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Genetic diversity of HPV-6 in concurrent multiple anogenital warts

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Abstract

Introduction: Anogenital warts (AGW) are the most common benign tumors in the anogenital area. They are etiologically associated with alpha human papillomaviruses (HPV), in more than 90% of cases with HPV-6 and HPV-11. AGW frequently displays a multifocal and multicentric appearance. However, it is not clear whether the occurrence of multiple AGW in a particular patient is a consequence of infection with single or multiple HPV genomic variants of a given HPV genotype.
Methods: Forty-five HPV-6 isolates from fresh-frozen AGW tissue specimens, obtained from 18 patients with concurrent multiple AGW, were included. The entire HPV-6 L1, E5a, E5b ORFs, and LCR genomic region was sequenced.
Results: Fourteen different HPV-6 L1-LCR-E5a-E5b genomic variants were identified among 18 patients with concurrent multiple AGW. In 17 out of 18 patients, a single identical HPV-6 L1-E5a-E5b-LCR genomic variant was identified in all concurrent multiple AGW collected in an individual patient. Co-infection with two different HPV-6 genomic variants was identified in one patient.
Discussion: The presence of an identical HPV genomic variant in all concurrently present multiple AGW within an individual patient supports the hypothesis that the occurrence of multiple concurrent AGW is a consequence of infection with a single HPV-6 genomic variant, rather than infection with multiple genomic variants of HPV-6.

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Introduction

Anogenital warts (AGW) are the most common clinical manifestation of infection with low-risk human papillomavirus (HPV) genotypes of genus alpha, particularly HPV types 6 and 11, which affect both genders equally (1). Because the most common route of HPV transmission is through sexual contact, the most significant risk factors for infection with HPV and the occurrence of AGW is closely related to risky sexual behavior; for example, early age at onset of sexual activity, greater number of lifetime sexual partners, lack of condom use, and multiple concurrent partners (2, 3). AGW rarely result from other modes of transmission, such as auto- or heteroinoculation from other anogenital lesions or contaminated objects (4, 5).

AGW usually manifest as exophytic papillomatous lesions on the external genitals and in the perianal region. They may also emerge in the anal canal, vagina, cervix, and urethra (6). The incidence of anal warts is growing rapidly, especially in men that have sex with men. Recently, an increasing incidence of anal warts has been also reported in a population of heterosexual men and women that deny practicing receptive anal intercourse (7).

Despite their benign nature, AGW cause significant morbidity, including frequent recurrence and a multifocal and multicentric appearance that requires complex and long-term treatment, and they pose serious physical discomfort and psychosocial and emotional distress for the patient and a financial burden for the healthcare system (2, 8). Multiple lesions in small groups or plaques are characteristic for AGW and may affect a wide area of the anogenital region (9, 10). In rare occasions, AGW may occur outside the anogenital region (11).

In our recent study of a representative population of Slovenian patients with AGW, multiple warts were detected at various anatomical locations within the anogenital region in 3% of patients (12). It has been demonstrated that in patients with concurrent multiple AGW the same HPV genotype was present in all simultaneously collected anogenital wart lesions. However, it is not clear whether the occurrence of multiple AGW in a particular patient is a consequence of infection with single or multiple HPV genomic variants of a given HPV genotype. Therefore the purpose of this study was to investigate and clarify this issue using genetic viral variant analysis. To the best of our knowledge, this study represents the first investigation of concurrent multiple AGW at a genetic level below the viral genotype.

Materials and methods

A total of 45 HPV-6 isolates from fresh-frozen AGW tissue specimens obtained from 18 Slovenian patients (10 men, 8 women) with concurrent multiple AGW were included in the study. All 18 patients were randomly selected from a cohort of 71 patients with simultaneously collected, HPV-6 induced, multiple AGW, which originated from our recent study (12).

The number of concurrent multiple AGW per patient ranged from two to four. AGW were located at various anatomic sites: the anal canal, perianal skin, vagina, penis, vulva, pubis, inguinal fold, and femoral fold. All AGW were excised surgically and histologically confirmed as *condylomata acuminata*.

