

## Diversifying Selection in Human Papillomavirus Type 16 Lineages Based on Complete Genome Analyses

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**Human papillomavirus type 16 (HPV16) is the primary etiological agent of cervical cancer, the second most common cancer in women worldwide. Complete genomes of 12 isolates representing the major lineages of HPV16 were cloned and sequenced from cervicovaginal cells. The sequence variations within the open reading frames (ORFs) and noncoding regions were identified and compared with the HPV16R reference sequence (50). This whole-genome approach gives us unprecedented precision in detailing sequence-level changes that are under selection on a whole-viral-genome scale. Of 7,908 base pair nucleotide positions, 313 (4.0%) were variable. Within the 2,452 amino acids (aa) comprising 8 ORFs, 243 (9.9%) amino acid positions were variable. In order to investigate the molecular evolution of HPV16 variants, maximum likelihood models of codon substitution were used to identify lineages and amino acid sites under selective pressure. Five codon sites in the E5 (aa 48, 65) and E6 (aa 10, 14, 83) ORFs were demonstrated to be under diversifying selective pressure. The E5 ORF had the overall highest nonsynonymous/synonymous substitution rate ( $\omega$ ) ratio ( $M3 = 0.7965$ ). The E2 gene had the next-highest  $\omega$  ratio ( $M3 = 0.5611$ ); however, no specific codons were under positive selection. These data indicate that the E6 and E5 ORFs are evolving under positive Darwinian selection and have done so in a relatively short time period. Whether response to selective pressure upon the E5 and E6 ORFs contributes to the biological success of HPV16, its specific biological niche, and/or its oncogenic potential remains to be established.**

Papillomaviruses are a heterogeneous group of DNA viruses with closed circular double-stranded DNA genomes of about 8 kb in size that contain three general regions. An upstream regulatory region (URR) contains sequences that control transcription and replication, an early region contains genes (e.g., E6, E7, E1, E2, E4, and E5) involved primarily in enzymatic activities, and a structural region produces the L1 capsid protein and L2, which facilitates packaging of the viral DNA. Human papillomaviruses (HPVs) are classified by the sequence similarity of their genomes. A cloned HPV genome whose L1 open reading frame (ORF) displays less than 90% similarity to previously designated types is defined as a novel type. To date, more than 90 different genotypes of the HPV have been fully characterized (21). Intratypic variants and subtypes are defined as HPVs that vary by less than 10% in their L1 DNA sequences (5, 21).

HPVs are causally involved in the etiology of cervical cancer and its precursor lesions (16, 17, 43, 49). Of the high-risk HPV types associated with cervical cancer, HPV16 is the most prevalent and is found in approximately half of all cancers (7, 49). Numerous variants of HPV16 have been identified in different

geographic locations and ethnic groups (35, 42, 53, 68). Although all HPV16 isolates are closely related, previous studies inferred five distinct phylogenetic branches among HPV16 variants: European (E), Asian (As), Asian-American (As-Am), African-1 (Af-1), and African-2 (Af-2), corresponding to the geographic locations from which the samples were obtained (12, 34). Subsequent studies by sequence analyses of the HPV16 variants in other genomic regions (e.g., E6, L2, and L1) expanded and complemented this phylogenetic hypothesis (69).

Although HPV16 variants are an important focus of phylogenetic studies and the molecular variants of E2, L2, L1, the URR, and especially the E6 region have been described in detail previously (28), covariation among different ORFs belonging to the same lineage or isolate have not been studied in great detail. HPV16 variants have demonstrable differences in biological properties *in vitro* which may be responsible, in part, for differences in pathogenicity, carcinogenic risk, and perhaps immunogenicity (28). Furthermore, HPV16 variants are associated with different cervical cancer risks (6, 32, 65, 67). Although the diversifying selection in the HPV16 E6 and E7 oncogenes has been described recently (20), the evolutionary basis of the entire genome, coevolutionary mechanisms among different HPV16 genes, and their underlying biological significance remain unknown. Obtaining whole-genome sequences representative of the major HPV16 variants allowed us to determine with certainty nucleotide and amino acid sequence changes that are of potential evolutionary importance.

