Research Article

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Changes in DNA Level of Oncogenic Human Papillomaviruses Other Than Types 16 and 18 in Relation to Risk of Cervical Intraepithelial Neoplasia Grades 2 and 3

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Abstract

Background: Epidemiologic data addressing clinical relevance of viral load fluctuation of oncogenic types other than human papillomavirus (HPV) types 16 and 18 are limited.

Methods: A type-stratified set of infections by non-HPV16/18 oncogenic types that were detected at $\geq$2 visits was randomly selected from women who were enrolled in a clinical trial and followed every 6 months for 2 years for detection of HPV and cervical intraepithelial neoplasia grades 2 and 3 (CIN2/3). Type-specific viral load was measured on both first and last HPV-positive cervical swab samples.

Results: CIN2/3 was initially confirmed at the last HPV-positive visit for 67 of 439 infections. The increase in risk of CIN2/3 was associated with high, relative to low, viral load at both first and last positive visits [$OR_{adjusted}$ = 3.67; 95% confidence interval (CI), 1.19–11.32] and marginally associated with a change of viral load from low to high levels ($OR_{adjusted}$ = 3.15; 95% CI, 0.96–10.35) for infection by species group alpha-9 non-HPV16 oncogenic types but not species group alpha-5-7 non-HPV18 oncogenic types. Among women with an initial diagnosis of CIN2/3 at the first positive visit, CIN2/3 was more frequently redetected at the last positive visit for infections with, compared with without, high DNA load of species group alpha-9 non-HPV16 oncogenic types at both visits (P_{exact} = 0.04).

Conclusions: In agreement with data on baseline viral load, the viral load change–associated risk of CIN2/3 differs by HPV species groups.

Impact: These findings underscore the importance of distinguishing species groups in future studies of clinical relevance of HPV DNA load.

Introduction

Infection with oncogenic human papillomavirus (HPV) type is a necessary but not sufficient condition for the development of cervical cancer and its precursor lesions, cervical intraepithelial neoplasia grade 3 (CIN3, here, combined with rare cases of adenocarcinoma *in situ*) and, less stringently CIN2 (1–3). Most infections resolve spontaneously within a few years; only a fraction tends to persist and eventually progress to CIN2/3 (4). Identification of biomarkers that may signal an even higher risk in a population of women with a persistent infection helps build understanding of etiology of HPV-induced cervical cancer precursors.

In the past decades, efforts have been made to determine possible impacts of HPV DNA load on outcomes of the infection. Most of these studies have focused on HPV16 and HPV18, reporting an increased risk of CIN2/3 that was associated with high viral load of HPV16 but not HPV18 (5–12). However, epidemiologic data addressing the association of CIN2/3 with viral load of oncogenic types other than HPV16 and HPV18 (non-HPV16/18 oncogenic types) have been limited, yielding inconsistent results (13–17). A recent study of baseline viral load of 11 non-HPV16/18 oncogenic types among women enrolled in a clinical trial revealed that the viral load–associated risk of CIN2/3 appeared species group-dependent, with the association seen for types that are phylogenetically classified as species group alpha-9 (i.e., HPV16-related types) but not those within species group alpha-7 (HPV18-related types; ref. 18). A similar observation was reported from a recent cohort study (19). The viral load in these studies was assessed only at a single time point. Data on clinical relevance of repeated viral load measures are rare (20–22). If viral load is important mainly for species group alpha-9 types, one would expect that the finding would extend to longitudinal specimens and that an increase in viral load would increase risk of CIN2/3 for alpha-9 types.

The viral load and its longitudinal course might also be relevant following treatment of CIN2/3. U.S. consensus guidelines (23) have recommended that among women treated typically by loop electrosurgical excision procedure (LEEP) for CIN2/3, a

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continuous presence of the type of HPV that caused the precancer represents a failed "test of cure," that is, a risk factor for the posttreatment CIN2/3 (24–26). A semiquantitative analysis of pretreatment HPV DNA load has revealed somewhat associations of high viral load prior to treatment with increased risks of persistent infection and persistence or recurrence of posttreatment CIN2/3 (27, 28). Data on a change of HPV DNA load before and after treatment for CIN2/3 and subsequent risk of recurrent precancer are currently unavailable.

