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Association of HPV16 E6 variants with diagnostic severity in cervical cytology samples of 354 women in a US population

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Abstract

It has been suggested that DNA sequence variants of HPV16 contribute to differences in the behavior of individual cervical lesions. To address this question, we have analyzed the association of HPV16 variants with diagnostic severity in 354 HPV16-positive Oklahoman women. HPV16 variant status was determined by PCR amplification and DNA sequencing of the *E6* open reading frame. European sequences were identified in 86% of samples and 14% were non-European. Of the 51 non-European cases, 61% were Asian-American, 23% African and 16% were Native American variants. European prototype and related variants were present in comparable numbers (43% each) but the relative proportion of each differed with diagnostic category. In general, the proportion of European variants and non-European variants increased with diagnostic severity while the European prototype decreased. When adjusted for age and race (white, black or Hispanic), the increased risk for carcinoma/severe dysplasia for non-European variants was statistically significant with an odds ratio of 3.8 (1.3–10.7). However, the analogous comparison for the European variants, although also showing increased association with carcinoma/severe dysplasia, did not reach statistical significance (OR = 1.6 (95% CI 0.7–3.6). Overall, HPV16 European sequences (both prototype and related variants), were predominant in Oklahoman women including those with cancers. This suggests that while there appear to be differences among the HPV16-variant categories in risk for progression to invasive cancer, all variant categories are associated with the development of invasive cancer.

Keywords

human papillomavirus; cervical carcinogenesis; cervical intraepithelial lesions; HPV variants; cervical neoplasia

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Novelty/Impact Statement: There were differences in the distribution of HPV16 variants in the spectrum of cervical lesions in a US population. While there appear to be differences in neoplastic potential in lesions associated with different variant categories, all categories were present in invasive cancers suggesting that all HPV16 - variant categories are associated with the potential for malignant progression.

Introduction

Intraepithelial lesions and carcinomas of the cervix have been overwhelmingly demonstrated to be associated with human papillomaviruses, with a particularly strong association with HPV16 (1–3). However, the fact that only a minority of women infected with HPV16 develop invasive carcinoma suggests that there are as yet undefined factors that affect the biological outcome in the associated lesions. In addition to various host factors, there has been strong interest in the possible role of DNA sequence variation (and hence protein structure/function) in contributing to the biological progression of individual HPV associated lesions. Intratype HPV variants have been defined as showing less than 2% nucleotide variation in the *L1* ORF (4) from the original reference or prototype sequence (5). In addition to the *L1* sequence changes, the individual variant types have characteristic nucleotide changes in other parts of the viral genome. These have been particularly well characterized for the *E6* ORF (6–8) and LCR (9,10). For HPV16, the *E6* sequence changes have been found to accurately assign variant category to individual isolates (8,11). The first HPV16 sequence (5) identified was from a European woman and was termed “European prototype”. Studies performed on a worldwide basis have identified several other major variant sequences that have a rough, although imprecise, geographical relationship. These have been grouped in many studies as Non-European. A number of studies have reported an association between biological potential in HPV16 positive cervical lesions and the HPV16 variant category. Non-European variants(10,12–14) and European variants harboring the 350 T→G nucleotide pattern (**15, 16)** have been associated with increased risk for progression to high grade intraepithelial precursors (13,15,17) and invasive cervical cancer (18,19). In the present study we have determined the distribution of HPV16 *E6* variants for the spectrum of diagnostic categories including invasive cancer in a US population.

Methods and Materials

Patient Samples

The testing reported herein was performed using liquid-based cytologic samples with the approval of the Institutional Review Board of the University of Oklahoma Health Sciences Center. The cases include 115 HPV16 positive samples that were previously reported (20,21), 210 cases subsequently accrued to that study and 60 HPV16-positive control cases (N/L) from our clinic population that were enrolled in the Study to Understand Cervical Cancer Early Endpoints and Determinants (SUCCEED) (22). The SUCCEED samples were included to supplement the originally limited number of controls. As in our previous studies (20,21), high grade squamous intraepithelial lesions (HSIL) were subdivided into HSIL-moderate (HSIL-M) and HSIL-severe (HSIL-S) to reflect the traditional diagnostic categories for high grade dysplasia. This is because our earlier work suggested that HSIL-M and HSIL-S were associated with different HPV genotypes (20,21). Our criteria for distinguishing these cytologic patterns have been previously described (20,21) and relate primarily to increasing nuclear/cytoplasmic ratio with increasingly severe intraepithelial disease.

