Identical Human Papillomavirus (HPV) Genomic Variants Persist in Recurrent Respiratory Papillomatosis for up to 22 Years

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Seventy initial and 125 follow-up tissue specimens of laryngeal papillomas, obtained from 70 patients who had had recurrent respiratory papillomatosis for from 1 to 22 years, were investigated for the presence of human papillomavirus (HPV) DNA and HPV E5a, LCR and/or full-length genomic variants. HPV-6 was found in 130/195, HPV-11 in 63/195, and HPV-6/HPV-11 in 2/195 samples. Within 67/70 (95.7%) patients, all follow-up HPV isolates genetically matched completely initial HPV isolate over the highly variable parts of the genome or over the entire genome. Frequent recurrence of laryngeal papillomas is a consequence of long-term persistence of the identical initial HPV genomic variant.

Keywords. recurrent respiratory papillomatosis; HPV; persistence; HPV-6 and HPV-11; complete viral genome; genetic diversity.

Recurrent respiratory papillomatosis (RRP) is a relatively rare but debilitating disease characterized by repeated growth of squamous cell papillomas in the upper respiratory tract, with a predilection to the larynx [1]. Human papillomaviruses (HPV), most notably low-risk genotypes HPV-6 and HPV-11, are the etiological agents of virtually all cases of RRP [1,2]. Infection with HPV-11 and a younger age at onset of the RRP correlate with a more severe course of the disease [3]. Other HPV genotypes, such as high-risk HPV-16 and HPV-18, can occasionally be found in papilloma tissue specimens [1,2].

RRP can affect persons of all ages; however, a bimodal age distribution is usually observed, peaking in children younger than 5 years (juvenile onset RRP) and again between the ages of 20 and 40 years (adult onset RRP). Although benign, RRP cause significant morbidity, including occasional death [4]. RRP may persist for many years, even decades, resulting in serious physical and emotional sequelae and high medical costs due to repeated treatments [1,3].

The usual treatment for RRP is surgical removal of the papillomas, coupled with adjuvant therapy with various antiviral or immunoregulatory drugs in patients with moderate or severe disease; however, papillomas frequently recur even after complete surgical resection using a cold knife or laser [3]. It is not clear, though, whether the frequent recurrence of papillomas is the consequence of long-term persistent HPV infection in adjunct, apparently normal epithelia, or repeated re-infections with novel HPV strains. To the best of our knowledge, this study is the first to address this dilemma using in depth genetic viral variant analysis.

METHODS

Tissue specimens of 195 histologically confirmed laryngeal papillomas, 70 initial and 125 follow-up samples, obtained from 70 Slovenian patients (46 men, 24 women) who had had RRP for from 1 to 22 years (median = 4.0 years), were included in the study. Sixty-four samples were fresh-frozen tissue specimens and the remaining 131 archival formalin-fixed, paraffin-embedded (FFPE) tissue specimens. The number of follow-up specimens per patient ranged from 1 to 6.

Total DNA was extracted from papilloma tissue specimens using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), as described previously [2]. The integrity of each DNA sample was verified by real-time polymerase chain reaction (RT-PCR) amplification of a 268-bp portion of the human beta-globin gene, as described previously [2]. The identity of the initial and follow-up tissue specimens within 20 randomly selected
RRP patients was verified by matching their autosomal short tandem repeat (STR) profiles, as described recently [5].

All samples were tested for the presence of HPV DNA using an HPV-6 and HPV-11 specific RT-PCR assay, as described previously [6]. Briefly, to characterize HPV-6 and HPV-11 genomic variants, full-length genome amplification and sequencing was performed on viral isolates obtained from fresh-frozen tissue specimens, as described previously [7, 8]. Genomic variants of HPV-6 isolates from FFPE tissue specimens were determined by sequencing the HPV-6 highly divergent E5a gene (276-bp, nt 3887–4162) amplified with HPV6-E5aF-3858 (5′-TGTTATGTTGATAATACACTG-3′, nt 3858–3879) and HPV6-E5aR-4166 (5′-CATCATAAATTGACATGTTAGC-3′, nt 4186–4164) primers; in the event of this reaction failing, a shorter PCR amplicon (E5a-3′-TGGTATTGGTGTAATACAACTG-3′) was generated and sequenced using HPV6-E5aF-3914 (5′-GCAGGAACACGCAGATT-3′, nt 3914–3933) and HPV6-E5aR-4201 (5′-CCAGCCAGGTATCTCACA-3′, nt 4201–4182) primers. Genomic variants of HPV-11 isolates from FFPE tissue specimens were determined by sequencing the most variable part of the HPV-11 LCR genomic region (197-bp, nt 7402–7598) amplified with HPV11-LCRf-7372 (5′-TATGTATGATGTATGTATGTATGATG-3′, nt 7372–7401) and HPV11-LCRr-7618 (5′-GTGTATGTGTCACGGA-3′, nt 7618–7599) primers, and the most divergent part of the HPV-11 E5a gene (203-bp, nt 3944–4146) amplified with HPV11e5aF-3924 (5′-GCCTGTTGTTATGGCATTG-3′, nt 3924–3943) and HPV11e5aR-4169 (5′-TAAGTGACAGTTAACATCACA-3′, nt 4169–4147) primers. The obtained sequences were edited and analyzed using Vector NTI Advance 11 software (Invitrogen, Carlsbad, CA). The identification of HPV-6 and HPV-11 genomic variants was done as described previously [7, 8].