In the analysis described here, we measured viral load of non-HPV16/18 oncogenic types on a random set of the first and last type-specific HPV-positive samples selected from infections that were detected at $\geq$2 visits in a clinical trial setting. We sought to ascertain the impact of viral load fluctuation on both pre- and posttreatment outcomes: (i) on risk of newly developed CIN2/3 among women with a persistent infection and (ii) on redetection of CIN2/3 posttreatment.

Materials and Methods

Study subjects

Study subjects were women enrolled in the Atypical Squamous Cells of Undetermined Significance and Low-Grade Squamous Intraepithelial Lesion Triage Study (ALTS), a clinical trial designed to evaluate three strategies for management of women with equivocal or mildly abnormal cervical cytology. Women in ALTS were followed every 6 months for 2 years for detection of HPV infections and cervical lesions. A detailed description of the ALTS design and study population is available elsewhere (29).

For this study, we randomly sampled a type-stratified set of 25% of infections by non-HPV16/18 oncogenic types detected at two or more visits (including alpha-9 types 31, 33, 35, 52, and 58; alpha-7 types 39, 45, 59, and 68; alpha-5 type 51; alpha-6 type 56). We assayed viral load for the first and last type-specific HPV-positive cervical swab samples. Of 512 eligible infections selected from 463 women, 26 were excluded because of a lack of remaining sample for viral load testing at the first positive visit ($n = 15$), the last positive visit ($n = 10$), or both ($n = 1$). We additionally excluded one infection because of a negative β-actin result (a reference for viral load normalization) for one sample, leaving 485 type-specific infections (from 440 women) in analysis.

The clinical endpoint for this study was CIN2/3 histologically confirmed by the panel of expert pathologists immediately following the last type-specific HPV-positive visit. The analysis of the viral load change–associated risk of new CIN2/3 was restricted to infections from women who did not have a previous or prevalent diagnosis of CIN2/3 at the first HPV-positive visit. We additionally excluded four infections from women who had a diagnosis of CIN2/3 at an interval visit (i.e., between the first and last positive visit), leaving 439 in analysis. In ALTS, a treatment by LEEP was offered to women having CIN2/3 histologically confirmed at any time during the trial. The analysis of a relation between posttreatment CIN2/3 and viral load change was confined to infections ($n = 42$) from women who had an initial diagnosis of CIN2/3 at the first type-specific HPV-positive visit. Data on HPV results tested by PCR-based reverse-line blot assay, clinical diagnoses, and characteristics of study subjects were obtained from the ALTS database. The study protocol was approved by the institutional human subject review boards at the NCI and at the University of Washington (Seattle, WA).

Quantification of type-specific HPV DNA load

Type-specific HPV DNA copy number and cellular DNA amount (determined by testing for *β-actin* gene) were measured by RT-PCR in triplicate. A detailed procedure of the assay was described previously (30). Briefly, two log-phase 5-point standard curves were implemented in each set of the assay for absolute quantification, one for HPV and the other for cellular DNA. The number of viral copies was divided by the input amount of cellular DNA for normalization and then $\log_{10}$-transformed. The mean of log value of three measures (expressed as $\log_{10}$ [HPV copy number per 1 nanogram of cellular DNA]) was used for analysis. The normality of the distribution of the $\log_{10}$-transformed viral load was assessed by normal Q-Q plot. The points for the observed log values against values from a normal distribution were clustered around a straight line, suggesting that the distribution of $\log_{10}$-transformed values was approximately normal.

Type-specific HPV DNA was undetectable by RT-PCR for 29 samples that tested positive previously by PCR-based reverse-line blot assay. The negative result was not explained by a lack of sufficient sample input as the amount of cellular DNA between samples with and without detectable HPV DNA by RT-PCR was comparable (*t* test, $P = 0.35$). As described previously (18), a value of one viral copy per 1 nanogram of cellular DNA was arbitrarily assigned to each of these samples. Results remained similar when these samples were excluded from the analysis.

