Research Article

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Human Papillomavirus Genotypes in Cervical Intraepithelial Neoplasia Grade 3

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Abstract

Background: There are few large case series describing the human papillomavirus (HPV) genotypes found in women diagnosed with rigorously reviewed cervical intraepithelial neoplasia grade 3 (CIN3), cervical precancer.

Methods: The Atypical Squamous Cells of Undetermined Significance (ASCUS) and Low-Grade Squamous Intraepithelial Lesion (LSIL) Triage Study (ALTS) was a clinical trial to evaluate the best management strategies for women with equivocal (ASCUS) or mildly abnormal (LSIL) Pap tests. During enrollment and the 2-year follow-up, 608 women had a histopathologic diagnosis of CIN3 and PCR-based HPV genotyping results on cervical specimens. The genotyping results were ranked hierarchically according to cancer risk: HPV16 > other carcinogenic HPV > noncarcinogenic HPV > PCR negative.

Results: Among the 608 women diagnosed with CIN3, 601 (98.8%) cases were positive for any HPV genotype and 95.4% for any carcinogenic HPV. HPV16 (59.9%), HPV31 (18.1%), HPV52 (14.8%), HPV51 (14.0%), and HPV18 (13.2%) were the five most common HPV genotypes detected. Younger age, consensus histologic confirmation, smoking, and multiparity increased the likelihood of testing HPV 16 positive. Specifically, HPV16-positive CIN3 occurred at a younger age than CIN3 positive for other carcinogenic HPV genotypes (median of 23.5 years versus 25 years, respectively; $P = 0.0003$, Kruskal-Wallis).

Conclusions: HPV16-positive CIN3 was more commonly diagnosed in younger women (versus older women), with consensus diagnosis (versus some disagreement between reviewers), and in smokers (versus nonsmokers), and was less commonly diagnosed in multiparous women compared CIN3 positive for other carcinogenic HPV genotypes.

Impact: In populations vaccinated against HPV16 (and HPV18), the median age of CIN3 in women with ASCUS and LSIL cytology should shift to older ages, possibly permitting later age at first screening.
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Introduction

Relatively few large case series describe the human papillomavirus (HPV) genotypes found in women diagnosed with rigorously reviewed cervical intraepithelial neoplasia grade 3 (CIN3), the immediate precursor lesion to invasive cervical cancer. CIN3 is considered a definite precancer and is the best surrogate for cancer risk. It is a critical end point for evaluating the efficacy of primary and secondary prevention strategies.

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We had previously described in detail the HPV genotypes detected in enrollment specimens collected from women participants in the Atypical Squamous Cells of Undetermined Significance (ASCUS) and Low-Grade Squamous Intraepithelial Lesion (LSIL) Triage Study (ALTS; refs. 1-3). However, details about the HPV genotypes detected at the time of diagnosis of CIN3, i.e., cervical precancer, have not been published. Because the exact onset of CIN3 development cannot be known, our previous analyses relied on the assumption that the cases of CIN3 detected within the 2-year study were prevalent, some detected immediately whereas others were missed and found only during follow-up or exit colposcopy. We recognize, however, that a small percentage of cases of CIN3 in a 2-year study are incident (2, 4); moreover, HPV18/45-related CIN3 are more difficult to identify in a single screen (5). We therefore sought to describe in brief the HPV genotypes detected in cervical specimens at the time of CIN3 diagnosis, using all of our available HPV genotyping testing to maximize analytic sensitivity (6), and to identify any factors that alter the fraction of positives for HPV16,

the major genotype targeted by HPV vaccination that causes 50% or more of all cervical cancer (7).

Materials and Methods

Study design and population

The ALTS (1997-2001) was a multisite, randomized trial comparing three management strategies (immediate colposcopy, HPV triage, and conservative management) for women referred for ASCUS[4] (n = 3,488) or LSIL (n = 1,572) conventional cytology (10-14). The National Cancer Institute and local institutional review boards approved the study, and all participants provided written, informed consent.

At enrollment and follow-up visits over the 2-year duration, all women underwent a pelvic examination with collection of two cervical specimens; the first specimen in PreservCyt for ThinPrep cytology (Cytyc Corporation, now Hologic) and the second in specimen transport medium (STM; Digene Corporation, now Qiagen). Women in all three arms of the study were re-evaluated by cytology every 6 months during the 2 years and sent to colposcopy if cytology was HSIL. An exit examination with colposcopy was scheduled for all women. We refer readers to other references for details on randomization, examination procedures, patient management, and laboratory and pathology methods (10-14).

