JOURNAL OF CLINICAL MICROBIOLOGY, June 2003, p. 2763–2767 Vol. 41, No. 6
0095-1137/03/$08.00+0 DOI: 10.1128/JCM.41.6.2763–2767.2003
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Oncogenic Human Papillomavirus DNA Loads in Human Immunodeficiency Virus-Positive Women with High-Grade Cervical Lesions Are Strongly Elevated

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Received 12 July 2002/Returned for modification 29 November 2002/Accepted 6 March 2003

Human papillomavirus (HPV) DNA loads of six oncogenic HPV types were measured by real-time PCR in cervical scrapes of human immunodeficiency virus (HIV)-infected and uninfected women. In both groups, HPV loads increased with the grade of cervical disease. HIV infection did not affect HPV loads in low-grade lesions but was associated with significantly higher HPV loads in severe dysplasia; highest loads were found in advanced HIV disease. Our data reflect the aggressive course of HPV infection in HIV-positive women.

High-risk human papillomaviruses (HPV) induce cervical squamous intraepithelial lesions (SIL) classified as low grade (LSIL) or high grade (HSIL) in severity, which may progress to cervical cancer (21, 32, 47). Human immunodeficiency virus (HIV) infection is associated with a higher prevalence of HPV, a higher incidence of SIL, and an increased risk for cervical cancer (1, 7, 9, 13, 15, 18, 19, 28–30, 33, 34, 38, 39). Several studies of HIV-negative patients have indicated that higher HPV DNA loads in cervical scrapes are predictive of the severity of the underlying cervical lesion (3, 11, 16, 41, 42). HPV16 viral loads were shown to be positively correlated with the risk for developing cervical carcinoma in situ (23, 46). This could not be confirmed by Lorincz et al. when measuring cumulative loads of high-risk HPV types by Hybrid Capture without normalization on input cells (26).

We designed type-specific real-time PCR protocols for the quantification of the commonest high-risk HPV types (HPV16, -18, -31, -33, -45, and -56), which prevail in more than 80% of cervical cancers worldwide (5, 44). To evaluate the influence of HIV infection on HPV DNA load, we comparatively analyzed 305 cervical scrapes of HIV-positive and HIV-negative women with defined cervical disease stages (Table 1). All patients had a complete gynecologic examination. Cervical scrapes for cytology and HPV load determination were taken by one investigator (A.M.F.). Colposcopy was performed on the patients' first visit and at following visits if the first cytology had been above normal. For HIV-positive patients, the number of CD4 cells per microliter, the HIV type 1 RNA load in plasma (Cobas Amplicor HIV-1 Monitor 1.5; Roche, Mannheim, Germany), and Centers for Disease Control and Prevention stage (8) were determined. DNA isolation from cervical scrapes (QIAamp-DNA Mini-Kit; Qiagen, Hilden, Germany), β-globin gene PCR (2), HPV screening-PCR (45), and HPV typing (22) were performed as previously described. Sample characteristics and HPV DNA prevalence data are given in Table 1. Quantification of HPV DNA was performed with the LightCycler (Roche) system as described before (45), with addition of 5% dimethyl sulfoxide to the PCR buffer. Sequences of type-specific primers and probes as well as PCR parameters are given in Table 2. HPV DNA copy numbers were determined in duplicate by using standard curves, generated in the same PCR run with HPV-plasmid dilutions in a human placental DNA solution (40 ng/μl) (4, 6, 12, 25, 31, 37). Sensitivity was 5 to 10 copies of HPV DNA. Quantification was linear from 10 to 10^9 copies for all HPV types. Type specificity could be shown by testing 500,000 copies of heterologous HPV types (HPV6, -11, -16, -18, -31, -33, -45, and -56) instead of the target type, which never yielded signals above background. Reliability of quantification in the presence of other HPV types was tested by spiking six identical samples containing 1,000 copies of the target HPV type with 100,000 copies of frequent genital HPV types (HPV6, -11, -16, -18, -31, -33, -45, and -56), respectively. The test was designated reliable when spiked samples yielded copy numbers within the threefold standard deviation of six unspiked samples. To correct for PCR efficiency and DNA integrity and to determine the number of input cell equivalents, the single-copy-number gene β-globin was quantified by using the LightCycler Control Kit DNA (Roche). HPV DNA load was defined as the number of HPV DNA copies/β-globin gene copy. Statistical analyses (17) were performed with SPSS 10.0.7 (SPSS Inc., Chicago, Ill.). Odds ratios (OR), 95% confidence intervals (CI), Fisher's exact tests, and Mann-Whitney U tests were based only on one sample per patient (the first HPV-positive sample collected).

