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Evidence of Diversifying Selection in Human Papillomavirus Type 16 E6 But Not E7 Oncogenes

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Abstract. Human papillomavirus type 16 is a common sexually transmitted pathogen capable of giving rise to cervical intraepithelial neoplasia and invasive carcinoma through the expression and activity of two adjacent oncogenes: E6 and E7. Naturally occurring amino acid variation is commonly observed in the E6 protein but to a much lesser extent in E7. In order to investigate the evolutionary mechanisms involved in the generation and maintenance of this variation, we examine 42 distinct E6-E7 haplotypes using codon-based genealogical techniques. These techniques involve estimation of the ratio of nonsynonymous to synonymous substitutions (d_n/d_s) and allow testing for directional (positive) natural selection. Positive selection was detected for four codon sites within the E6 oncogene but not in any E7 codons. The amino acid compositions and locations of selected sites are described. Possible sources of natural selection including antiviral immune pressure and polymorphism of host cellular proteins are discussed.

Key words: Adaptive evolution — Diversifying selection — DNA virus — Codon substitution model — Phylogeny

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Introduction and Background

Human papillomavirus type 16 (HPV16) is a double stranded DNA virus (genus *Papillomavirus*, family *Papovaviridae*) that codes for eight ORFs including two oncogenes: E6 and E7. The virus is the main etiologic agent of cervical cancer, being found in approximately 60% of such tumors (Bosch et al. 1995). Cervical cancer is the second most common malignancy among women, responsible for 500,000 new cases and 200,000 deaths annually (Syrjänen and Syrjänen 2000).

HPV16 replication primarily takes place in the stratified squamous epithelium and virus maturation is intimately linked to the differentiation path of developing keratinocytes. Replication begins with cellular induced transcription of E6 and E7 in basal cells. The proteins are responsible for alteration of the normal cell cycle and inhibition of differentiation making viral DNA replication possible. E6 facilitates degradation of the cellular tumor suppressor protein p53 via the ubiquitin pathway. This degradation requires complex of the E6 protein with p53 and cellular E6-associated protein (E6-AP) (Huibregtse et al. 1991). Direct p53 binding by E6 inhibits p53-mediated transcriptional repression (Lechner et al. 1992) and activation (Pim et al. 1994). E7 is responsible for binding to and inactivating retinoblastoma protein p105RB. This releases cellular transcription factor E2F, thought to be involved in cell proliferation. During typical HPV16 replication, transcription of E6 and E7 is downregulated by the viral E2 protein.

This leads to the resumption of p53 and p105RB function and subsequent normal cellular differentiation and replication.

Overexpression of E6 and/or E7 may lead to permanent inactivation of tumor suppressor proteins that ultimately results in cellular transformation and neoplasia (Hawley-Nelson et al. 1989, Münger et al. 1989, Lambert et al. 1993). This is most often brought about by integration of viral DNA into the host cell genome followed by unregulated, constitutive expression (Schwarz et al. 1985, Smotkin et al. 1986, Baker et al. 1987).

The E6 protein is composed of 158 amino acids, with a molecular mass of 18 kDa. It contains four Cys-X-X-Cys motifs that give rise to two well-conserved zinc fingers. The protein is known to perform additional functions and to interact with other cellular factors including paxillin (Tong et al. 1997) and hD1g/SAP97 (Lee et al. 1997), as well as participate in the upregulation of telomerase (Klingelhutz et al. 1996). The E7 protein is an acidic 21 kDa phosphorylated protein composed of 98 amino acids. It contains two Cys-X-X-Cys motifs forming one zinc finger. The E7 protein interacts with other pRB-related proteins (e.g. p107, p130) and with cellular factors involved in transcriptional control (e.g. AP-1, TBP) (see Zwerschke and Jansen-Dürr 2000).

Papillomaviruses rely on cellular DNA replication machinery and this results in slow synonymous substitution rates near 10^{-8}/site/year. Yet, naturally-occurring HPV16 polymorphism is demonstrable and has been the focus of numerous studies (Chan et al. 1992, Eriksson et al. 1999, Ho et al. 1991, Wheeler et al. 1997, Yamada et al. 1997). Amino acid variation in E6 and E7 has been examined in the context of clinical manifestations of infection, epidemiology, and host genetic makeup (Tornesello et al. 1997, Bontkes et al. 1998, Zehbe et al. 1998, Brady et al. 1999, van Duin et al. 2000, Berumen et al. 2001, Hu et al. 2001). At both the nucleotide and amino acid levels, E6 genes are considerably more variable than haplotypic E7 genes, although they are genomically adjacent and both encode nonstructural proteins required for viral replication (Tornesello et al. 1997, van Duin et al. 2000). HPV16 genetic diversity often entails variation in the biological properties of the virus (Stoppler et al. 1996), which is likely to result in differences in pathogenicity, carcinomic risk, and perhaps immunogenicity, especially with respect to the E6 and E7 genes (Xi et al. 1997, Yamada et al. 1997). The evolutionary basis of this variation has never been examined, however, and its underlying biological significance remains unknown.