Statistical analyses

The analysis of HPV DNA load in pairs of the first and last positive samples was performed at the infection level. Thus, a woman would be counted multiple times if she had two or more types of interest included. To illustrate a change of viral load straightforwardly, HPV DNA load at each time point for individual infections was dichotomized as high versus low levels with means of type-specific viral load as cut-off points. Accordingly, viral load fluctuations between two visits for individual infections were described as low-to-low, high-to-low, low-to-high, or high-to-high levels.

Unconditional logistic regression was used to estimate odds ratios (OR) and 95% confidence intervals (CI) for the association between risk of CIN2/3 and level of viral load at both visits. Considering the small number of CIN2/3 cases linked to individual types and our previous finding that the viral load–associated risk of CIN2/3 was seen only for types within species group alpha-9 (18), types were grouped as species group alpha-9 versus other species. Separate regression models were fit for each group. A variable indicating types within the group (i.e., HPV types 31, 33, 35, 52, and 58 in a group of species 9; HPV types 39, 45, 51, 56, 59, and 68 in a group of species 5–7) was always included as a covariate. The ORs were adjusted for number of interval visits (0, 1, and 2–3) between the first and last positive visit and a set of covariates that were previously selected for analysis of baseline viral load–associated risk of CIN2/3 (18) including self-reported race (Caucasian vs. non-Caucasian), current smoking status (yes vs. no), time of the first HPV-positive detection (enrollment vs. follow-up), and coinfection by other oncogenic types (yes vs. no for any oncogenic types except for the one evaluated). Robust variance estimates were used to count for correlation within subjects. The 95% CIs were computed using a parametric bootstrap method with 1,000 repetitions; the lower and higher bounds were given by the 25th and 975th bootstrap ORs, respectively. If one or more parameters could not be

estimated in ≥10% bootstrap replicates, the 95% CIs were estimated by jackknife logistic regression clustering on study subject identification (31). The logistic regression model was also used to estimate the association of CIN2/3 with differences in paired viral loads (i.e., viral load at the last positive visit minus viral load at the first positive visit) with and without an additional adjustment for viral load at the first positive visit.

A paired *t* test was used to compare viral loads between the first and last HPV-positive visit for individual types. Among women with a previous diagnosis of CIN2/3 at the first HPV-positive visit, Fisher exact test was used to compare frequencies of CIN2/3 recurrent diagnosis between infections with and without high viral load at both visits. Statistical analyses were performed using STATA version 11 (StataCorp); all tests were at the 5% two-sided significance level.

Results

The mean age of 440 study subjects was 23.9 years (SD, ±5.9) who provided 485 type-specific infections for this study including 251 with species group alpha-9 non-HPV16 oncogenic types and 234 with species group alpha-5–7 non-HPV18 oncogenic types. The median time between the first and last type-specific HPV-positive visit was 12.0 months (interquartile range, 6.41–18.25). Type-specific HPV DNA load between two visits was comparable for all types except for HPV68 (Table 1). Type-distributions were comparable across four categories of viral load fluctuation in either species group alpha-9 non-HPV16 oncogenic types ($P = 0.85$) or species group alpha-5–7 non-HPV18 oncogenic types ($P = 0.86$).