We observed an increase of median HPV16 load with the severity of cervical disease in HIV-negative and HIV-positive patients (normal cytology for HIV-negative patients/HIV-positive patients, <0.1/<0.1; LSIL, 0.6/1.3; and HSIL, 14.6/63.2). For each cytological grade, strong load variations were ob-

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TABLE 1. Patient data and HPV DNA prevalence in cervical scrapes

HIV status	No. of patients analyzed	No. of samples analyzed[b]	Patients' mean age (yr) at 1st visit (range)	Cytology result[c] (no. of positive samples/no. of samples analyzed [%])[e]					No. of scrapes with multiple infections[d]/no. of HPV$^+$ samples (%)
				Normal	ASCUS	LSIL	HSIL	Cervical cancer	All	
HIV$^+$	152	353	32.5 (17.0–64.2; SD = 6.7)	122/212 (57.5)	11/18 (61.1)	33/37 (89.2)	83/85 (97.6)	1/1 (100)	250/353 (70.8)	145/250 (58.0)
				53/122 (43.4)	5/11 (45.5)	25/33 (75.8)	68/83 (81.9)	1/1 (100)	152/250 (60.8)	
HIV$^-$[a]	285	349	33.2 (15.8–72.9; SD = 9.7)	89/196 (45.4)	22/32 (68.8)	27/33 (81.8)	82/85 (96.5)	3/3 (100)	223/349 (63.9)	99/223 (44.4)
				45/89 (50.6)	13/22 (59.1)	24/27 (88.9)	68/82 (82.9)	3/3 (100)	153/223 (68.6)	

[a] HIV-negative women were referred to the university hospital with suspicion of dysplasia. An HIV infection was excluded by Axsym HIV1/2 MEIA (Abbott, Wiesbaden, Germany).
[b] For patients in whom more than one sample was collected, the time interval between sample collections was 6 to 12 months.
[c] Cytology was classified both by the Bethesda system (shown above) and by the Papanicolaou (Pap) classification system (24, 27, 40). The majority of PapIIID/HSIL and all PapIV/HSIL and cervical cancer diagnoses were confirmed by colposcopy-directed histology.
[d] Infections with multiple HPV types are defined as infections with ≥2 HPV types.
[e] The second and fourth rows give the number of samples positive for HPV16, -18, -31, -33, -45, or -56/number of HPV$^+$ samples as indicated in the first and third rows.

TABLE 2. Primers, hybridization probes, and PCR conditions for the quantification of DNA of HPV16, -18, -31, -33, -45, and -56 by real-time PCR

HPV type	$T_{ann.}$[a] (°C)	[Mg^{2+}] (mM)	Sequences[b,c] (5′→3′)
16	56	4.25	fw: GCACAGGGCCACAATAAT bw: GACCAAAATTCCAGTCCTC So1: TCAACTGTGCAAAATAACCTTAACTGC So2: ACGTTATGACATACATACATTCTATGA ATTCCACT
18	55	3.5	fw: GCACAGGGTCATAACAATGG bw: CAAAGTTCCAATCCTCTAA So1: TTGGTAGCATCATATTGCCCAGGTACA So2: AGACTGTGTAGAAGCACATATTGTT AAATTGG
31	55	4.5	fw: GCTCAGGGACACAATAATGG bw: CAAAATTCCAATCTTCCAAA So1: AATTACTACTTTTAAATGTAGTATCA CTGT So2: TGCAATTGCAGCACAAACAGACAT ATTG
33	54	3.0	fw: GCACAAGGTCATAATAATGG bw: CAAATTGCCAATCTTCTAA So1: ATATGTACTGTCACTAGTTACTTGTGT GCAT So2: AGTCATATTAGTACTGCGAGTGGTATCT
45	54	4.5	fw: GCCCAGGGCCATAACAATGG bw: CAAAATTCCAATTTTCTAA So1: AACTTAGTAGGGTCATATGTACTTGGC So2: CAGGATTTTGTGTAGAGGCACATAATG
56	54	4.0	fw: GCCCAAGGCCATAATAATGG bw: CAATATTCCAGTCCTCCAG So1: GCATCATATTTACTTAACTGTTCT GTAGC So2: GTACTAATAGTCATGTTAGTACT TCTAGT AGTATC