Diagnostic groups used in this analysis were N /L for infected women with diagnoses of LSIL or less, HSIL -M, HSIL-S and invasive cancer. All cases were entered in the cancer category based on the histologic diagnosis of invasion in concurrent tissue samples or biopsies performed within 6 months after the cytologic sample. Similarly, 5 HPV16-positive cases with AIS were entered into this study based on corresponding histologic diagnoses and were included in the HSIL-S category.

Variant Analysis

DNA was extracted from residual PreservCyt® (Hologic, Inc, Bedford, MA) samples and subjected to HPV genotyping using the Roche reverse line blot and successor, the LINEAR ARRAY® Genotyping Test (Roche Molecular Systems, Roche Diagnostics, Mannheim, Germany), as previously described (20,21). DNA from 385 HPV16-positive samples was further analyzed for HPV16 *E6* variant category by using PCR amplification and DNA sequencing. Approximately, 1 μl of DNA from each sample was amplified in a final reaction mixture (25 μl total volume) consisting of 2.5 μl of 1X Reaction Buffer (Promega), 2 μM $MgCl_2$, 200 μM dNTPs, 5 ng/μl each primer (*E6* and β-globin) and 1 U *Taq* DNA polymerase. *E6* ORF primers (nt 83–559) comprised 5′-TGA ACC GAA ACC GGT TAG TA-3′ and 5′-CAT GCA ATG TAG GTG TAT CTC C-3′ and β-globin primers comprised 5′-ACA CAA CTG TGT TCA CTA GC 3′ and 5′-CAA CTT CAT CCA CGT TCA CC 3′. Thermal cycling conditions consisted of an initial 3 minute denaturation at 94 °C, followed by 40 cycles of 94 °C for 1 minute, 55 °C for 1 minute, and 72 °C for 2 minutes, and a final extension at 72° C for 7 minutes. Each PCR run included HPV16-positive and DNA-negative controls. PCR products were initially fractionated using 1.5–2% agarose gels stained with ethidium bromide then purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA) prior to sequence analysis.

HPV16 variant status of the samples was determined by bidirectional PCR-based fluorescent dideoxy chain termination sequencing of the *E6* ORF (performed by the core facility at the Oklahoma Medical Research Foundation, Oklahoma City, OK) using the same primers used for initial amplification. HPV16 from CaSki and SiHa cell lines (23) was also sequenced to verify the accuracy of our results. Sequence alignments were performed using CLUSTALW (24). Only nucleotide changes verified as occurring on both strands were accepted. A variant category was assigned for each case relative to the prototype nucleotide sequence (5) which has been subsequently modified as HPV16R (25), as well as published HPV16 *E6* sequence patterns that define the different variants (6–8). European categories included EP and EV patterns based on a small number of nucleotide differences, while the NE categories included AA, NA-1, AF-1 and AF-2. The As variant which differs from the EP by one nucleotide (178 T→G) (6) was grouped with EV. Diagnostic category, age, stated race and number of HPV types were also recorded for each patient.

Twenty-three cases (6%) failed to amplify using the *E6* primers. The PCR products from each of the remaining 362 cases were purified and submitted for sequencing. Eight cases yielded sequences that could not be aligned using the HPV16 *E6* reference sequence. The remaining 354 cases (92%) form the population for this study. All data, including diagnoses, were collected in a blinded fashion so that the molecular analysis and patient information were accrued independently and without knowledge of the other results.

Statistical analyses, including tabular analysis using chi square, Armitage tests for trend, one way analysis of variance (ANOVA) with planned comparisons for linear trend, and logistic regression analysis, were performed using NCSS 2007 (Kaysville, UT). P-values ≤ 0.05 were considered to indicate statistical significance.