HPV testing

Line blot assay was done on enrollment STM specimens as previously described (15) for the detection of 27 of 38 individual HPV genotypes (16, 17).

Aliquots of archived STM specimens from women diagnosed with CIN3 were retested using linear array, which tests for 37 of 38 HPV genotypes detected by line blot assay (excluding HPV57) as previously described (3). HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 were defined as the carcinogenic HPV genotypes (18). We have previously shown that HPV genotype detection by linear array on specimen aliquots stored for approximately 8 to 12 years after they were collected was (*a*) well correlated with, but analytically more sensitive than, detection by line blot assay (3), and (*b*) the clinical performance for detection of CIN3 of linear array (for carcinogenic HPV in aggregate) was comparable with hc2 (19).

Statistical analyses

Any histologic CIN3 diagnosis by either the clinical center pathologists or the quality control pathology review during the 2-year study was included in this analysis. Thus, every CIN3 had a second diagnosis (by the other pathology review) that was categorized as CIN3, CIN2, or <CIN2. When the two reviews agreed, the CIN3 was called a "consensus diagnosis." We calculated the prevalence of any HPV genotype, any carcinogenic genotype, the prevalence of multiple HPV genotypes and multiple carcinogenic HPV genotypes (among HPV positives), and the individual 37 HPV genotypes detected by line blot assay, linear array, or by either assay. We note that linear array detects HPV52 by inference, which we previously found to underestimate the presence of HPV52 (3). However, we observed in this set of test results that there was an excess of inferred HPV52-positive results that was associated with testing HPV16 positive (data not shown). We therefore relied on the line blot assay detection of HPV52 only.

Multiple HPV genotypes were so commonly detected in the cervical specimens collected from this population that we classified the HPV genotype detection hierarchically according to cancer risk (HPV risk group): HPV16, else HPV16 negative but positive for other carcinogenic HPV genotypes, else negative for all carcinogenic HPV genotypes but positive for noncarcinogenic HPV genotypes, else PCR negative. We used standard contingency table methods with a Fisher's exact or χ^2 test to assess possible univariate associations of categorical variables with HPV risk group. The relationships of continuous variables (e.g., age at diagnosis) with HPV risk group were assessed with nonparametric ANOVA and Kruskal-Wallis test.

Because the numbers of CIN3 cases in the categories of noncarcinogenic HPV and PCR negative were rare, we excluded these cases from the following analyses. A logistic regression model was used to calculate the odds ratios (OR) and 95% confidence intervals (95% CI) as a measure of association of relevant parameters with HPV16 (versus carcinogenic HPV genotypes excluding HPV16). Variables from the univariate analysis with a P value of ≤ 0.2 were considered as possible covariates and were added into the logistic regression model until a parsimonious model was achieved. Dose-response relationships (P_{trend}) were assessed in logistic regression models by treating ordinal variables as continuous (which assumes a linear trend).

P values of <0.05 were considered statistically significant. STATA version 9.0 was used for statistical analyses.

Results

Of the 621 CIN3 diagnosed during the 2-year duration of the ALTS, cervical specimens collected at the screening visit immediately prior to diagnosis from 596 (96.0%) women were tested by line blot assay, 588 (94.7%) by linear array, and 576 (93.8%) by both tests; 608 (97.9%) were tested by line blot assay or linear array and defined our analytical set. The timing of diagnosis of the CIN3 was 54% at enrollment, 8% at 6 months, 6% at 12 months, 6% at 18 months, and 26% at 24 months. Of the 608 CIN3 included in this analysis, 601 (98.8%) cases were positive for any HPV genotype and 95.4% for any carcinogenic

[4]ASCUS under the 1991 Bethesda system (8) was slightly more inclusive, particularly of probable reactive changes and ASC-H (atypical squamous cells, cannot rule out high-grade intraepithelial lesion), than the ASC-US category of the 2001 Bethesda system (9).

Table 1. HPV genotypes detected by line blot assay, linear array, and either line blot assay or linear array, for all and single HPV genotypes in cervical specimens from women diagnosed with CIN3