CIN2/3 was first detected at the last HPV-positive visit for 67 (15.3%) of 439 infections including 39 (17.2%) of 227 infections from 217 women with species group alpha-9 non-HPV16 oncogenic types and 28 (13.2%) of 212 infections from 207 women with species group alpha-5–7 non-HPV18 oncogenic types. With an adjustment for type within the group, race, current smoking status, time of the first HPV-positive detection, coinfection with other oncogenic types, and number of interval visits, the increase in risk of CIN2/3 was statistically significantly associated with high, relative to low, viral load at both visits ($OR_{adjusted} = 3.67$; 95% CI, 1.19–11.32; $P = 0.02$), and marginally associated with a change of viral load from low to high levels ($OR_{adjusted} = 3.15$; 95% CI, 0.96–10.35; $P = 0.06$) for infection by species group alpha-9 non-HPV16 oncogenic types. The viral load change–associated risk of CIN2/3 was not evident for infection by species group alpha-5–7 non-HPV18 oncogenic types; estimates were well within the limits of chance given no true relation (Table 2). Results remained similar to those presented in Table 2 when the viral load was dichotomized according to medians of type-specific viral load or when infections from women with a previous or prevalent diagnosis of CIN2/3 were included (see Supplementary Data). Two hundred and thirty-one type-specific infections had other oncogenic type(s) simultaneously detected at the last positive visit. When the analysis was restricted to infections without coexistence of any other oncogenic types at the last positive visit, women with, compared with without, high DNA load of species group alpha-9 non-HPV16 oncogenic types at both visits were at an even higher risk of CIN2/3 ($OR_{adjusted} = 6.67$; 95% CI, 1.67–26.75; $P = 0.01$). The same analysis was not performed for infection by species group alpha-5–7 non-HPV18 oncogenic types because of a small number of CIN2/3 diagnoses linked to infections without coinfection by other oncogenic types at the last positive visit (2/33 with high viral load at both visits and 3/62 without).

Table 3 shows differences in paired viral loads between women with and without CIN2/3 at the last positive visit. With an additional adjustment for viral load at the first positive visit (besides a set of variables including type within the group, race, current smoking status, time of the first HPV-positive detection, coinfection with other oncogenic types, and number of interval visits), the association of CIN2/3 with per 1 log-unit increase in viral load at the last, compared with the first, positive visit was statistically significant for infection by species group alpha-9 non-HPV16 oncogenic types ($OR_{adjusted} = 1.45$; 95% CI, 1.06–1.98; $P = 0.02$) but not for infection by species group alpha-5–7 non-HPV18 oncogenic types ($OR_{adjusted} = 1.23$; 95% CI, 0.90–1.69; $P = 0.19$). The association was apparently attenuated if the OR was not additionally adjusted for viral load at the first positive visit ($OR_{adjusted} = 1.10$; 95% CI, 0.87–1.40; $P = 0.43$ for infection by species group alpha-9 non-HPV16 oncogenic types; $OR_{adjusted} = 1.06$; 95% CI, 0.84–1.35; $P = 0.62$ for infection by species group alpha-5–7 non-HPV18 oncogenic types).

CIN2/3 was redetected at the last positive visit for 4 (9.5%) of 42 type-specific infections from women who had an initial diagnosis of CIN2/3 at the first HPV-positive visit including 3/22 from 20 women with species group alpha-9 non-HPV16 oncogenic types and 1/20 from 18 women with species group

Table 1. HPV DNA load at the first and last type-specific HPV-positive visit

Alpha HPV		No. of	Mean (SD) of log_{10} HPV copies per 1 nanogram of cellular DNA at			
Species	Oncogenic type	Women[a]	Both visits[b]	The first positive visit	The last positive visit	P^c
5	51	50	3.55 (±1.66)	3.40 (±1.64)	3.70 (±1.69)	0.27
6	56	31	4.04 (±1.17)	4.04 (±1.12)	4.04 (±1.24)	0.99
7	39	49	3.22 (±1.55)	3.16 (±1.56)	3.29 (±1.56)	0.62
7	45	37	2.87 (±1.51)	2.98 (±1.42)	2.75 (±1.62)	0.45
7	59	42	2.81 (±1.51)	2.76 (±1.51)	2.86 (±1.52)	0.74
7	68	25	2.42 (±1.47)	2.96 (±1.26)	1.87 (±1.48)	0.002
9	31	53	3.49 (±1.38)	3.54 (±1.25)	3.45 (±1.51)	0.67
9	33	23	3.26 (±1.06)	3.08 (±0.97)	3.44 (±1.13)	0.15
9	35	41	3.01 (±1.28)	3.08 (±1.14)	2.93 (±1.41)	0.58
9	52	84	3.00 (±1.45)	3.04 (±1.45)	2.97 (±1.46)	0.71
9	58	50	3.39 (±1.35)	3.38 (±1.17)	3.40 (±1.52)	0.93

[a]A woman was counted multiple times if she was positive for ≥2 non-HPV16/18 oncogenic types. Infection with 2, 3, and 4 types of interest was detected in 38, 2, and 1 women, respectively.
[b]Used for dichotomization of type-specific viral load at each time point for individual infections.
[c]Paired *t* test for differences in viral loads between the first and last HPV-positive visit.