Results

Population Characteristics

The population consisted of 354 HPV16-positive women (mean age= 31.1 years; range 15 – 82 years) of all diagnostic categories who had cervical cytology and/or histology performed as part of routine patient care. The population characteristics are shown in Table 1. The modal age group was 21–30 years containing 42.9% of the women. The population with data for race was largely white (71.9%), (reflecting the population demographics served by

the University of Oklahoma Health Sciences Center), with 14.7% African American, 7.6% Hispanic, 2.9% American Indian, 1.8% “other” and 1.1% Asian. Race data were missing on 76 (21.5%) of the 354 women. The race/ethnicity information was unknown primarily in women in all categories of intraepithelial lesions who were seen on an outpatient basis only, while the data were most complete (97.6%) for women with cancers who were treated as inpatients. While there is no reason to believe that there is a difference in the women in each diagnostic category with known race/ethnicity from those in which it is unknown, we cannot exclude this possibility.

Because of referral patterns and demographic influences on the incidence of cancer in Oklahoma, cancer cases in this study population tended to be poorly-screened rural white women who were referred to our institution for cancer treatment from a large part of the state. The women with intraepithelial lesions, part of a variably screened population, were largely from the Oklahoma City metropolitan area. This group overlapped with the cancer population but included a more heterogeneous ethnic background. In general, the screened population at our institution is comprised of predominantly younger women who seek attention for pregnancy and contraception. Because of these variations, we performed preliminary statistics to evaluate our population. The mean ages for each diagnostic category differed significantly as follows: N/L = 24.1 ± 1.1 (SEM); HSIL-M = 27.4 ± 1.2; HSIL-S = 29.8 ± 1.0; cancer = 43.9 ± 1.1. There was a highly significant association between mean age and diagnostic category (p for trend < .001) indicating an increasing mean age with increasing severity of cytologic diagnosis. We also found a significant association between race and diagnosis using the 278 subjects whose race was known (p < .001). This indicated that black women in our population were at greater risk. For these reasons we controlled for age and race in the analysis.

Variant Analysis

The distribution of HPV16 *E6* sequence variants according to diagnostic category in our population is shown in Table 2. European categories (EP and EV) accounted for 303 (86%) women in this study. The reference sequence, European prototype (EP), the single most frequent HPV16 *E6* sequence identified over all, was found in 151 (43%) of women. European sequences showing minor nucleotide differences from EP, i.e. EV, also accounted for 43% of subjects. The 350T→G (350G) variant was identified in 73 (21%) women in the European category, while 47 (13%) other European cases had the 350G nucleotide variation with one or more additional nucleotide changes. Thus, a total of 120 (34%) of European cases showed the 350G nucleotide variation, while the prototype EP pattern (350T) was more common in our population. Notably, the same 350G nucleotide variation is also a characteristic finding in all AA and NA-1 sequences. As a result, the 350T→G pattern was found in 159 or 45% of the total population. Only seven cases (2%) showed the As pattern which is included in the EV category. Interestingly, all of the samples with As variants were diagnosed with HSIL-S or cancer.

Overall, NE variants represented a minority of cases (51 or 14%) and the AA-variant was the most common of these (31 or 9% overall). The AA cases represented 31 (61%) of 51 NE variant cases in this study. Interestingly, 8 cases (2% overall and 16% of NE) harbored uncommonly reported NA-1 variants (6) which included 3 subjects with squamous carcinoma, 2 with AIS, 2 HSIL-S and 1 with HSIL-S+ AIS).

Trend comparisons of EV or NE to EP were significant, suggesting that EP variants pose the lowest risk of the variant groups. Compared with EP, NE variants were enriched in the cancers relative to less severe diagnoses (p for trend ≤ .01). A similar pattern was present when EP and EV cases were compared; the proportion of EV increased with the severity of diagnosis relative to EP (p for trend =.03).