	Line blot assay (*n* = 596)	Linear array (*n* = 588)	Line blot assay or linear array	
			All (*n* = 608)	Single (*n* = 128)
	%	%	%	%
Any HPV	96.0	98.1	98.9	100
Any carcinogenic HPV	92.1	94.4	95.4	89.8
Multiple HPV genotypes	64.7	77.2	77.8	
Multiple carcinogenic HPV genotypes	51.3	61.3	60.0	
HPV6	2.0	4.3	4.4	1.6
HPV11	1.0	1.7	1.6	0.0
HPV16	56.5	58.8	59.9	47.7
HPV18	11.2	12.4	13.2	7.0
HPV26	1.0	1.7	1.8	0.8
HPV31	14.8	17.0	18.1	10.9
HPV33	7.7	8.8	10.0	3.9
HPV35	9.2	8.8	9.4	4.7
HPV39	7.7	10.2	10.5	0.8
HPV40	1.5	1.9	2.1	0.0
HPV42	3.0	5.4	5.6	0.8
HPV45	6.7	8.3	8.4	2.3
HPV51	10.1	13.4	14.0	0.8
HPV52*	14.8	23.5†	14.8	5.5
HPV53	7.1	11.1	11.2	0.0
HPV54	6.7	9.7	10.0	0.0
HPV55	3.9	5.4	5.4	0.8
HPV56	5.0	7.0	7.2	0.8
HPV57	0.2	n/a	0.2	0.0
HPV58	7.9	9.7	9.9	5.5
HPV59	6.0	8.3	8.1	0.0
HPV61‡	4.5	7.1	6.9	0.0
HPV62‡	5.2	9.2	9.1	0.8
HPV64‡	0.5	0.0	0.3	0.0
HPV66	3.9	6.1	6.3	2.3
HPV67‡	2.9	2.4	3.0	1.6
HPV68	3.5	4.6	4.6	0.0
HPV69‡	0.9	1.0	1.0	0.0
HPV70‡	4.8	4.3	4.9	0.0
HPV71‡	1.4	0.7	1.2	0.0
HPV72‡	2.0	2.2	2.5	0.0
HPV73	2.5	3.2	3.3	0.0
HPV81‡	2.5	4.1	4.0	0.8
HPV82	3.5	3.7	3.6	0.8
HPV82v‡	0.0	0.5	0.5	0.0
HPV83	4.7	6.0	6.3	0.0
HPV84	3.4	8.0	8.2	0.0
HPV89‡	4.3	6.1	6.4	0.0

*HPV52 for line blot assay or linear array was based only on the line blot assay result because of the tendency of linear array to overestimate the presence of HPV52.

†Inferred based on testing positive for a pool of probes.

‡These HPV genotypes were not detected in all enrollment specimens tested by line blot assay.

HPV genotype as detected by either or both HPV genotyping assays (Table 1). HPV16 (59.9%), HPV31 (18.1%), HPV52 (14.8%), HPV51 (14.0%), and HPV18 (13.2%) were the five most common HPV genotypes detected. When restricted to cases in which only one HPV genotype was detected (n = 128), HPV16 (47.7%), HPV31 (10.9%), HPV18 (7.0%), HPV52 (5.5%), and HPV58 (5.5%) were the five most common HPV genotypes detected; HPV66 (2.3%) was the most common noncarcinogenic HPV genotype detected singly. The median age of women who had multiple HPV genotypes detected was younger than those with a single HPV genotype (24 years versus 26 years, respectively, P = 0.0001).

When categorized according to HPV risk groups, 364 (59.9%) were HPV16 positive, 216 (35.5%) were positive for other carcinogenic HPV genotypes, 21 (3.5%) were positive for noncarcinogenic HPV genotypes, and 7 (1.2%) were PCR negative. The seven PCR-negative cases of CIN3 were older on average than the rest (median age of 40 years). Only one of these seven PCR-negative cases was a consensus diagnosis of CIN3, with three second diagnoses of <CIN2 and three of CIN2. Three of the seven PCR-negative cases had normal cytology as read on both pathology reviews, and two of six tested positive for carcinogenic HPV by Hybrid Capture 2 (Qiagen; data not shown).

Age at diagnosis (P = 0.0003), second diagnosis (P < 0.0005), cigarette smoking (P = 0.005), oral contraceptive use (P = 0.04), parity (P = 0.03), and the cytologic interpretation at the time of the CIN3 diagnosis (P < 0.0005) were crudely associated with the HPV risk group (Table 2). In particular, HPV16 was more common when the second diagnosis was also CIN3 (68%) compared

Table 2. Characteristics of CIN3 by hierarchically defined HPV risk group: HPV16 > other carcinogenic HPV genotypes (Carc. excl. HPV16) > noncarcinogenic HPV > PCR negative