Comparison of synonymous (silent;  $d_s$ ) and nonsynonymous

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TABLE 1. Clinical information on specimens

Sample	Age of patient (yr)	HPV16 class	Cervix pathology <sup>a</sup>	Human immunodeficiency virus infection status <sup>b</sup>	Study location or name	Length (bp)	GenBank accession no.
CR-1	37	As-Am	Invasive cancer	NT	Costa Rica	7,906	AF402678
CR-2	35	As-Am	ASCUS	NT	Costa Rica	7,906	AY686579
CR-3	47	As-Am	CIN grade 2	NT	Costa Rica	7,907	AY686582
CR-4	28	E	CIN grade 2	NT	Costa Rica	7,906	AY686580
CR-5	32	E	CIN grade 1	NT	Costa Rica	7,906	AY686581
CR-6	22	E	ASCUS	NT	Costa Rica	7,907	AY686583
CR-7	36	E	Normal	NT	Costa Rica	7,906	AY686584
HAPI-1	19	Af-1	CIN grade 1	NT	HAPI	7,908	AF472508
HAPI-2	27	Af-2	Normal	NT	HAPI	7,904	AF472509
WIHS-1	30	E (G131)	ASCUS	+	WIHS	7,904	AF536179
WIHS-2	24	Af-1	N/A	+	WIHS	7,906	AF536180
WIHS-3	45	E (As)	Normal	+	WIHS	7,905	AF534061

<sup>a</sup> ASCUS, atypical squamous cells of undetermined significance; N/A, not available.

<sup>b</sup> NT, not tested.

(amino acid-changing;  $d_N$ ) substitution rates in protein-coding genes provides an important means for investigating the forces of molecular evolution (72). The nonsynonymous/synonymous rate ratio ( $\omega = d_N/d_S$ ) measures selective pressure at the protein level. Nonsynonymous rates that are significantly higher than synonymous rates are accepted as evidence for molecular adaptation (71, 72). This criterion has been used to identify a number of genes under adaptive molecular evolution (45).

In this report, the complete nucleotide sequences of and codon variations within HPV16 genomes representing the five major lineages were determined. By examining the ratio of  $d_N$  to  $d_S$  substitutions per site, diversifying selection acting on all of the eight protein-coding regions was evaluated. In addition, complete genome sequences for these variants allow us to reconstruct their genealogical relationships with unprecedented precision.

MATERIALS AND METHODS

**Clinical specimens and sequencing.** Cervicovaginal samples were obtained from women participating in a number of different studies involving HPV genotyping in our laboratory, including a population-based study in Costa Rica (30), the Women’s Interagency HIV Study (WIHS) (52), and a study of the natural

history of HPV in young women (HAPI) (9, 33). Samples found to contain HPV16 DNA by MY09/MY11 PCR and dot blot analyses were further subclassified into HPV16 variant lineages by sequencing the URR and/or the E6 region from PCR products (32).

Sequence analysis of the URR revealed that HPV16 genomes were distributed into five previously defined lineages of HPV16 (69): As-Am, Af-1, Af-2, E, and As. Clinical information for each sample, together with the GenBank accession numbers of the HPV16 DNA sequences, are listed in Table 1. HPV16 whole genomes were amplified by overlapping PCR (58, 59). Two sets of primers for nested PCR were designed to amplify the entire genome in two overlapping fragments of 4,108 bp and 3,863 bp. Oligonucleotide primer sequences used in these studies are available from the authors. For overlapping PCR, an equal mixture of AmpliTaq Gold DNA polymerase (Perkin-Elmer Applied Biosystems, Foster City, CA) and Platinum Taq High Fidelity DNA polymerase (Invitrogen, Rockville, MD) were utilized as previously described (58, 59). The estimated rate of error using high-fidelity Taq polymerase is approximately  $1 \times 10^{-6}$  errors per bp (18, 47). PCR products were separated by electrophoresis in agarose gels, stained with ethidium bromide, and visualized under UV illumination. After confirmation of the appropriate product size, each PCR product was purified (QIAGEN gel extraction kit; QIAGEN, Valencia, CA), ligated into the pGEM-T Easy vector (Promega, Madison, WI), and sequenced on an ABI 3700 sequencer in the Einstein Sequencing Facility. Several additional primers were used to obtain supplemental sequences to clarify sequence ambiguities. Isolates within the same lineages were included to evaluate intralinesage variation. HPV16 nucleotide and amino acid positions were numbered according to the