Table 2. Risk of CIN2/3 associated with levels of HPV DNA load at the first and last type-specific HPV-positive visit

Alpha oncogenic HPV		Levels of HPV DNA load at the first/last positive visit[a]	No. of women[b]	No. (%) of CIN2/3	OR_{crude} (95% CI)	$OR_{adjusted}$ (95% CI)[c]	P^d
Species	Types						
5–7	Non-HPV18	Low/low	71	8 (11.3)	1.0	1.0	
5–7	Non-HPV18	High/low	39	4 (10.3)	0.83 (0.19–3.62)	0.92 (0.17–4.98)	0.92
5–7	Non-HPV18	Low/high	38	5 (13.2)	1.17 (0.29–4.76)	1.03 (0.24–4.33)	0.97
5–7	Non-HPV18	High/high	64	11 (17.2)	1.89 (0.67–5.36)	2.64 (0.74–9.40)	0.13
9	Non-HPV16	Low/low	64	7 (10.9)	1.0	1.0	
9	Non-HPV16	High/low	41	3 (7.3)	0.64 (0.12–3.50)	0.93 (0.17–5.15)	0.93
9	Non-HPV16	Low/high	46	11 (23.9)	2.66 (0.90–7.87)	3.15 (0.96–10.35)	0.06
9	Non-HPV16	High/high	76	18 (23.7)	2.64 (0.93–7.45)	3.67 (1.19–11.32)	0.02

[a]Dichotomized with means of type-specific viral load as cut-off points. The mean (SD) of $\log_{10}$ viral copies per 1 nanogram of cellular DNA at the first/last positive visit was 1.95 (±0.95)/1.98 (±1.01), 4.36 (±0.77)/1.75 (±1.08), 2.03 (±0.82)/4.32 (±0.87), and 4.50 (±0.95)/4.61 (±1.03) for categories of low/low, high/low, low/high, and high/high levels, respectively, for infection by species group alpha-5–7 non-HPV18 oncogenic types. The corresponding values for infection with species group alpha-9 non-HPV16 oncogenic types were 2.08 (±0.85)/1.96 (±0.77), 3.97 (±0.52)/1.94 (±0.87), 2.18 (±0.95)/4.09 (±0.61), and 4.30 (±0.70)/4.48 (±0.82), respectively.

[b]Excluded were 46 infections from 40 women who had a diagnosis of CIN2/3 prior to the last HPV-positive visit. A woman was counted multiple times if she was positive for ≥ 2 non-HPV16/18 oncogenic types.

[c]Adjusted for type within the group, race, current smoking status, time of the first HPV-positive detection, coinfection with other oncogenic types, and number of interval visits.

[d]Two-sided Wald test for a null hypothesis of lack of the association.

alpha-5–7 non-HPV18 oncogenic types. The DNA load of species group alpha-9 non-HPV16 oncogenic types increased from 3.93 logs (SD, ±0.56) at the first positive visit to 5.09 logs (SD, ±0.65) at the last positive visit for women with a recurrent diagnosis of CIN2/3 and decreased from 3.66 logs (SD, ±1.06) at the first positive visit to 2.61 logs (SD, ±1.64) at the last positive visit for those without. As shown in Table 4, CIN2/3 was more frequently redetected at the last positive visit for women with, compared with without, high DNA load of species group alpha-9 non-HPV16 oncogenic types at both visits (3/8 vs. 0/14, $P_{exact} = 0.04$).

Discussion

In this analysis of DNA load of non-HPV16/18 oncogenic types in pairs of the first and last type-specific HPV-positive visit, we found that the increase in risk of CIN2/3 was statistically significantly associated with high, relative to low, viral load at both visits, and marginally associated with a change of viral load from low to high levels for infection by species group alpha-9 non-HPV16 oncogenic types. A similar association was not seen for infection by species group alpha-5–7 non-HPV18 oncogenic types. The lack of association for infection by species group alpha-5–7 non-HPV18 oncogenic types could not be simply attributable to insufficient statistical power as its sample size did not differ substantially from infection by species group alpha-9 non-HPV16 oncogenic types.