When considering the individual sequences, EP (E-350T) was the single most common DNA sequence in all diagnostic categories including cancers (n=32) although the largest number of cancer cases had EV sequences (n=36), a heterogeneous category with respect to nucleotide changes (Table 2). The E-350G pattern (n=16) was less common in cancers than EP (n=32) even when cases with this sequence and additional nucleotide changes were considered (n=11). We found no association between E-350G and increasing diagnostic category (p for trend =.32) compared with E-350T. Other than the E-350G variant, the EV category showed a variety of nucleotide changes with no consistent pattern.

The logistic regression model for the relationship of HPV16 variants to diagnostic category is shown in Table 3. We used this model to compare women with cancer or HSIL-S with a control population composed of women with cytologic diagnoses of N/L. HSIL-M cases were excluded in this model because they represent an equivocal intermediate diagnosis and possibly a heterogeneous group of acute infections and incipient HSIL-S. Compared to EP, unadjusted logistic regression analysis showed a significant association for HPV 16 *E6* EV (odds ratio [95% confidence interval] =2.18 [1.12 –4.26]) and NE (odds ratio =2.67 [1.06 – 10.66]) variant categories with risk for carcinoma and its immediate precursor. When adjusted for age and race/ethnicity, these results confirmed the increased risk for carcinoma and immediate precursor lesions in women with NE variants (odds ratio = 3.76 [1.32 – 10.66]) although this was not the case for the EV category. Only a non-significant increased risk of carcinoma was found when comparing European variants to European prototypes (odds ratio =1.55 [0.68 – 3.55]). Age was significantly associated with increased risk of HSIL-S/Cancer. African American race was also associated with increased risk for HSIL-S/Cancer but this association did not reach statistical significance. However, given the small number (11.6%) of African American women in this population, this finding is intriguing and merits further study.

Discussion

HPV variants are defined by a small number of nucleotide differences in the primary DNA sequence when compared with the original reference. For HPV16, the original reference sequence (EP) was isolated from a cervical cancer in a German woman (5). Although generally identified as the prototype sequence, this sequence was simply the first identified and the designation as prototype does not imply a particular biological importance. A number of other HPV16 sequences with specific patterns have been identified that have a geographical association (7,9). For example, EP and related variants predominate in European and related populations, while there is increased prevalence of AA in Latin America and Asia and AF-variants in African populations. While the US population is quite diverse, HPV16 European sequences have dominated most variant analyses (6–8,12,19,26). Given that the prevalence of cervical cancer varies in different regions and countries, a number of studies have addressed the possible association of HPV16 variant status with different risks for progression to malignancy. This interest was based on the association of amino acid differences with some DNA sequences, suggesting the possibility that tertiary structure changes in viral proteins or alterations in control elements in the LCR can affect the biological activity or immunologic characteristics of associated lesions.

While there have been conflicting reports of the biological potential of European variants (11,15,16,27–31), a number of studies have shown consistent evidence of increased association of HPV 16 NE variants and increasingly severe cervical neoplasia (10,12,13,18,26) compared with the European categories. In the US, Xi et al (26) demonstrated strong evidence of increased association of NE variants, particularly AA, with CIN3 using the ALTS population (26) that was derived from four different centers in the US. (Our institution was one of the institutions that contributed subjects to the ALTS

population. However, the cases reported here were accrued after closure of the ALTS trial.) Our current data support this finding and add a population of invasive cervical cancers to the spectrum of cervical lesions. Thus the association of NE variants, particularly AA, with increasingly severe cervical neoplasia in the US involves not only progression to CIN3 but extends to invasive cancer as well. It is interesting to note that the eight cases of NA-1 variant reported here were invasive cancers, HSIL-S or AIS. NA-1 is closely related to the AA variant, with the *E6* sequence differing only at nt532. The AA variants show an A→G change while the NA-1 has the prototype A-residue at this location. Although the number of cases is small, these data and the similarity of the NA-1 sequence to that of AA, suggest that NA-1 carries a similar risk as AA. HPV16 AF variants have also been reported to have increased risk for cancer in some (32,29) but not all (33) reports. The HPV16 AF-2 variant (26) has been reported to have increased biological risk for progression in the US. However, as there were few cases harboring AF variants in our population, our data do not address this question.