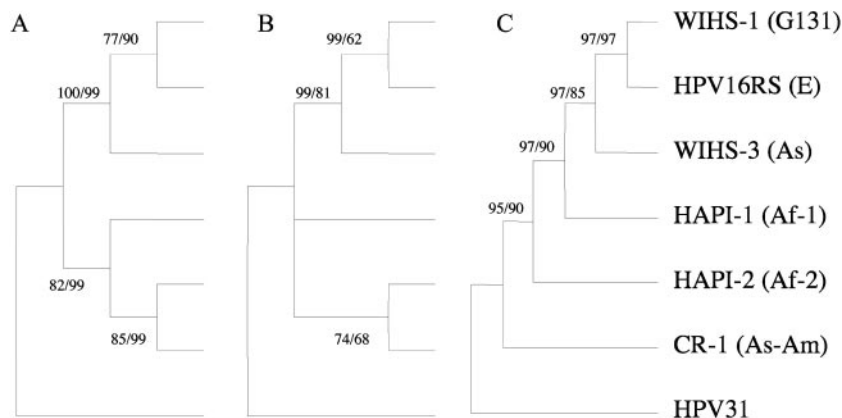


FIG. 1. Phylogenetic trees inferred from the complete genomes of representative HPV16 variants. Trees were generated using multiple algorithms. (A) Tree created using Bayesian construction and MP based on the nucleotide sequences of the eight ORFs and the URR. (B) Tree created using Bayesian construction and ML based on the nucleotide sequences of the eight ORFs and the URR. (C) Tree created using Bayesian construction and MP based on the amino acid sequences of the eight ORFs. The parsimony tree based on the concatenated amino acid and nucleotide sequences was similar to the tree shown in panel C (data not shown). The numbers on or near branches are support indices for the algorithms described for each tree.







TABLE 4. Comparison of nucleotide and amino acid sequence variability within HPV16 genomes

ORF or region	No. of nucleotide sequences	No. of variable nucleotide positions (%) <sup>a</sup>	Total no. of nucleotide variations (%) <sup>b</sup>	No. of amino acids	No. of variable codons (%)	No. of variable aa positions (%) <sup>c</sup>	No. of synonymous changes (%) <sup>d</sup>	Nonsynonymous/synonymous change ratio <sup>e</sup>
E6 <sup>f</sup>	456	15 (3.29)	16 (3.51)	151	12 (7.95)	5 (3.31)	7 (4.64)	0.71
E7	297	6 (2.02)	6 (2.02)	98	6 (6.12)	1 (1.02)	5 (5.10)	0.20
E1	1,950	55 (2.82)	56 (2.87)	649	55 (8.47)	21 (3.24)	34 (5.24)	0.62
E2	1,098	53 (4.83)	55 (5.01)	365	47 (12.88)	32 (8.77)	15 (4.11)	2.13
E4	288	18 (6.25)	19 (6.60)	95	17 (17.89)	4 (4.21)	13 (13.68)	0.31
E5	252	15 (5.95)	17 (6.75)	83	14 (16.87)	8 (9.64)	6 (7.23)	1.33
NCR	142	29 (20.42)	31 (21.83)					
L2	1,422	58 (4.08)	63 (4.43)	473	56 (11.84)	22 (4.65)	34 (7.19)	0.65
L1	1,596	36 (2.26)	37 (2.32)	531	36 (6.78)	12 (2.26)	24 (4.52)	0.50
URR	833	48 (5.76)	49 (5.88)					
Aggregate <sup>g</sup>	7,908	313 (3.96)	328 (4.15)	2,452	243 (9.91)	105 (4.29)	138 (5.63)	0.76

<sup>a</sup> Number and percentage of positions showing nucleotide variations among 13 HPV16 complete genomes.

<sup>b</sup> Number of nucleotide variations, including multiple changes per position.

<sup>c</sup> Number and percentage of changed amino acid positions per total number of amino acids/codon in each ORF (nonsynonymous changes).

<sup>d</sup> Number and percentage of synonymous/silent changes per total number of amino acids/codon in each ORF.

<sup>e</sup> Per codon.

<sup>f</sup> The first ATG start codon of the HPV16 E6 ORF is predicted to be at nt 104.

<sup>g</sup> Each nucleotide position is counted only once.

ported variants were also detected in our sequenced isolates. This is the first report to analyze taxa within an HPV type in the context of the complete genome.