One concern on analysis of viral load of a species group of types is that types within the group may differ in their neoplastic potentials. The association might have resulted from differences in neoplastic potentials rather than a change of viral load, had a more or less oncogenic type been preferably linked to certain patterns of viral load fluctuation. The comparable-type distributions across four categories of viral load fluctuation, however, make it doubtful that it had a substantial impact on the study results. Also, a variable indicating types within the group was always included as a covariate in analysis of the viral load change–associated risk of CIN2/3.

A second concern is the possibility that not all non-HPV16/18 oncogenic types detected at the time concurrent to a diagnosis of CIN2/3 were lesion-related. Coexistence of multiple types is common in natural history of HPV infection. As shown by studies of HPVs in microdissected cervical tissue samples (32, 33), most CIN2/3 cases positive for multiple types had only one type detected in the case-defining high-grade lesion. It is therefore possible that for some cases, the lesion detected at the last positive visit could be attributable to types other than the one evaluated. While this potential misclassification cannot be dismissed, the analysis was additionally restricted to infections without coexistence of other oncogenic types at the last positive visit. The high

Table 3. Risk of CIN2/3 associated with a change of HPV DNA load between the first and last type-specific HPV-positive visit

Alpha HPV		Paired differences in $\log_{10}$ HPV copies per 1 nanogram of cellular DNA between the first and last HPV-positive visit[a] for women						
		Without CIN2/3		With CIN2/3				
Species	Oncogenic types	No. of women[b]	Mean (SD)	No. of women[b]	Mean (SD)	OR_{crude} (95% CI)	$OR_{adjusted}$ (95% CI)[c]	P^d
5–7	Non-HPV18	184	−0.07 (±1.94)	28	0.28 (±1.30)	1.22 (0.95–1.57)	1.23 (0.90–1.69)	0.19
9	Non-HPV16	188	−0.01 (±1.65)	39	0.32 (±1.07)	1.36 (1.06–1.74)	1.45 (1.06–1.98)	0.02

[a]Paired differences = viral load at the last positive visit minus viral load at the first positive visit. The mean (SD) of $\log_{10}$ viral copies per 1 nanogram of cellular DNA at the first/last positive visit was 3.27 (SD. ±1.44)/3.55 (SD, ±1.40) and 3.16 (SD, ±1.54)/3.09 (SD, ±1.68) for women with and without CIN2/3, respectively, for infection by species group alpha-5–7 non-HPV18 oncogenic types. The corresponding values for infection with species group alpha-9 non-HPV16 oncogenic types were 3.42 (SD, ±1.34)/3.75 (SD, ±1.16) and 3.13 (SD, ±1.28)/3.12 (SD, ±1.45) for women with and without a diagnosis of CIN2/3, respectively.

[b]Excluded were 46 infections from 40 women who had a diagnosis of CIN2/3 prior to the last positive visit. A woman was counted multiple times if she was positive for ≥ 2 non-HPV16/18 oncogenic types.

[c]Adjusted for type within the group, race, current smoking status, time of the first HPV-positive detection, coinfection with other oncogenic types, number of interval visits, and viral load at the first positive visit.

[d]Two-sided Wald test for a null hypothesis of lack of the association.

