 5-mL peripheral blood sample was collected (into a red-top tube) from the intravenous port that was established for anesthesia. Cells from the surface of JORRP-affected anatomical sites were obtained before instrumentation of the site, by an otolaryngologist (N.W.T.), using a cytobrush. The cytobrush was then placed in phosphate-buffered saline collection media (0.01M phosphate-buffered saline, pH 7.4, and 0.5mM EDTA). Tissue samples from representative lesions were collected by cup forceps and placed in sterile transport medium (Dulbecco-modified Eagle medium with fetal bovine serum, penicillin G, streptomycin sulfate, and gentamicin sulfate). All samples were stored at 4°C until transported to a Centers for Disease Control and Prevention laboratory. Serum was separated from peripheral blood and stored at −20°C in 0.5-mL aliquots until withdrawal for antibody testing. Cells from the cytobrush were dislodged by vortex mixing, made into pellets, washed once by resuspension in phosphate-buffered saline, and then made into pellets again. Cell pellets were stored at −70°C. Half of each tissue sample was fixed in formalin and processed to paraffin, and the other half was frozen at −70°C.

Total nucleic acids were extracted from tissue and cell pellets using a purification kit (MasterPure Complete DNA and RNA Purification Kit; Epicenter Technologies, Madison, Wis). Frozen tissue samples were mechanically disrupted with a homogenizer before extraction. The total nucleic acids were resuspended in 50-µL TE buffer (10mM Tris hydrochloride, pH 8.0, and 1mM EDTA).

**LABORATORY TESTING**

We used type-specific, direct, virallike particle enzyme-linked immunosorbent assays, as previously described, to detect IgG and IgA antibodies against HPV-6 and HPV-11.\textsuperscript{6} Negative control serum samples (n = 9) were obtained from a panel of samples from Egleston Children’s Hospital, Atlanta (n = 6), and from a panel of samples obtained from nuns (n = 3). Results from these serum samples were used to set the optical density cutoff value for seropositivity at 0.4, 2 times the average optical density value for negative control serum samples.

We determined copy numbers of HPV-6, HPV-11, and β-globin by using real-time quantitative fluorescent polymerase chain reaction, as described previously.\textsuperscript{2} Viral load was expressed as copies of virus per cell equivalent (based on 2 β-globin copies per cell). Children were considered infected if they had at least 1 sample positive for HPV-6 or HPV-11.

**STATISTICAL ANALYSES**

Mixed models with a spatial power covariance structure were used to conduct repeated-measures analysis of the associations between continuous measures of log-transformed HPV-6 and HPV-11 viral loads measured in tissue specimens and demographic and clinical variables.\textsuperscript{10} Two-tailed P values obtained in unadjusted mixed models are reported as indicators of statistically significant associations; P < .05 was required for statistical significance. Dichotomous levels of continuous clinical factors were based on median values for all children. Spearman rank correlation coefficients were computed to measure correlations of HPV-6 and HPV-11 viral copy numbers measured within and between tissue types and also between HPV-6 and HPV-11 copy numbers and continuous values of clinical factors. The Wilcoxon rank sum test was used to compare median HPV-6 and HPV-11 viral copy numbers within and between specimen types. The Wilcoxon rank sum test was also used to compare median values of continuous clinical markers by HPV-6 and HPV-11 infection status. Coefficients of variation were computed as indicators of the variation in HPV-6 and HPV-11 viral load measurements for each child, based on 100 × (standard deviation/mean), using log-transformed (natural log) values of viral loads. SAS statistical software, version 9.0 (SAS Institute Inc, Cary, NC), was used to conduct all statistical analyses.

Sixteen children were enrolled following parental informed consent; however, 1 child was omitted from this analysis because of missing clinical data. The 15 children were enrolled at a median age of 3.9 years (range, 1.5–13.8 years). Most (n = 11) were girls and had a low socioeconomic status (11 had Medicaid insurance coverage, 3 did not have Medicaid insurance, and 1 had a missing insurance status). There were 7 black children and 7 white children, 1 child’s race was unknown.
The median age at diagnosis was 2.4 years (range, 0.5-5.7 years), and the median duration of illness at enrollment was 8 months (range, 1.2 months to 12 years). Twelve patients had lesions limited to 1 site (11 in the larynx and 1 in the trachea).