Although our data confirm the increased association of NE variants with high grade cervical lesions, in fact, most of our cancer cases harbored EP or EV sequences, as has been reported by others in US (7,11,19) and European populations (7,26,34,35). The most frequent HPV16 *E6* pattern reported to date in cancers from China (36,37) and Japan (38) is the As variant that is closely related to EP with a wide prevalence in Asia. Those populations reporting large numbers of women with cancers harboring AA variants, such as Mexico (18) and other areas of Central and South America (7) in fact have high prevalence of AA variants overall (39). A unique sequence variation related to AA has been reported in cancers from Indonesia (35). Similarly, there is a report of increased association of HPV16 AF variants in cancers studied from Africa (8). In sum, these findings suggest that all HPV16 variants carry some level of risk for progression to invasion and that, in general, the association of the individual variants in a cancer population likely reflects to some extent the prevalence of variants in that population as a whole. This can also explain the variability in the differing reports of risk for the various E-variants. For example, some studies (15,17,18,28,40) found that E-350G was the dominant E-sequence in cancers while others (11,26,31,33), like ours, found little difference in the distribution of the E-350 patterns. The implications of this conclusion would be that HPV16 *E6* variant analysis of cases of intraepithelial lesions, with the hopes of detecting cases with high and low risk for progression (14), may be difficult at this time. At the current level of understanding, there does not appear to be reason to treat a lesion with one HPV16 variant differently than another, since all variants appear to have the capacity to progress. Similarly, the fact that all major HPV16 *E6* categories are found in cancers suggests that variances in the HPV16 *E6* sequence alone are probably not key to the progression from intraepithelial lesion to invasion.

This proposal does not negate the aggregate evidence that NE variants, particularly AA, have an increased risk for persistence and progression to cancer. The reason for this difference is not clear and may relate to differences in the DNA sequence related to other regions of the genome (41). For example, HPV16 AA and AF variants characteristically show additional sequence differences in other parts of the HPV16 genome (28,35) that may contain the key to the increased association with progression to cancer. Thus, while HPV16 *E6* sequence alterations alone do not appear to account for the differences in biological behavior, the enhanced risk for progression to carcinoma associated with NE-variants remains an interesting biological question.

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Abbreviations

HPV	human papillomavirus
ORF	open reading frame
LCR	long control region
N/L	negative for epithelial lesion/ low grade intraepithelial lesion (CIN1)
SUCCEED	Study to Understand Cervical Cancer Early Endpoints and Determinants
HSIL	High grade squamous intraepithelial lesion
HSIL-M	Moderate dysplasia/CIN2
HSIL-S	Severe dysplasia/CIN3
AIS	Adenocarcinoma in situ
EP	European prototype
EV	European sequence with small number of sequence differences from prototype
NE	non-European variants
AA	Asian-American variant
NA-1	North American variant
AF-1 and AF2	African variants
As	Asian variant

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Table 1

Distribution of Traits in HPV-16 Positive Oklahoman Women in this Population (N=354)

	Category	Number	Per Cent
DIAGNOSIS	Negative /LSIL	88	24.9
	HSIL-Moderate	74	20.9
	HSIL-Severe/AIS	107	30.2
	Cancer	85	24.0
AGE (YEARS)	≤20	65	18.4
	21–30	151	42.7
	31–40	60	16.9
	41–50	49	13.8
	≥51	27	7.6
	Unknown	2	0.6
RACE	Asian	3	0.9
	Black	41	11.6
	Caucasian	200	56.5
	Hispanic	21	5.9
	Native American	8	2.3
	Other	5	1.4
	Unknown	76	21.5