RESULTS

A 268-bp fragment of the human beta-globin gene, which served as an internal quality and amplification control, was successfully amplified from all 195 RRP tissue specimens included in the study. Within each of the 20 selected patients with RRP, including all with the detected HPV genomic variant switch, an identical autosomal STR profile was obtained in initial and all follow-up tissue specimens. Using an HPV-6 and HPV-11 specific RT-PCR assay, HPV DNA was detected in all 195 RRP tissue specimens: HPV-6 DNA in 130/195 (50 initial and 80 follow-up specimens) and HPV-11 DNA in 63/195 (19 initial and 44 follow-up specimens) samples. In two samples from the same patient, one initial and one follow-up sample, co-infection with HPV-6 and HPV-11 was detected and was additionally confirmed by the INNO-LiPA HPV Genotyping Extra test (Innogenetics, Gent, Belgium).

All HPV-6 and HPV-11 isolates were successfully amplified and sequenced across the E5a and/or LCR region (FFPE isolates) and the entire genome (fresh-frozen tissue isolates). The HPV-6 and HPV-11 whole genome nucleotide sequences are deposited in the DDBJ, EMBL and GenBank databases under the following accession numbers - HPV-6: HE599226-HE599246, HE962026-HE962032, and FR751320, FR751323, FR751324, FR751329, FR751331-FR751333 and FR751338 (reported previously [7]); HPV-11: HE611258-HE611274, HE962023-HE962025, HE962365-HE962368, and FN907957 and FN907964 (reported previously [8]).

Altogether, nine HPV-6 E5a genomic variants were identified among 50 patients with HPV-6 induced RRP (Supplementary alignment 1). HPV-6 E5a sequences from the follow-up sample(s) matched completely (100%) the HPV-6 E5a sequence from the initial sample in 49 patients, while in one patient a change of HPV-6 E5a genomic variant was observed during the 15-year course of RRP (Supplementary Table 1; Supplementary alignment 1: patient no. 6). The follow-up period for patients in whom HPV-6 genomic variants were identified by E5a gene sequencing ranged from 1 to 22 years (median = 3.5 years). For 18 HPV-6 positive RRP patients, HPV-6 full-length genome sequences were determined and 17 HPV-6 genomic variants were identified. Similarly, HPV-6 genome sequences were identical in two or more consecutive papilloma samples in 16/17 patients, while in one patient a change of the full-length HPV-6 genomic variant was observed during the three-year course of the disease (Supplementary Table 1; Supplementary alignment 1: patient no. 35); one patient was excluded from the matching analysis since the HPV-6 full-length genome sequence was available only from one follow-up RRP specimen. The follow-up period for 17 patients in whom HPV-6 genomic variants were identified by viral full-length genome sequencing ranged from 1 to 10 years (median = 3.0 years).

In total, four HPV-11 LCR and two HPV-11 E5a genomic variants were identified among 19 patients with HPV-11 induced RRP (Supplementary alignment 2). Within an individual patient, HPV-11 LCR and HPV-11 E5a sequences obtained from the follow-up sample(s) matched completely the HPV-11 LCR and HPV-11 E5a sequences from the initial sample. The follow-up period for patients in whom HPV-11 genomic variants were identified by LCR and E5a gene sequencing ranged from 1 to 18 years (median = 4.0 years). HPV-11 full-length genome sequences were determined for 10 HPV-11 RRP patients; altogether, seven HPV-11 genomic variants were identified. Within 9/10 patients, HPV-11 full-length genome sequences were identical in two or more consecutive papilloma samples (Figure 1); one patient was excluded from the matching analysis since a HPV-11 full-length genomic sequence was available only from one follow-up RRP specimen. The follow-up period for nine patients in whom
Figure 1. Full-length genomic variants of HPV-11 identified in consecutive papilloma specimens of nine patients with HPV-11 induced RRP. For an individual HPV-11 isolate, mutations detected over the entire genome compared to the corrected (modified) HPV-11 reference sequence (Maver et al., 2011) are presented. Within an individual patient, the initial HPV-11 isolate is terminally marked with 0, while follow-up isolates are marked with numbers representing years from the initial sampling. In patients nos. 63, 65 and 66, initial viral isolates could not be fully-sequenced due to the poor DNA quality (FFPE).