We have investigated E6 and E7 with an emphasis on the role of natural selection using 42 different haplotypes. Diversifying selection acting on a protein-coding region can be estimated by examining the ratio of nonsynonymous (d_n) to synonymous substitutions (d_s) per site (Hughes and Nei 1988, 1989). We employed genealogical, codon-based models developed by Nielsen and Yang (1998) and Yang et al. (2000) that incorporate maximum likelihood estimates of d_n and d_s.

Materials and Methods

E6 and E7 Haplotype Sequences

In the summer of 1998 cervical swabs were collected as part of an epidemiological study in Oaxaca, Mexico. Dacron-tipped swabs containing exfoliated cervical cells were stored in 800 μL of specimen transport medium (Digene, Beltsville, MD). DNA was then extracted using QIAmp tissue extraction kits (Qiagen, Valencia, CA) according to the manufacturer's protocol. The PCR amplification of E6 was as described by Alvarez-Salas et al. (1995); the amplification of E7 was as described by Fujinaga et al. (1994). E6 and E7 PCR products were sequenced directly using the dye dideoxy-termination method on an ABI 377 DNA sequencer (PE Applied Biosystems, Foster City, CA). The primers for sequencing reactions were the same used for the PCR amplifications. Both strands were sequenced and the five newly obtained sequences have been deposited in GenBank under accession numbers AY089951–AY089955.

Thirty-seven of the E6-E7 DNA sequences analyzed here are from Seedorf et al. (1985), Tornesello et al. (1997), Zehbe et al. (1998), and van Duin et al. (2000). We only use nonidentical E6–E7 haplotype sequences. The 42 total E6-E7 haplotypes were aligned by eye using GeneDoc version 2.5 (Nicholas and Nicholas 1997). There are no insertions/deletions. We used E6 codons 9–158 (corresponding to nucleotide numbers 110–556) and E7 codons 2–97 (corresponding to nucleotide numbers 565–855). Stop codons are not included in the analysis. Alignments are available from VRD upon request.

Phylogenies

Phylogenies were reconstructed separately for E6 and E7. We used both the neighbor-joining algorithm (Saitou and Nei 1986) as implemented by the program PAUP version 4.0b3 (Swofford 1999), and a maximum-likelihood algorithm (Felsenstein 1981) as implemented by the program FastDNAml with transition/transversion ratio set to 2. All available pairwise distance estimation methods were used for implementing the neighbor-joining algorithm. Likelihood scores for all trees were compared following Hasegawa et al. (1991) and the gene tree with the highest likelihood score was used in the analysis. For the E6 gene, this tree is generated using FastDNAml; for E7, this tree is generated using the neighbor-joining method with distance estimation performed under the general reversible model. Tree topologies are available from VRD upon request.

Analysis of Positive Selection

In order to determine whether positive selection impacts the evolution of E6 or E7, seven codon-substitution models were used. These models view the codon as the fundamental unit of evolutionary change and take into account genealogic history when calculating parameters. Log likelihood scores evaluate the quality of the fit of the input data to the conditions of the model. In these models, d_n/d_s was estimated for separate classes of codons that are assumed to evolve independently of one another.

Table 1. Codon-based models used (after Yang et al. 2000)

Model	Parameters	$d_n/d_s > 1$?
M0 (Invariant)	d_n/d_s	Yes
M1 (Neutral)	p_0	No
M2 (Selection)	p_0, p_1, d_n/d_s	Yes
M3 (Discrete)	p_0, p_1, … p_{K-2}; $d_n/d_s(0)$ $d_n/d_{s(1)}$,…$d_n/d_{s(K-1)}$	Yes
M5 (Gamma)	α, β	Yes
M7 (Beta)	p, q	No
MS (Beta&ω)	p_{beta}, p, q, d_n/d_s	Yes

$d_n/d_{s(i)}$ = Per site ratio of nonsynonymous substitutions to synonymous substitutions for site class i.
p_n = Frequency of codons falling into site class n.
α, β = Gamma distribution shape parameters.
p, q = Beta distribution shape parameters.
p_{beta} = Frequency of codons falling into beta 0, 1 site class

When $d_n/d_s = 0$ for a particular codon site, it is assumed that nonsynonymous (i.e. amino acid) substitutions are eliminated by natural selection. When $d_n/d_s = 1$ it is assumed that amino acid changes have approximately the same probability of fixation as synonymous substitutions, which are selectively neutral with respect to protein evolution, so that the site is not subject to natural selection. When $d_n/d_s > 1$ it is assumed that nonsynonymous substitutions have a greater probability of fixation than synonymous substitutions and the site is evolving under the influence of diversifying selection, so that amino acid changes are adaptive. When $0 < d_n/d_s < 1$, it is assumed that amino acid changes are slightly deleterious (nearly neutral) and their fate is determined by a mixture of selection, mutation, and drift.