Table 4. Frequencies of CIN2/3 redetection by levels of HPV DNA load at the first and last type-specific HPV-positive visit

Alpha HPV		Levels of HPV DNA loads at the first/last positive visit[a]	No. of CIN2/3 cases[b]	No. (%) with CIN2/3 redetection	P^c
Species	Oncogenic types				
5–7	Non-HPV18	Low/low	2	0	
5–7	Non-HPV18	High/low	3	0	
5–7	Non-HPV18	Low/high	4	0	
5–7	Non-HPV18	High/high	11	1 (9.1)	0.55
9	Non-HPV16	Low/low	4	0	
9	Non-HPV16	High/low	8	0	
9	Non-HPV16	Low/high	2	0	
9	Non-HPV16	High/high	8	3 (37.5)	0.04

[a]Dichotomized with means of type-specific viral load as cut-off points. The mean (SD) of $\log_{10}$ viral copies per 1 nanogram of cellular DNA at the first/last positive visit was 1.08 (±1.52)/1.63 (±1.19), 3.10 (±0.14)/0.51 (±1.00), 2.50 (±0.58)/3.97 (±1.23), and 4.36 (±0.67)/4.21 (±0.85) for categories of low/low, high/low, low/high, and high/high levels, respectively, for infection by species group alpha-5–7 non-HPV18 oncogenic types. The corresponding values for infection by species group alpha-9 non-HPV16 oncogenic types were 2.31 (±0.65)/1.87 (±1.30), 4.18 (±0.54)/1.56 (±1.15), 2.61 (±0.07)/5.18 (±0.93), and 4.18 (±0.76)/4.31 (±0.87), respectively.

[b]Included were infections from women who had a previous diagnosis of CIN2/3 at the first positive visit. A case was counted multiple times if she was positive for ≥ 2 non-HPV16/18 oncogenic types. Four cases (mean age of 21.5 years, 2 Caucasians and 2 non-Caucasians, one current smoker) with CIN2/3 redetected at the last positive visit were positive for HPV31, 33, 35, and 51, respectively, with the infection initially detected at study entry; all of them had 3 interval visits and a coinfection of other HPV types.

[c]Fisher exact test for differences in frequencies of CIN2/3 redetection between cases with and without high viral load at both visits.

DNA load of species group alpha-9 non-HPV16 oncogenic types at both visits remained associated with increased risk of CIN2/3.

Lastly, our findings pertain to generally young women who had a cytologic diagnosis of ASC-US or LSIL within 6 months prior to enrollment into ALTS. The viral load detected might not be generalizable to that in the general populations. In this study, a persistent infection was defined as infection(s) detected by Roche Linear Array at ≥2 visits among women who were followed every 6 months for 2 years. It is possible that viral load or even positive status may change within the intervals. Thus, our findings might not be generalizable to women with transient infections and even those with different follow-up intervals. However, no evidence suggests that this lack of generalizability would affect the validity for assessment of the viral load change–associated risk of CIN2/3. Replication studies ideally would examine the association among population-based screening women.

Use of high versus low viral load at the first and last positive visit to reflect viral load fluctuation is straightforward. A similar approach was reported by others showing the association of high-grade CIN with repeat moderate-high viral load (34). However, a selection of cut-off points for dichotomization is somewhat arbitrary. Although estimates of the association remained similar when the analysis was repeated with medians of type-specific viral load as cut-off points, we showed only the impact of trends of viral load fluctuation on risk of CIN2/3. In a second analysis, therefore, a change of viral load between two visits was treated as a continuous variable. The quantitative analysis of viral load change was also reported by others, in which a change of viral load was measured by linear regression slope and coefficient of determination; differences in slopes were used to distinguish transient infections from infections leading to the development of CIN3 (20–22). We used here a statistic of paired differences in $\log_{10}$-transformed viral load between two visits, the approach that avoids a strong assumption of linear increase or decrease in viral load during a study period.

In agreement with results of the dichotomized viral load, a statistically significant association of CIN2/3 with per 1 $\log_{10}$-unit increase in viral load at the last, compared with the first, positive visit was seen for infection by species group alpha-9 non-HPV16 oncogenic types but not by species group alpha-5–7 non-HPV18 oncogenic types. One observation meriting mention is that the association of CIN2/3 with the magnitude of viral load change would be substantially attenuated, if the OR was not additionally adjusted for viral load at the first positive visit, suggesting an effect of negative cofounding by the baseline viral load. Intuitively, given the same amount of viral load increase, risks of CIN2/3 are likely to augment as increasing initial viral load, if the viral load truly plays a role in the development of cervical precancer.