Total DNA was extracted from tissue specimens using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), as described previously (12). In order to investigate the sequence diversity of selected HPV-6 isolates, the entire HPV-6 L1, E5a, E5b ORFs, and LCR genomic regions were sequenced. Altogether the HPV-6 L1, E5a, E5b ORFs, and LCR genomic regions represent approximately 30% of the HPV-6 genome. In all 45 HPV-isolates first two overlapping DNA fragments, covering the entire HPV-6 genome, were generated using long-template PCR, as described previously (13): the 4,511-bp fragment contained the complete E6, E7, E1, E2, E4 and E5 ORFs, and the second 4,908-bp fragment contained complete L2 and L1 ORFs and the LCR genomic region. The complete L1 ORF (1,503 bp) was amplified by PCR using primer pair HPV6-L1F

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and HPV6-L1R, the entire E5a and E5b ORFs by primer pair HPV6-E5S and HPV6-E5R, and the LCR region by primers HPV6-LCRF and HPV6-LCRS2R, as described previously (13). HPV-6 genomic variants were identified with the BioEdit Sequence Alignment Editor v7.0.9.0 program (North Carolina State University, Raleigh, NC, USA), using the corrected genome sequences of prototype HPV-6 isolate (GenBank acc. no. X00203) as standard for comparisons and nucleotide position numbering (13).

Results

A 4,511 and 4,908-bp long fragments were successfully amplified and sequenced across the entire L1, E5a and E5b ORFs and LCR genomic region in all 45 HPV-6 isolates included in the study.

Using L1, LCR, E5a, and E5b genomic comparison, altogether 14 different HPV-6 L1-LCR-E5a-E5b genomic variants were identified among 18 patients with HPV-6-induced concurrent multiple AGW. Twelve HPV-6 genomic variants corresponded to non-prototypic genetic lineage HPV-6vc and two genomic variants to prototypic genetic lineage HPV-6b. None of the HPV-6 L1-LCR-E5a-E5b genomic variants identified were identical to the HPV-6 reference sequence.

In 17 out of 18 patients, a single identical HPV-6 L1-E5a-E5b-LCR genomic variant was identified in all concurrent multiple AGW collected in an individual patient. The most frequently present HPV-6 genomic variant, identified in AGW specimens of four patients (three females, one male) was the HPV-6 genomic variant that corresponded to non-prototypic genetic lineage HPV-6vc. Other 13 HPV-6 genomic variants were each detected in a single patient only (four females, nine males).

Co-infection with two different HPV-6 genomic variants was identified in one 64-year-old female patient from whom warts from perianal skin, the pubic region, and femoral fold skin were collected at the time of surgery. Both HPV-6 L1-LCR-E5a-E5b genomic variants corresponded to non-prototypic genetic lineage HPV-6vc. The HPV-6 L1-LCR-E5a-E5b genomic variant, identified in perianal warts and warts removed from pubic region, matched completely in selected L1-LCR-E5a-E5b genomic regions, whereas the genomic variant identified in AGW, removed from the femoral fold, was distinct in the HPV-6 E5a genomic region in two nucleotides and in the HPV-6 LCR region in one nucleotide (Figure 1).

Discussion

A previous study showed that the same HPV genotype is present in multiple warts of an individual patient (11). In addition, there have been reports of *condylomata acuminata* lesions in children caused by the same HPV genotypes as found in the AGW of their mothers (14). Our study, which was the first performed at a genetic level below the viral genotype, showed that the occurrence of multiple concurrent AGW is a consequence of infection with a single HPV-6 genomic variant rather than infection with multiple genomic variants of HPV-6. Namely, among 17 of the 18 patients included in the study, the presence of a single and identical HPV-6 L1-LCR-E5a-E5b genomic variant was determined in all concurrent multiple AGW specimens collected from each individual patient.

In one female patient, with AGW removed from the perianal skin, pubic region, and femoral fold, co-infection with two HPV-6 L1-LCR-E5a-E5b genomic variants was identified. Perianal and pubic warts contained identical HPV-6 L1-E5a-E5b-LCR genomic variant, which differed from the HPV-6 genomic variant obtained from femoral fold AGW by two nucleotides in the E5a genomic region and by one nucleotide in the LCR noncoding region. Because the HPV-6 genome changes very slowly over time (13, 15), we believe that the presence of two HPV-6 genomic variants is not a consequence of mutations in these genomic areas, but the result of independent infections with two different HPV-6 genomic variants.