There was no evidence for recombination between HPV16 variants. A deep branch was determined between non-European and European variants, which can be inferred from linked amino acid variations throughout the HPV16 genome; 21 such positions distributing to six HPV16 ORFs represent a primordial change in the non-European taxa ancestor (in bold-face in Table 3). Previous studies suggested that non-European isolates might confer a higher risk of developing high-grade cervical intraepithelial neoplasia (CIN)/cancer compared to so-called prototype-like (European) variants based on a limited set of nucleotide alterations (28). However, the array of common variants likely contains a complex set of biological changes related to a higher risk of cervical cancer. Further immunological and biochemical analyses focusing on these variable sites are needed.

**Diversifying selection within HPV16 oncogenes.** In this study, the nonsynonymous/synonymous rate ratio ( $\omega = d_N/d_S$ ) was used to determine whether positive selection impacts the evolution of HPV16 variants within different ORFs. The average  $d_N/d_S$  ratio of each HPV16 ORF was less than 1, indicating that all HPV16 ORFs are under purifying selection pressure. However, in agreement with a previous report (20), the E6 oncogene contains three codon sites that are evolving under the influence of diversifying selection (HPV16 E6 aa 10, 14, 83). HPV16 E6 aa 27 was also suggested to be under selective pressure (20), but this site was not identified in the present study. In addition, we demonstrate that E5 contains two sites under diversifying selection (HPV16 E5 aa 48 and 65), which suggests that HPV16 E6 and E5 have recently evolved through positive selection pressure.

Immune selection is likely to be a force driving the evolution of HPV16 genomes. The main immune response to HPV infection is cell mediated. The presentation of viral peptides to T cells in the context of human leukocyte antigen (HLA) class I and II molecules is influenced by genetic polymorphisms of

both HPV and HLA. These variations in the immune response to different viral isolates are influenced by the heterogeneity of the host immune response and influence the outcome of HPV infection. Recent suggested that the common E6 variant, L83V, is predominately associated with three distinct HLA class I alleles, namely, B\*44, B\*51, and B\*57, for the E6 epitope stretching from aa 74 to 83 (SEYRHYCYSL/V) (1, 11, 75). The immunologic relevance of the HPV16 E6 N-terminal region and variant positions E6 aa 10 and 14 is supported by the demonstration of an endogenously processed HLA A\*0201-restricted E6 peptide (KLPQLCTEL; E6 aa 11 to 19) as well as of an overlapping HLA B-7-restricted E6 peptide (RPRKLPQL; E6 aa 8 to 15) in this region (3, 23). In another study, the HPV16 E6 variant R10G was demonstrated to alter a B\*07 binding epitope such that it may influence immune recognition by cytotoxic T lymphocytes (23). Certain HLA class I alleles, in concert with specific HPV variants, could be associated with a predisposition for cervical cancer development, whereas others may be protective. The variable positions predicted to be under Darwinian selection (HPV16 E6 aa 10, 14, and 83) might thus be under immune selection.

Additional functional characteristics of the sites under selection may be related to modification of E6 protein-protein interaction domains. A substantial proportion of the changes found in HPV16 E6 were located in the amino half of the protein, which represents the binding region of the cellular protein E6-AP and has a role in both cell-mediated and humoral host immune responses (26, 40, 41). Moreover, a recent report indicates that variations in codons 14 and 27 substantially altered the E6 antigenic index and may affect interactions with E6-AP (66). This provides biochemical support for selection at HPV16 E6 aa 14. In addition, the cellular tumor suppressor protein p53, usually targeted and degraded by HPV E6, is polymorphic in human populations and varies according to latitude and ethnicity (4). A p53 polymorphism at codon 72, exon 4, resulting in either a proline (Pro) or an arginine (Arg), has been identified as a possible risk factor for the development of cervical carcinoma (55). A few studies have suggested