[a] $T_{ann.}$, annealing temperature.
[b] fw, forward primer; bw, backward primer; So1, 5′ hybridization probe (3′ end labeled with fluorescein); So2, 3′ hybridization probe (5′ end labeled with LightCycler-Red-640 and 3′ end phosphorylated).
[c] Primers used were at 0.5 μM; hybridization probes were at 0.15 μM.

served. We, like others (41, 46), were therefore not able to define a clear cutoff predictive of HSIL. However, HPV16 loads that were > 1 were significantly associated with HSIL for HIV-negative ($P < 0.001$, OR, 8.3; CI, 2.8 to 24.5; and $n = 76$) and HIV-positive ($P < 0.001$, OR, 41.2; CI, 2.1 to 778.7; and $n = 31$) patients. In four scrapes from patients with cervical cancers, HPV DNA loads were above 1. The distribution of HPV16 loads in non-HSIL and HSIL scrapes is shown in Fig. 1 (non-HSIL includes normal cytology, atypical squamous cells of undetermined significance [ASCUS], and LSIL). Graphs for non-HSIL scrapes of HIV-negative and HIV-positive patients are almost identical. For HPV18, -31, -33, -45, and -56 as well, no remarkable load differences could be seen in non-HSIL scrapes of HIV-negative and HIV-positive patients. In contrast, HPV16 loads were significantly higher in HSIL scrapes from HIV-positive patients than in those from HIV-negative patients ($P = 0.041$; HIV$^+$ $n = 14$; and HIV$^-$ $n = 37$) (Fig. 1). The same tendency was seen for HPV18, -31, -45, and -56 but not for HPV33 (Table 3).

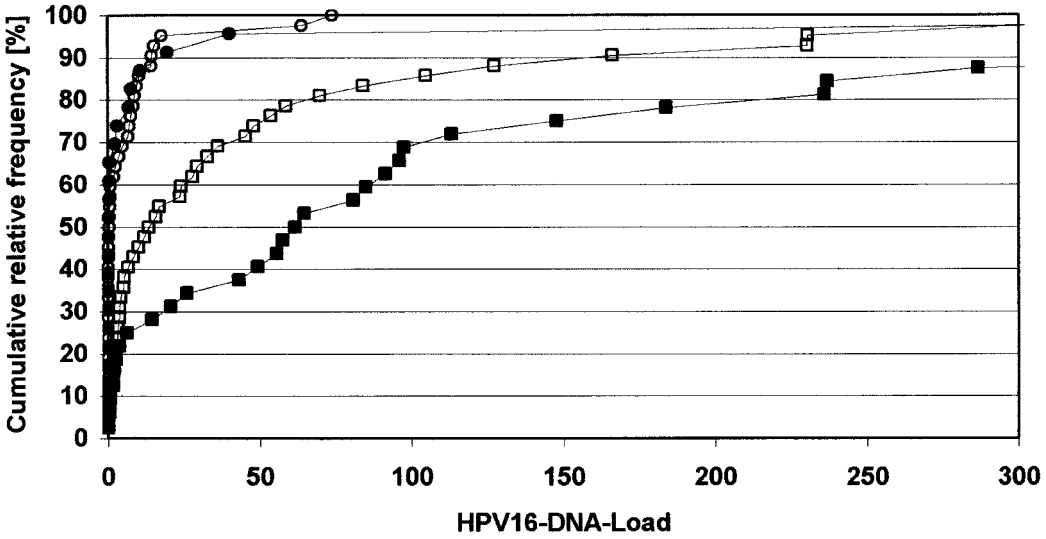

FIG. 1. Cumulative relative frequency of HPV16 DNA loads in cervical scrapes of HIV-negative and HIV-positive patients. Filled circles, HIV positive, non-HSIL (normal cytology, $n = 12$; ASCUS, $n = 1$; and LSIL, $n = 10$); empty circles, HIV negative, non-HSIL (normal, $n = 24$; ASCUS, $n = 8$; LSIL, $n = 10$); filled squares, HIV positive, HSIL ($n = 27$); and empty squares, HIV negative, HSIL ($n = 40$). HPV16 DNA loads above 300, which were only found in HSIL patients, are not shown (HIV^+, $n = 5$; and HIV^-, $n = 2$).

In patients infected with more than one of the investigated six high-risk types, viral loads of individual types varied by up to 3 orders of magnitude. Interestingly, median HPV16 loads found in HSIL lesions infected with more than one HPV type were twofold (HIV positive: 72.8 versus 35.0; $n = 32$) and fourfold (HIV negative: 23.7 versus 6.7; $n = 42$) higher than those in HSIL lesions infected with HPV16 alone.