Characteristics at diagnosis	*n* (%)	HPV16	Carc. (excl. HPV16)	Noncarcinogenic	PCR negative	*P*
		n = 364	*n* = 216	*n* = 21	*n* = 7	
Median age (y)	608 (100)	23.5	25	24	40	0.0003
	n (%)	Row %	Row %	Row %	Row %	
Second diagnosis						
<CIN2	42 (7)	36	48	10	7	<0.0005
CIN2	236 (39)	53	42	4	1	
CIN3	330 (54)	68	29	2	0	
Smoking status						
Never	247 (41)	55	41	3	2	0.005
Former	80 (13)	54	39	4	4	
Current	281 (46)	66	30	4	0	
Oral contraceptive use						
Never	156 (26)	56	40	3	1	0.04
Former	127 (21)	58	34	6	0	
Current	273 (45)	66	32	2	0	
Missing	52 (9)	44	46	6	0	
Parity						
Never pregnant	162 (27)	67	29	4	0	0.03
0 births	76 (13)	66	33	1	0	
1 birth	159 (26)	60	37	3	1	
≥2 births	211 (35)	52	40	5	3	
Worst cytologic interpretation						
Negative	45 (7)	40	42	11	7	<0.0005
ASCUS	70 (12)	54	36	6	4	
LSIL	107 (18)	63	35	2	1	
HSIL/ASC-H	385 (63)	63	35	3	0	
Study arm						
Immediate colposcopy	227 (37)	57	35	6	2	0.1
HPV triage	163 (27)	60	38	2	1	
Conservative management	218 (36)	63	34	2	1	

Abbreviations: HSIL, high-grade squamous intraepithelial lesion; ASC-H, atypical squamous cells, cannot rule out HSIL.

Table 3. Logistic regression model for detecting HPV16 versus other carcinogenic HPV genotypes in cervical specimens from women diagnosed with CIN3

Characteristics at diagnosis	*n* (%)	OR (95% CI)	*P*
Age (per year)	580 (100)	0.97 (0.94-1.0)	0.04
Second diagnosis			
<CIN2 (ref.)	35 (6)	1.0 (—)	
CIN2	224 (39)	1.8 (0.85-3.8)	0.1
CIN3	321 (55)	3.1 (1.5-6.4)	0.003
Parity			
Never pregnant (ref.)	156 (27)	1.0 (—)	
0 births	75 (13)	0.95 (0.52-1.7)	0.9
1 birth	154 (27)	0.69 (0.43-1.1)	0.1
≥2 births	195 (34)	0.57 (0.35-0.93)	0.03
Smoking status			
Never (ref.)	236 (41)	1.0 (—)	
Former	74 (13)	1.0 (0.59-1.8)	0.9
Current	270 (47)	1.7 (1.2-2.5)	0.005

NOTE: Odds ratios are mutually adjusted for all variables in the table.

with when the second diagnosis was CIN2 (53%) or <CIN2 (38%).

We used a logistic regression model to evaluate factors independently associated with being HPV16 positive versus other carcinogenic HPV genotypes (n = 580; Table 3). A consensus diagnosis of CIN3 was strongly associated with being a HPV16-positive CIN3 (OR, 3.1; 95% CI, 1.5-6.4; P = 0.003); there was an increasing trend to find HPV16 associated with increasing severity of the second diagnosis (P_{trend} < 0.0005). Younger age at diagnosis was associated with testing HPV16 (OR, 0.97 per year; 95% CI, 0.94-1.0; P_{trend} = 0.04); using quartiles of age at diagnosis (≤21, 22-24, 25-28, and 29-70) yielded a similar pattern (P_{trend} = 0.001). Current smoking (versus never smoked, OR, 1.7; 95% CI, 1.2-2.5; P = 0.03) was positively associated and multiparity (versus never pregnant, OR, 0.57; 95% CI, 0.35-0.93; P = 0.005) was negatively associated with testing HPV16 positive; among women who were ever pregnant (n = 424), increasing number of births marginally decreased the likelihood of testing HPV16 positive (P_{trend} = 0.06), but few women in this relatively young study population had many births. Inclusion of the timing of the CIN3 diagnosis confirmed as previously shown (5) that HPV16-related CIN3 was diagnosed earlier than CIN3 related to other carcinogenic HPV genotypes but did not appreciably alter the association of other covariates with being HPV16 positive (data not shown). Oral contraceptive use was strongly, negatively associated with parity (P < 0.001) but was not significantly associated with being HPV16 positive after adjusting for other covariates and was excluded from the final analysis. Likewise, study arm and worst cytologic interpretation were not associated with being HPV16 after adjusting for the other covariates and were excluded from the final analysis.