TABLE 5. Phylogenetic analysis by ML estimation for HPV16 genes

ORF	Model	Log likelihood	$d_N/d_S^a$	Parameters
E6	M0 (one-ratio)	-750.939522	0.4125	$\omega = 0.4125$
	M1 (neutral)	-744.238187	0.1477	$p_0 = 0.85229 p_1 = 0.14771$
	M2 (selection)	-739.714214	0.4073	$p_0 = 0.94403 p_1 = 0.00000 p_2 = 0.05597 \omega_2 = 7.27647$
	M3 (discrete)	-739.714190	0.4073	$p_0 = 0.12144 p_1 = 0.82259 p_2 = 0.05597 \omega_0 = 0.00001 \omega_1 = 0.00001 \omega_2 = 7.27677$
	M7 (beta)	-744.540127	0.1000	$p = 0.00195 q = 0.02108$
	M8 (beta and $\omega$ )	-739.714078	<b>0.4073</b>	$p = 0.00100 q = 1.54547 p_0 = 0.94403 (p_1 = 0.05597) \omega = 7.27644$
E7	M0 (one-ratio)	-442.311583	0.1736	$\omega = 0.1736$
	M1 (neutral)	-440.244427	0.0763	$p_0 = 0.92366 p_1 = 0.07634$
	M2 (selection)	-438.921563	<b>0.1577</b>	$p_0 = 0.98457 p_1 = 0.0000 p_2 = 0.01543 \omega_2 = 10.21907$
	M3 (discrete)	-438.921757	0.1577	$p_0 = 0.01594 p_1 = 0.96863 p_2 = 0.01543 \omega_0 = 0.00001 \omega_1 = 0.00001 \omega_2 = 10.21925$
	M7 (beta)	-440.277261	0.1000	$p = 0.00165 q = 0.02011$
	M8 (beta and $\omega$ )	-438.921563	0.1577	$p = 0.00100 q = 1.47425 p_0 = 0.98457 (p_1 = 0.01543) \psi = 10.21900$
E1	M0 (one-ratio)	-2937.078929	0.1538	$\omega = 0.1538$
	M1 (neutral)	-2935.147538	0.1613	$p_0 = 0.83868 p_1 = 0.16132$
	M2 (selection)	-2935.131457	0.1577	$p_0 = 0.82138 p_1 = 0.00000 p_2 = 0.17862 \omega_2 = 0.88286$
	M3 (discrete)	-2935.130917	<b>0.1577</b>	$p_0 = 0.49174 p_1 = 0.32967 p_2 = 0.17860 \omega_0 = 0.00001 \omega_1 = 0.00001 \omega_2 = 0.88293$
	M7 (beta)	-2935.139255	0.1573	$p = 0.01252 q = 0.06910$
	M8 (beta and $\omega$ )	-2935.131324	0.1577	$p = 0.00294 q = 1.19859 p_0 = 0.82138 (p_1 = 0.17862) \omega = 0.88284$
E2	M0 (one-ratio)	-1876.140617	0.5218	$\omega = 0.5218$
	M1 (neutral)	-1872.769407	0.4232	$p_0 = 0.57684 p_1 = 0.42316$
	M2 (selection)	-1871.302950	<b>0.5611</b>	$p_0 = 0.75842 p_1 = 0.000 p_2 = 0.24158 \omega_2 = 2.32256$
	M3 (discrete)	-1871.303083	0.5611	$p_0 = 0.56211 p_1 = 0.19631 p_2 = 0.24158 \omega_0 = 0.00001 \omega_1 = 0.00001 \omega_2 = 2.32256$
	M7 (beta)	-1873.007264	0.5000	$p = 0.00450 q = 0.00441$
	M8 (beta and $\omega$ )	-1871.302950	0.5611	$p = 0.00100 q = 1.68952 p_0 = 0.75842 (p_2 = 0.24158) \omega = 2.32255$
E4	M0 (one-ratio)	-499.426327	0.1601	$\omega = 0.1601$
	M1 (neutral)	-498.066176	0.1510	$p_0 = 0.84896 p_1 = 0.15104$
	M2 (selection)	-498.010196	<b>0.1658</b>	$p_0 = 0.88391 p_1 = 0.0000 p_2 = 0.11609 \omega_2 = 1.42792$
	M3 (discrete)	-498.010222	0.1658	$p_0 = 0.28572 p_1 = 0.59821 p_2 = 0.11607 \omega_0 = 0.00001 \omega_1 = 0.00001 \omega_2 = 1.42799$
	M7 (beta)	-498.153076	0.1588	$p = 0.01166 q = 0.06421$
	M8 (beta and $\omega$ )	-498.036494	0.1607	$p = 0.00213 q = 0.01934 p_0 = 0.95917 (p_1 = 0.04083) \omega = 1.58742$
E5	M0 (one-ratio)	-435.001494	0.6705	$\omega = 0.6705$
	M1 (neutral)	-432.604998	0.3664	$p_0 = 0.63363 p_1 = 0.36637$
	M2 (selection)	-429.272907	0.8071	$p_0 = 0.59008 p_1 = 0.37250 p_2 = 0.03743 \omega_2 = 11.61198$
	M3 (discrete)	-429.254476	0.7965	$p_0 = 0.26115 p_1 = 0.69348 p_2 = 0.04537 \omega_0 = 0.32659 \omega_1 = 0.32660 \omega_2 = 10.68596$
	M7 (beta)	-432.625891	0.4000	$p = 0.00201 q = 0.00326$
	M8 (beta and $\omega$ )	-429.254456	<b>0.7966</b>	$p = 48.11157 q = 99.00000 p_0 = 0.95469 (p_1 = 0.04531) \omega = 10.69102$
L2	M0 (one-ratio)	-2270.264783	0.1330	$\omega = 0.1330$
	M1 (neutral)	-2265.762403	0.1433	$p_0 = 0.85669 p_1 = 0.14331$
	M2 (selection)	-2265.545031	0.1315	$p_0 = 0.00000 p_1 = 0.10056 p_2 = 0.89944 \omega_2 = 0.03446$
	M3 (discrete)	-2265.081776	<b>0.1406</b>	$p_0 = 0.55727 p_1 = 0.42184 p_2 = 0.02089 \omega_0 = 0.08231 \omega_1 = 0.08232 \omega_2 = 2.87503$
	M7 (beta)	-2265.639112	0.1330	$p = 0.03159 q = 0.20124$
	M8 (beta and $\omega$ )	-2265.548829	0.1326	$p = 0.00667 q = 0.01316 p_0 = 0.33537 (p_1 = 0.66463) \omega = 0.04454$
L1	M0 (one-ratio)	-2392.492442	0.1308	$\omega = 0.1308$
	M1 (neutral)	-2391.381730	0.1350	$p_0 = 0.86500 p_1 = 0.13500$
	M2 (selection)	-2391.376118	<b>0.1330</b>	$p_0 = 0.85231 p_1 = 0.00000 p_2 = 0.14769 \omega_2 = 0.90078$
	M3 (discrete)	-2391.376120	0.1330	$p_0 = 0.68619 p_1 = 0.16614 p_2 = 0.14766 \omega_0 = 0.00001 \omega_2 = 0.00001 \omega_2 = 0.90089$
	M7 (beta)	-2391.392774	0.1317	$p = 0.01192 q = 0.07147$
	M8 (beta and $\omega$ )	-2391.376158	0.1330	$p = 0.00186 q = 2.45210 p_0 = 0.85232 (p_1 = 0.14768) \omega = 0.90083$