Table 2

Distribution of HPV16 Variants According to Diagnostic Category

VARIANT	NEG/LSIL N (%)*	HSIL-M N (%)*	HSIL-S N (%)*	Cancer N (%)*	TOTAL N (%)*
EUROPEAN					
EP (350T)	*47 (53)*	*33 (45)*	*39* (36)	*32* (38)	*151 (43)*
EV (Total)	*30* (34)	*36 (49)*	*50 (47)*	*36 (42)*	*152 (43)*
*350G***	*16* (18)	*21* (28)	*20* (19)	*16* (19)	*73* (21)
As (178G)	-	-	3 (2)	4 (5)	*7* (2)
131G	-	*1* (1)	-	*1* (1)	*2* -
212C	*1* (1)	-	*4* (3)	-	*5* (1)
137A	-	*1* (1)	*1* (1)	*1* (1)	*3* (1)
186G	*1* (1)	-	*2* (2)	-	*3* (1)
268G	*1* (1)	-	-	*1* (1)	*2* -
132A	-	*1*(1)	-	*1* (1)	*2* -
134C	-	-	*1* (1)	-	*1* -
154G	-	*1*(1)	-	-	*1* -
173T	-	*1*(1)	-	-	*1* -
185G	-	-	*1* (1)	-	*1* -
416T	-	-	*1* (1)	-	*1* -
310G	-	-	-	*1* (1)	*1* -
335T	-	-	*1* (1)	-	*1* -
534C	-	*1*(1)	-	-	*1*-
109C, 350G	*6* (7)	*4* (5)	*6* (6)	*2* (2)	*18* (5)
176A, 350G	-	-	-	*1* (1)	*1* -
176C, 350G	-	-	*1* (1)	-	*1* -
205T, 350G	-	*1*(1)	-	*1*(1)	*2* -
257G, 350G	-	-	-	*2* (2)	*2* -
113A, 278G	-	-	*1* (1)	-	*1* -
110G, 350G	-	-	*1* (1)	-	*1* -
256T, 350G	-	*1* (1)	-	-	*1* -
131G, 350G	*5* (6)	*3* (4)	*5* (5)	*2* (2)	*15* (4)

VARIANT	NEG/LSIL N (%)*	HSIL-M N (%)*	HSIL-S N (%)*	Cancer N (%)*	TOTAL N (%)*
131G, 182A, 350G	-	-	-	*1* (1)	*1* -
131G, 350G, 448A	-	-	*1* (1)	-	*1* -
310G, 350G	-	-	*1* (1)	-	*1* -
141G, 350G	-	-	-	*1* (1)	*1* -
315G, 350G	-	-	-	*1* (1)	*1* -
European (Total)	**77 (88)**	**69 (93)**	**89 (83)**	**68 (80)**	**303 (86)**
NON-EUROPEAN					
AA	*7* (8)	*3* (4)	*10* (9)	*11* (13)	*31* (9)
NA-1	*0*	*0* -	*5* (5)	*3* (4)	*8* (2)
AF-1	*2* (2)	*1* (1)	*3* (3)	*1* (1)	*7* (2)
AF-2	*2* (2)	*1* (1)	*0* -	*2* (2)	*5* (1)
Non-European (Total)	**11 (12)**	**5 (7)**	**18 (17)**	**17 (20)**	**51 (14)**
TOTAL CASES	**88**	**74**	**107**	**85**	**354**

*Percent of total cases in column; Less than 1% left blank

**The number represents the nucleotide location followed by the base change relative to the EP sequence (see refs 8,26).

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Table 3

Logistic regression model of the relationship of HPV 16 variants to diagnostic category (N=214)*.

Independent Variables	Odds Ratio (95% CI) Cancer/HSIL-S compared to N/L	
	Crude	**Adjusted**
Age in years		1.20 (1.13 –1.28)
Race/ethnicity		
White		1.00 (Referent)
African American		1.87 (0.62 – 5.60)
Hispanic		0.84 (0.23 – 3.07)
HPV 16 Variant		
16-European Prototype	1.00 (Referent)	1.00 (Referent)
16-European Variant	2.18 (1.12 – 4.26)	1.55 (0.68 – 3.55)
16-Non-European Variant	2.67 (1.06 – 6.71)	3.76 (1.32 –10.66)

* For this model, American Indian, Asian, Other and Unknown races were excluded