This study is an extension of our previous report of type-dependent, viral load–associated risk of CIN2/3 (18). In that study, baseline DNA load of non-HPV16/18 oncogenic types was found to be associated with concurrent and subsequent risk of CIN2/3 for alpha-9 types but not others. This study lends further support to previous findings by showing the species group-dependent, viral load change–associated risk of CIN2/3 among women with a persistent infection. The consistency of the results between these studies strongly supports a notion of the species group disparity in the viral load–associated risk of CIN2/3. Although reasons for this are currently unclear, findings from this and previous studies (18, 19) provide clues for further research into the causes and mechanisms by which risks of CIN2/3 increase as increasing DNA load of species group alpha-9 oncogenic types but not species group alpha-5–7 oncogenic types. As discussed previously (18), whether alpha-9 types differ from types in other species groups in tropism for the host cells, behavior of the virus in these cells, and location of the HPV-related lesion deserves consideration.

Few studies of HPV DNA load in consecutive specimens have reported that serial viral load measures might be predictive for outcome of the infection for all high-risk HPV types and even a low-risk type of HPV6 and could be useful for HPV-based cervical cancer screening (20–22). Results from this study suggest that clinical value of screening for underlying CIN2/3 by measuring a change of viral load between two visits alone appears limited as risk of CIN2/3 was not significantly associated with viral load change for infection by species group alpha-5–7 non-HPV18 oncogenic types. Although high DNA load of species group alpha-9 non-HPV16 oncogenic types at both visits signaled an elevated risk of CIN2/3, approximately 11% of women with low viral load at both visits had also a diagnosis of CIN2/3. Nevertheless, the finding of the species group disparity in the viral load change–associated risk of CIN2/3 is of value that enriches our understanding of HPV-related pathogenesis and underlines the necessity of distinguishing species groups in future studies of clinical relevance of HPV DNA load.

In this study, CIN2/3 was redetected at the last HPV-positive visit for 9.5% of infections from women who had an initial diagnosis of CIN2/3 at the first positive visit. A possible link between continuous presence of HPV infection after treatment for high-grade CIN and redetection of the lesion has been noted in some clinical observations (24–26). There are few data available showing that women with high, compared with low, viral load prior to the treatment were more likely to have a persistent HPV

infection (35) and a diagnosis of posttreatment lesion (27, 28); cervical lesions associated with high viral load were more likely to persist than those associated with low viral load (36). To the best of our knowledge, this report is one of the first, if not the first, to show a relation between posttreatment CIN2/3 and viral load before and after treatment. Among women with an initial diagnosis of CIN2/3 at the first positive visit, those with, compared with without, high DNA load of species group alpha-9 non-HPV16 oncogenic types at both visits were more likely to have CIN2/3 redetected at the last positive visit, the finding that was consistent with the viral load change–associated risk of incident CIN2/3.

As noted, all cases with CIN2/3 redetected at the last positive visit had high viral load at both visits. This finding, although limited by the small number of cases, offers some support for a hypothesis that posttreatment CIN2/3 may be more likely to arise as a result of continuous exposure to high HPV DNA load before and after treatment. If this finding can be further confirmed in large-scale studies and in different study populations, measuring viral load change over time may have a potential utility in the evaluation of response to therapeutic interventions. Although we were unable to tell whether the redetected lesion resulted from new occurrence, recurrence, or persistence of the previous lesion, from the view of clinical management of women treated for cervical precancers, the implication of these results is the same. Knowing a relation between risk of posttreatment CIN2/3 and a change of viral load is important in terms of patient counseling and clinical management.

Taken together, data from this study indicate that risks of CIN2/3 associated with a change of viral load differ by HPV species groups, the findings that underscore the importance of distinguishing species groups in studies of clinical relevance of HPV DNA load. Because of a small number of study subjects, a link between posttreatment CIN2/3 and high viral load at both first and last HPV-positive visits should be viewed only as a hypothesis worthy subsequent testing.

Disclosure of Potential Conflicts of Interest

D.A. Galloway is a consultant/advisory board member for and reports receiving a commercial research grant from Merck. No potential conflicts of interest were disclosed by the other authors.

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