<sup>a</sup> The  $d_N/d_S$  ratios in boldface indicate the "best" models interpreted from the maximum log likelihood value.

that the combination of HPV16 E6 aa L83 and the p53 Arg/Arg genotype might represent an interactive risk factor for cervical cancer (8, 63). Therefore, selection pressure driven by protein-protein polymorphic interactions may facilitate the life cycle and pathogenicity of HPV-associated disease.

HPV16 E5 is a highly hydrophobic membrane-bound protein of 83 amino acids associated with the Golgi apparatus, endoplasmic reticulum, and nuclear membrane in infected cells. Recently, the E5 protein has been suspected of acting to alter levels of cell-cycle regulators and to play a role during the productive/early stage of the HPV16 life cycle (25, 27). Previ-

ous studies have indicated that amino acid changes within HPV16 E5 might alter the transforming activity of the protein by affecting the interactions with the epidermal growth factor receptor, the 16-kDa subunit of H<sup>+</sup>-ATPase and, potentially, other cellular proteins (19, 56). An amino acid change, L48V, was noted between HPV16 E5 amino acids 46 and 50, a stretch of hydrophobic residues potentially representing a transmembrane domain. HPV16 E5 protein also stimulates the nuclear oncogenes c-jun, junB, and c-fos. E5 induction of c-jun is through an activator protein-1 binding site, and E5-activation of c-fos is via nuclear factor-1 (NF-1) binding sites. Therefore,