Four had lesions involving multiple sites (the larynx and trachea in 2 patients; the nasal, oral, and pharynx sites in 1 patient; and the larynx and right vocal chord in 1 patient). Three patients (20%) had involvement of the trachea. The median time between treatments among all children was 2.9 months (range, 0.5-9.9 months) (4 children had data on only 1 clinic visit; thus, they had values of 0 for time between treatments). The median number of treatments per year was 3.3 (range, 0.5-10.8).

We examined associations between demographic factors and clinical indicators. Girls were significantly younger than boys at enrollment (median age, 2.7 vs 6.5 years; \( P = .01 \)). Because age at enrollment was highly correlated with duration of illness (\( r = 0.67, P = .008 \)), girls also had a significantly shorter median duration of illness at enrollment compared with that of boys (0.4 vs 3.0 years; \( P = .02 \)). In addition, girls had a shorter median interval between treatments (3.5 vs 10.7 months; \( P = .02 \)). No other demographic factors were associated with clinical factors.

All patients were HPV positive, 4 were infected with HPV-6 only, 4 were infected with HPV-11 only, and 7 were coinfected with HPV-6 and HPV-11. Children infected with HPV-11 only had the same median level of annual surgical procedures as those coinfected with HPV-11 and HPV-6 (median, 3.6 annual surgical procedures). Children infected with HPV-6 only had fewer annual surgical procedures (median, 2.5). The difference in median annual surgical procedures between children with HPV-11 infection (single or coinfection) and children infected with HPV-6 only was statistically significant (\( P = .02 \)) (Figure). Although the magnitude of the difference in median number of surgical procedures was the same when the comparison was restricted to those with HPV-11 only, compared with those with HPV-6 only, the few children in these groups (\( n = 4 \)) resulted in statistical nonsignificance (\( P = .18 \)). Neither HPV-11 nor HPV-6 infection status was associated with other clinical markers of disease severity (data not shown).

The HPV viral loads measured in total nucleic acids from biopsy and cytobrush samples on the same procedure dates were highly correlated (\( r = 0.88 \) and \( r = 0.87 \) for HPV-6 and HPV-11, respectively; \( P < .001 \) for both). Median values of viral loads were significantly higher in biopsy than cytobrush samples (HPV-6: 7.5 vs 0.0 log copies, respectively [\( P = .001 \)]; and HPV-11: 16.1 vs 14.7 log copies, respectively [\( P < .001 \)]). The HPV viral loads for HPV-6 and HPV-11 measured in tissue were stable over follow-up for most children. For HPV-11, the coefficient of variation was 15% or less for 5 (71%) of 7 children with multiple measurements; and the coefficient of variation for HPV-6 was less than 9% for 7 (78%) of 9 children who had multiple measurements. The HPV-6 and HPV-11 viral loads measured in cytobrush samples had similar coefficients of variation (data not shown).

Among the 7 patients with coinfections, the HPV-6 tissue viral load levels were inversely correlated with those for HPV-11, such that patients with low levels of HPV-6 had high levels of HPV-11 and vice versa (\( r = -0.80, P < .001 \)). Among these 7 children, HPV-6 viral loads predominated in 4, HPV-11 viral loads predominated in 2, and similar levels of HPV-6 and HPV-11 were detected in 1. For the children in whom 1 viral load predominated, a consensus polymerase chain reaction assay detected only the predominant type (data not shown).

Evaluation of associations between repeated measures of HPV-6 or HPV-11 viral loads in biopsy samples and indicators of clinical severity was conducted separately among HPV-6-infected (and coinfection) children and HPV-11–infected (and coinfect) children. In repeated-measures analysis, neither HPV-11 nor HPV-6 viral loads were associated with any demographic factors, including age at enrollment, sex, race, and Medicaid insurance status (data not shown). In addition, these viral loads were not associated with any indicators of clinical severity, including longer duration of illness, having RRP lesions at more than 1 anatomical site, having lesions on the trachea, average number of annual surgical procedures, months between treatments, or age at diagnosis (Table).

None of the children had detectable antibodies against HPV-6, and only 3 had detectable antibodies against HPV-11 virallike particles, representing 27% of those with HPV-11 DNA and 20% of all children in the study. These 3 children had 3 of the 4 highest levels of HPV-11 viral load in the study; 2 of these 3 children had the highest average number of annual surgical procedures.