Among those with any carcinogenic HPV genotype, the median age at diagnosis was younger among the HPV16 positive versus those positive for other carcinogenic HPV genotypes (23.5 versus 25 years, respectively, P = 0.0003). A similar pattern was observed when analysis was restricted to a single HPV genotype detected (24 versus 28 years, respectively, P = 0.01) and to only those with consensus CIN3 diagnoses (24 versus 25 years, respectively, P = 0.04). HPV16-positive CIN3 occurred at a younger age in all randomization arms compared with other carcinogenic HPV-positive CIN3. The median time since sexual debut to CIN3 diagnosis was significantly shorter for HPV16-positive compared with other carcinogenic HPV-positive CIN3 (7 versus 9 years, respectively, P = 0.004).

Discussion

CIN3 is the critical intermediate end point targeted by cervical cancer screening programs and in HPV vaccine trials. We were interested in determining the HPV genotypes detected by screening immediately before the CIN3 diagnosis and any factors that influence the distribution. We used a combination of HPV genotyping tests to maximize the analytic sensitivity. Virtually all were HPV positive (98.9%), and most of those that were HPV positive were positive for at least one of the carcinogenic HPV genotypes (96.5%). The high prevalence of multiple HPV genotypes detected in the cervical specimens highlighted the major limitation in this analysis: HPV genotyping was not done on the diagnostic tissue. Restricting to single HPV genotypes probably led to an underestimation, and hierarchically categorizing the HPV genotypes according to cancer risk probably led to an overestimation, of the fraction of CIN3 caused by HPV16.

Despite this limitation, we made several important observations. First, we found that the certainty of the CIN3 diagnosis influenced the proportion of women who tested positive for HPV16 at the visit proximal to the CIN3 diagnosis, confirming an earlier report from this study population based on detection of HPV16 in enrollment specimens restricted to those referred for an ASCUS Pap smear (20). This suggests that HPV genotype attribution of cervical precancer (7) will depend somewhat on its definition (4, 20, 21), with an increasing percentage of HPV16 positives as diagnostic certainty of precancer increases from HSIL cytology to CIN2, CIN2/3, CIN3, and finally confirmed CIN3 histology.

Smoking and parity influenced the proportion of HPV16 positives but in opposite directions: current smokers had more HPV16-related CIN3 and multiparous women had less. Both factors are considered cofactors for

the development of cancer (22), and smoking has been shown to be associated with the development of precancerous lesions (23-25). There is no previous evidence, however, that these factors differentially influence HPV16 natural history versus other carcinogenic HPV genotypes in this population (24, 26). Smoking duration or intensity did not alter the association with being HPV16 positive (data not shown). These associations might be due to chance or related to an unknown effect of smoking or parity on the detection and diagnosis of HPV16-related CIN3 versus other carcinogenic HPV genotypes (27).

Finally, we observed that HPV16-positive CIN3 were diagnosed at a younger age than were other carcinogenic HPV-positive CIN3, even in the artificial circumstances of a clinical trial involving aggressive screening and management that led to early detection of very small CIN3 lesions (28, 29). Even when controlling for the study arm, which was related to the aggressiveness of the enrollment evaluation, the age of diagnosis was significantly younger for HPV16-positive CIN3 versus HPV16-negative CIN3. Likewise, the median time from age of sexual debut to the CIN3 diagnosis was shorter for HPV16-positive CIN3 than for the CIN3 positive from other carcinogenic HPV genotypes. An earlier study reported that the proportion of CIN2 and CIN3 related to HPV16 was greater in younger women than in older women (30). Another study found that the mean age of HPV16- and HPV18-related diagnoses of invasive cancer was five years younger than the mean age for cancers caused by other carcinogenic HPV genotypes, and the mean age of HPV16- and HPV18-related CIN3/carcinoma *in situ* diagnosis was not significantly lower than the mean age for CIN3/carcinoma *in situ* caused by other carcinogenic HPV genotypes (31). These findings are consistent with the unique carcinogenic potential of HPV16. Although HPV16 is more prevalent than any other single HPV genotype at any age, it is less prevalent than all other carcinogenic HPV genotypes combined, and therefore it seems an unlikely argument that the earlier diagnosis of HPV16-positive CIN3 compared with non–HPV16-positive CIN3 is due to the former being more common and therefore being acquired earlier after sexual debut. Alternatively, colposcopists may more easily identify HPV16-related lesions than lesions caused by other HPV genotypes (27). Or it could be some combination of the two: HPV16-related CIN3, being more aggressive, become larger sooner after the acquisition of the causal HPV16 infection and easier to identify at colposcopy at a younger age.

In conclusion, in populations vaccinated against HPV16 (and HPV18), the median age of CIN3 in women with ASCUS and LSIL cytology may shift to older ages. This suggests that cautious consideration of raising the age limit of first screen in vaccinated populations might be considered in the United States.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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