TABLE 6. Likelihood ratio tests for positive selection of amino acid sites

ORF	Model 1	Model 2	LRT statistic <sup>a</sup>	$d_N/d_S > 1$ detected?	Positively selected codons <sup>b</sup>
E6	M1	M2	<b>9.047946</b>	Yes	10 R 1.0000** 14 Q 1.0000** 83 L 1.0000**
	M1	M3	<b>9.047794</b>	Yes	10 R 1.0000** 14 Q 1.0000** 83 L 1.0000**
	M7	M8	<b>9.652098</b>	Yes	10 R 1.0000** 14 Q 1.0000** 83 L 1.0000**
E7	M1	M2	2.647728	Yes	None
	M1	M3	2.645340	Yes	None
	M7	M8	2.711396	Yes	None
E1	M1	M2	0.032162	No	None
	M1	M3	0.033242	No	None
	M7	M8	0.015862	No	None
E2	M1	M2	2.932914	Yes	None
	M1	M3	2.932648	Yes	None
	M7	M8	3.408628	Yes	None
E4	M1	M2	0.111960	Yes	None
	M1	M3	0.111908	Yes	None
	M7	M8	0.233164	No	None
E5	M1	M2	<b>6.664182</b>	Yes	48 L 0.6823 65 I 0.9945**
	M1	M3	<b>6.701044</b>	Yes	48 L 0.8923 65 I 0.9998**
	M7	M8	<b>6.742870</b>	Yes	48 L 0.8909 65 I 0.9998**
L2	M1	M2	0.434744	No	None
	M1	M3	1.361254	Yes	None
	M7	M8	0.180566	No	None
L1	M1	M2	0.011224	No	None
	M1	M3	0.011220	No	None
	M7	M8	0.033232	No	None

<sup>a</sup> LRT statistics follow a  $\chi^2$  distribution with degrees of freedom equaling 2 when values were  $>5.99$  and  $P$  values were  $<0.05$  (in boldface).

<sup>b</sup> \*\*, positively selected sites with posterior probability ( $P$ ) values of  $>0.99$  (2).

E5 may influence HPV gene expression via the activation of activator protein-1 and NF-1 (13, 14). Since several binding sites of transcription factors (e.g., NF-1) located in the HPV URR are variable, selective pressure driven by the ability of HPV16 E5 protein to active viral gene expression via these transcription factors is possible. Although mutation analyses indicated that the transforming activity of mutant E5 proteins was similar to that of wild-type E5 (37), the oncogenicity of specific HPV variants may vary geographically, possibly due to genetic differences between populations. Since the biochemical and biological functions of HPV16 E5 protein are not well characterized, efforts are still needed to decipher the complicated network existing between E6, E7, E5, and other cellular proteins.

**Diversifying selection within the HPV16 E2 gene.** It is worth noting that the E2 gene might be under selection pressure, since it has a relatively high  $\omega$  ratio (Table 5). The HPV16 E2 protein is involved in gene-expression regulation and replication of the HPV genome and appears to be the target of both humoral and cellular immune responses (22, 44). Several humoral epitopes are known in the E2 protein, one in the trans-activation domain (aa 121 to 140), three in the hinge region (aa 181 to 200, 241 to 260, and 271 to 290), and one in the DNA binding domain (aa 328 to 346) (22). In this work, all but one (i.e., aa 181 to 200) of these epitopes had changes in the E2

proteins of non-European variants, suggesting the potential for different humoral responses to HPVs of the European and non-European taxa (Table 3). Since the disruption of the E2 gene during viral integration contributes to tumor progression, the variant HPV16 E2 proteins of non-European variants (e.g., the As-Am isolate) may be better adapted for deregulating the expression of viral oncogenes (10). Hence, the high level of diversity among HPV16 E6 and E5 proteins may be a potential force driving the evolutionary selection of the E2 gene. For instance, HPV16 E2 variants frequently cosegregated with the E6 T350G (L83V) variant that has been associated with viral persistence (10). This cosegregation was proposed to act as an additional risk factor for the development of cervical cancer (46, 60). Another reason that the HPV16 E2 gene contained a relatively high level of nonsynonymous substitutions might be explained by the evolutionary constraint imposed upon it from the overlapping gene, HPV16 E4. Accordingly, synonymous substitutions of the E2 gene might be constrained by the relatively conserved E4 gene (39, 48). However, no specific sites were detected by the LRTs. Yang and Swanson (74) suggest that these tests are highly prone to sampling bias and that more variants of HPV16 E2 gene should therefore be included in future molecular evolutionary analyses.

This study provides new data demonstrating lineage fixation of variants in complete HPV16 genomes. Analysis of a representative set of HPV16 genomes identified diversifying sites under selective pressure within the HPV16 E6 and E5 ORFs. These HPV16 proteins are known to have transforming activities. Nevertheless, more effort is needed to examine the evolutionary dynamics of HPV genomes, especially those associated with high-risk HPV types, in order to understand the genetic basis of biological success and niche adaptation that probably indirectly lead to cancer causation.

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