### Comment

In our study of 15 children with JORRP who were clinically followed up for a median of 5.4 months, 7 were coinfected with HPV-6 and HPV-11, 4 were infected with HPV-6 alone, and 4 were infected with HPV-11 alone. The levels of HPV-6 and HPV-11 viral loads were stable for most children throughout follow-up. The proportion of dually infected children is higher than in other reports. This may be because we used 2 separate typespecific assays to detect and quantitate HPV. Unless copy
In this study, children with JORRP due to infection with either HPV-11 only or HPV-11 and HPV-6 coinfection required more frequent surgical interventions (ie, recurrences were more frequent and required extirpation more often) than those with HPV-6 infection only, suggesting that infection with HPV-11 resulted in more severe manifestations of disease. Our study found that association was statistically significant only when comparing the children with HPV-11 and HPV-6 coinfection with those with HPV-6 infection only. A comparison of median annual surgical procedures between the few children infected with HPV-11 only and HPV-6 only precluded statistical significance. Previous reports disagree about the association of disease severity with the type of HPV infection. Among a series of 20 children with JORRP seen at the Red Cross War Memorial Children’s Hospital, 75% were infected with HPV-11 only, although HPV-6 predominated among children with the most severe manifestations of disease.13 However, others have reported a predominance of HPV-11 in association with disease severity in patients with JORRP. In reports on 2 series of pediatric patients having similar numbers of children infected by HPV-6 and HPV-11, only HPV-11 was associated with disease severity, including having a tracheotomy12 or a higher average frequency of treatments per year, a longer duration of disease activity, active disease at the time of study, and younger age at diagnosis.7 In addition, an extended disease course into adulthood was associated with HPV-11 infection among a series of 59 patients in Manchester, England, who were followed up for 18 years.13

Antibody responses to HPV have not been well studied in subjects with JORRP. In prior studies, only HPV-11 or HPV-6 and HPV-11 coinfection were studied, and in only 1 was response to virallike particles measured, even though they are the most accepted measure of type-specific antibody response.1416 None of the children in our study had detectable levels of antibodies to HPV-6, including those with predominately or only HPV-6 in the lesion, and only 27% of children with HPV-11 DNA in their tissues had a detectable seropositive response to HPV-11. The observation that seropositivity to HPV-6 and HPV-11 was associated with patients with the most surgical procedures has been reported previously for HPV-6 and HPV-11.13 The findings in that study were based on a synthetic peptide (L2) assay and argue that the mucosa is an immunologically protected site until surgical intervention. This low seroresponse to HPV-causing papillomas (warts) in the respiratory tract stands in stark contrast to the nearly 50% response to HPV-617 (58%) or HPV-1118 (46%) in persons with genial condylomas. Some of the difference may be because of accumulated exposure to multiple HPV types among adults with condylomas. In addition, genital condylomas may be exposed to more local trauma, resulting in greater immune stimulation.

We investigated the reliability of cytobrush sampling of papillomas to detect HPV that was found in tissue. Cytobrush sampling is noninvasive and could be used to study HPV in the larynx of asymptomatic individuals if the sampling method is validated. We measured concordance between the 2 types of samples (cytobrush and tissue), and high correlations were detected in the viral loads of cytobrush and tissue samples collected at the same time from individuals (HPV-6, r = 0.88; and HPV-11, r = 0.87). The absolute viral loads observed for the cytobrush samples were significantly lower than those observed for the tissue samples. It is possible that the cytobrush included cells from nonlesional sites, resulting in a dilution effect, or that the brushing was so gentle (no bleeding occurred at any of the sites) as to acquire only the most superficial aspect of the lesion. Type concordance is generally found between exfoliated cells and tumor tissue, but comparison of viral loads has not been reported.10

The main limitation of this study is the few individuals studied. The study was intended as a pilot to study the feasibility of different sampling approaches, to assess the need for multiple samples to characterize virus type and viral load, and to evaluate the relevance of HPV serologic features. The size of our pilot study limited the power to detect associations between HPV infection status and age at diagnosis along with most other clinical markers of severity that were examined.

In conclusion, we found a significant association of HPV-11 infection status and more annual surgical procedures, a marker of clinical severity of RRP disease, in a pilot study of 15 children. Multiple samples do not contribute additional information about HPV type and viral load. Cytobrush samples may substitute for tissue biopsy specimens in HPV detection and typing, but not for absolute viral load determinations. Human papillomavirus seropositivity was low in these children and correlated with multiple surgical procedures. Additional studies with larger samples sizes are needed to examine the association of HPV viral load and clinical disease severity.

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REFERENCES