By determining the nucleotide sequences in four selected HPV-6 genomic areas, exceptional genetic diversity for low-risk alpha HPVs was identified in this study. Altogether, 14 different HPV-6 L1-LCR-E5a-E5b genomic variants were identified among 18 patients. The prevalence of genomic variants of non-prototypic family HPV-6vc was almost eight times more frequent in comparison to the genomic variants of prototypic family HPV-6b. Our results are in line with the results of a recent study in which we showed great genomic diversity of HPV-6 in AGW (16).

HPV infection is typically multicentric and infection is often not limited to the initial site of viral entrance (17); therefore, AGW's multicentric and multifocal appearance can be explained by the existence of the HPV also in tissues surrounding the wart. Recent studies have shown that individuals with AGW have detectable or latent HPV infection in the surrounding, clinically normal appearing genital epithelium and/or hair follicles in the absence of clinical signs of disease (18–20). In addition, it was recently shown by our group that a particular HPV genomic variant can persist in recurrent respiratory papillomas in unchanged form for up to 22 years (21). Latent and/or persistent infection can remain subclinical for a long period or can be activated as a result of wounding, immunosuppression, or exposure to exogenous factors (17, 22).

In conclusion, due to the detection of an identical HPV-6 genomic variant in all concurrently present multiple AGW within an individual patient, this study supports the hypothesis that the occurrence of multiple concurrent AGW is a consequence of infection with a single HPV-6 genomic variant rather than infection with multiple genomic variants of HPV-6.

Patient no./ AGW anatomical location	E5a variant	E5a genomic positions													LCR variant	LCR genomic positions																			
		3	3	4	4	4	4	4	4	4	4	4	4	4		7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	0
		8	9	0	0	1	1	1	1	1	1	1	1	1		3	4	4	4	5	5	6	6	6	6	6	6	7	7	8	8	9	0	
		9	3	0	4	0	2	2	2	3	4	4	4	4		4	0	5	6	1	1	1	1	2	2	6	9	0	4	1	9	5	2	
		8	4	3	8	0	3	5	6	6	1	7	8	9		9	0	2	7	3	4	7	8	3	8	1	6	0	8	2	3	4	0	
Ref-6b-X00203	Ref-6b-E5a	G	C	G	T	C	A	T	G	T	T	T	A	C	Ref-6b-LCR	G	C	C	G	C	C	A	A	C	C	A	T	C	C	C	T	G	T	
Ref-6a-L41216	Ref-6a-E5a	A	A	C	C					C	C			A	Ref-6a-LCR		A	A	T	A		C		A	T	G	G	G	A	I2		A	I3	
Ref-6vc-AF092932	Ref-6vc-E5a	A	A	C	C	T	T	G	T	C	C	G		A	Ref-6vc-LCR	T	A	A	T	A	I1	C	C	A	T	G	G	G	A	I2		A	I3	
P127-perianal AGW	6-E5a-8	A	A	C	C		T	G	T	C	C	G		A	6-LCR-3	T	A	A	T	A	I1	C	C	A	T	G	G	G	A	I2	G	A	I3	
P127-femoral AGW	6-E5a-9	A	A	C	C	T	T	G	T	C	C	G	C	A	Ref-6vc-LCR	T	A	A	T	A	I1	C	C	A	T	G	G	G	A	I2		A	I3	
P127-pubic AGW	6-E5a-8	A	A	C	C		T	G	T	C	C	G		A	6-LCR-3	T	A	A	T	A	I1	C	C	A	T	G	G	G	A	I2	G	A	I3	

Figure 1 | **HPV-6 L1-LCR-E5a-E5b genomic variants identified in AGW collected from different anatomical locations of a female patient co-infected with two different HPV-6 genomic variants.** The genomic variant identified in perianal and pubic AGW differs from the genomic variant determined in femoral AGW at nucleotide positions 4,100 nt and 4,148 nt in the E5a genomic region and at nucleotide position 7,893 nt in the LCR genomic region. Insertions: I1 = TACATTATTGTATA; I2 = ATATGTTTATTGCCACTGCA; I3 = T.

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