Figure 2. HPV genotyping and HPV genomic variant sequencing results of two consecutive RRP specimens (nos. 108 and 117) from patient no. 4, taken five years apart. Using INNO-LiPA-based genotyping, persistent infection with HPV-6 and HPV-11 was observed (left). Following direct sequencing of the highly variable parts of the viral genome, two nucleotide changes in the initial HPV-6 E5a genomic variant (above right, E5a sequence P52-LP108-O) were observed in the follow-up specimen (above right, E5a sequence P52-LP117-5) while the analyzed parts of the HPV-11 genome remained completely unchanged (below right, E5a + LCR sequences P52-LP108-0 and P52-LP117-5).
HPV-11 genomic variants were identified by viral full-length genome sequencing ranging from 1 to 8 years (median = 3.0 years).

In a patient co-infected with HPV-6 and HPV-11 (Supplementary alignments 1 and 2: patient no. 4) persistent infection with the same HPV-11 LCR and E5a genomic variant and switch of the HPV-6 E5a genomic variant was determined during the five-year course of RRP (Figure 2).

**DISCUSSION**

Laryngeal papillomas are the most frustrating benign lesions in the head and neck region, due to their multiplicity, frequent recurrence and the propensity to spread to adjacent areas [9]. It is now well established that HPV-6 and HPV-11 are the main cause of RRP, accounting for more than 90% of cases [1,2].

It was already shown in the early 1980s and 1990s that HPV-6 and HPV-11 persist in sequential recurrent papillomas of an individual patient [10–12]. However, to the best of our knowledge, no previous studies have investigated viral persistence on a genetic level below the viral genotype.

Using partial and full-length genome sequencing, we investigated the genomic diversity of HPV-6 and HPV-11 isolates obtained from sequential recurrent laryngeal papillomas of 70 patients with RRP. We showed for the first time that the recurrence of papillomas in RRP patients is due to the persistence of a single initial viral genomic variant rather than repeated re-infections with novel HPV strains. Namely, within 67/70 (95.7%) RRP patients included in the study, all follow-up HPV isolates, obtained from one to a maximum of 22 years after the onset of RRP, fully genetically matched the initial HPV isolate over the highly variable part of the genome (LCR, E5a) or over the entire genome. In contrast, in two patients (Supplementary Table 1; Supplementary alignment 1: patients nos. 4 and 6) all follow-up samples contained identical HPV E5a genomic variants that differed from the initial variant by two nucleotide exchanges. Similarly, in one patient (Supplementary Table 1; Supplementary alignment 1: patient no. 35) HPV-6 full-length genomic variants determined in both follow-up samples were identical but differed from the initial variant by two nucleotide exchanges and one 41-bp insertion in the E2 gene. Altogether, nine HPV-6 E5a and 17 HPV-6 full-length genomic variants, and four HPV-11 LCR, two HPV-11 E5a and seven HPV-11 full-length genomic variants were identified among 70 RRP patients, indicating a high discriminatory power of our genomic variant typing method.

The observation that the HPV-6 or HPV-11 genomic variant detected in recurrent papillomas remained genetically identical in 95% of the investigated RRP patients, suggests that re-infection is endogenous and probably represents reactivation of a latently present virus in the clinically normal surrounding laryngeal epithelium. HPV present in apparently normal tissue surrounding overt papillomas, as well as in more distant tissues in the airway, may serve as a viral reservoir responsible for the repeated growth of papillomas [13, 14]. The triggers for viral reactivation are currently unknown. It has been suggested that the wound healing process after removal of the papillomas, which is characterized by increased cell proliferation, may possibly promote replication of latently present viruses in the surgical margins or nearby squamous tissues [1, 14, 15].

In conclusion, the presence of an identical and unique HPV genomic variant within an individual patient with RRP in initial and follow-up RRP sample(s) obtained from 1 to 22 years later supports the hypothesis that frequent recurrence of laryngeal papillomas is a consequence of the long-term persistence of a single viral genomic HPV variant, rather than of repeated re-infections with novel HPV strains.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyrighted. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Note**

_Potential conflicts of interest._ B. J. K. has received consultancy fees from Abbott. M. P. has been an advisory board member/consultant for Abbott, GlaxoSmithKline and Roche, has received speaker’s honoraria and travel grants from Abbott, GlaxoSmithKline, Merck and Co. and Roche, and research grants from Abbott, Merck and Co. and Roche. All other authors report no potential conflicts of interest.

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