Stratification by CD4 cell count, AIDS status, and HIV RNA loads (Table 4) revealed that severe immunosuppression or high HIV RNA levels were generally associated with increased HPV loads in HSIL patients. These results extend preliminary data by Heard et al. (20), who have found large HPV DNA amounts more frequently in severely immunosuppressed HIV-positive patients when comparing results from differently sensitive Southern blot and PCR analyses.

Elevated HPV DNA loads found in HIV-positive patients with HSIL could result from a higher proportion of infected cells shed from larger lesions and/or from enhanced HPV replication. The impaired immunity in HIV-positive patients will lead to a less efficient elimination of HPV-infected keratinocytes (10, 36) and thus to an expansion of lesions. Increased HPV DNA replication could be the consequence of direct or indirect effects of HIV (14, 43), of synergistic effects of infections with multiple HPV types (see above), or of coinfections with other sexually transmitted agents, such as herpes simplex viruses (35). An increased risk of malignant conversion can be expected both from the larger pool of HPV-infected cells in expanded lesions and from elevated gene doses, leading to higher concentrations of viral oncoproteins. All these mechanisms could contribute to the more aggressive course of cer-

TABLE 3. DNA loads[a] of HPV16, -18, -31, -33, -45, and -56 in cervical scrapes of HIV-negative and -positive patients with HSIL

HIV status	Type of statistic	DNA load for:					
		HPV16	HPV18	HPV31	HPV33	HPV45	HPV56
HIV^-	Median	14.6 (42)[b]	<0.1 (5)	1.6 (15)	0.8 (6)	<0.1 (4)	30.2 (6)
	80th per.[c]	75.5	12.2	629.3	27.6	0.4	68.6
HIV^+	Median	63.2 (32)	15.4 (18)	86.3 (11)	1.1 (20)	0.5 (15)	266.1 (24)
	80th per.	236.4	261.0	1,317.6	26.5	4,717.8	785.5

[a] HPV loads are defined as numbers of HPV DNA copies/β-globin gene copy.
[b] Numbers of analyzed samples are given in parentheses.
[c] 80th per., 80th percentile HPV DNA load.

TABLE 4. Median and 80th percentile HPV DNA loads of HPV16, -18, -31, -33, -45, and -56 in HIV-positive patients with HSIL differentiated by CD4 cell count, AIDS status, and HIV RNA load

Patient category	DNA load for:					
	HPV16	HPV18	HPV31	HPV33	HPV45	HPV56
CD4 count $\geq$ 200/μl	57.5[a]/215.1[b] (21)[c]	1.1/12.5 (8)	5.9/200.0 (5)	0.6/22.2 (13)	1.9/10,830 (8)	227.0/785.5 (14)
CD4 count $<$ 200/μl	80.9/435.8 (11)	157.6/3,153 (10)[d]	554.6/1,746.2 (6)	3.5/206.1 (6)	$<$0.1/8.0 (7)	276.8/815.5 (10)
AIDS neg. (CDC A and B)	17.5/183.8 (14)	1.8/15.6 (7)	119.5/239.0 (2)	0.3/20.8 (14)	1.7/3.3 (2)	232.9/1,241.4 (8)
AIDS pos. (CDC C)[e]	72.8/303.3 (18)	132.5/2,435.7 (11)	86.3/1,526.8 (9)	7.0/223.6 (5)	0.5/6,424.4 (13)	270.9/845.8 (16)
HIV RNA count $<$ 10^4/ml	59.6/236.4 (22)	5.8/277.7 (15)	65.2/1,599.9 (8)	$<$0.1/6.9 (15)	0.5/7,486.7 (11)	272.1/584.4 (19)
HIV RNA count $\geq$ 10^4/ml	85.0/2,214.5 (7)[d]	132.5/182.8 (3)	239.0/1,003.7 (3)	153.5/241.2 (3)	1.4/15.8 (4)	450.8/1,764.1 (4)

[a] Median HPV DNA load.
[b] Given here is 80th percentile HPV DNA load.
[c] Numbers of analyzed samples are given in parentheses.
[d] $P < 0.05$ in Mann-Whitney U test.
[e] CDC, Centers for Disease Control and Prevention; neg., negative; and pos., positive.

vical HPV infections in HIV-positive patients. The observed elevated HPV DNA loads, due to whatever mechanism, thus reflect the increased risk for cervical cancer in HIV-positive women.

This work was supported by the Wilhelm Sander-Stiftung, project number 2000.065.1.

We thank Monika Junk for excellent technical assistance.

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