These seven codon models estimate different sets of parameters using different assumptions (see Table 1). M0 assumes that all codons have the same d_n/d_s, which is averaged over all sites (see Goldman and Yang 1994). M1 assumes that all sites fall into one of two classes (see Nielsen and Yang 1998). The first class is at frequency p_0 and has $d_n/d_{s(0)} = 0$, (no amino acid variation is tolerated), and the second class is at frequency $p_1 = 1 - p_0$ and has $d_n/d_{s(1)} = 1$. Model M2 allows for an additional class of codons at frequency p_2 for which d_n/d_s may take any value between 0 and infinity. M3 separates all codons into K different site classes, so that for each class d_n/d_s can be between 0 and infinity and codons may fall into each class at any frequency (Yang et al. 2000). We started with $K = 3$ and increased the number of classes by one until the log likelihood did not improve. M5 assumes a discrete gamma distribution for d_n/d_s among sites in the interval $(0, \infty)$ with shape parameter α (Yang et al. 2000). In this model, d_n/d_s can also vary from 0 to infinity. M7 assumes a discrete beta distribution for d_n/d_s among sites in the interval (0, 1) with shape parameters p and q (Yang et al. 2000). This model only allows $0 \leq d_n/d_s \leq 1$. M8 is a more general version of M7 that allows for an additional class of sites with $d_n/d_s > 1$ and thus uses two site classes (Yang et al. 2000). Codons in site class 1 (at frequency p_0) are assumed to have beta-distributed d_n/d_s values between 0 and 1, and codons in site class 2 (at frequency $1 - p_0$) are assumed to have the same d_n/d_s that can vary between 0 and infinity. All models were implemented using the program CODEML in the PAML software package version 3.0c (Yang 2001).

In order to assess the influence of positive selection on a particular coding region, a likelihood ratio test (LRT) is performed to compare nested models (Nielsen and Yang 1998, Yang et al. 2000). All models involve estimates of genealogy branch lengths, transition/transversion rate ratio (κ), and nucleotide frequencies. Specific models estimate additional parameters such as d_n/d_s, site class frequencies, and distribution shape parameters. Some models are less general equivalents of others, since they estimate subsets of parameters (e.g. those that do not allow for positively selected sites are less general than those that do). In this case, twice the log likelihood difference between the two models follows a χ^2 distribution with degrees of freedom equal to the difference in the number of parameters estimated between the models. For instance, M2 and M3 are more general versions of M1. When d_n/d_s estimates are greater than 1 in the more general models and these have significantly better likelihood scores, the existence of positively selected sites is probable (i.e. a null hypothesis of no positively selected sites is rejected). Likewise, M7 is nested within M8. When $d_n/d_s > 1$ in M8, the LRT tests the alternative hypothesis of the presence of positively selected sites. Anisimova and colleagues (2001) have analyzed the effectiveness of this approach and found that the LRT is powerful when used under similar conditions including sequence length, number of taxa, and level of divergence.

Results

Table 2 shows the results of the likelihood analysis, including parameter estimates for different models. Table 3 gives the results of the positive selection LRTs comparing nested models within each gene. All tests for positively selected sites within E6 rejected a null hypothesis of no such sites. All tests for positively selected sites within E7 fail to reject a null hypothesis of no such sites. Results from M3 with $K = 3$ site classes are given since $K > 3$ did not improve the likelihood.

For E6, M3 with $K = 3$ gave the highest likelihood. This model indicates that approximately 3.22% of the E6 codon sites fall into a site class with $d_n/d_s \approx 9.8994$. Furthermore, both M5 and M8 indicate that positively selected sites are present in E6. Four codon sites were consistently shown to have $d_n/d_s > 1$. These are E6 codons 17, 21, 34, and 90 (see Figure 1). Table 4 displays the amino acids observed in those positions, their frequencies, and their biochemical characteristics. For E7, M8 gave the highest likelihood, but no sites with $d_n/d_s > 1$ were detected under any model. Additionally, the M8 likelihood score is not significantly better than for any other model.

In order to examine qualitative amino acid diversification at codon sites identified by PAML as likely to be under positive selection in E6, we employed the diversification method of Hughes et al. (1990). This method characterizes nonsynonymous substitutions

Table 2. Results of PAML estimation for E6 and E7 genes

Gene	Model	ln Likelihood	Parameter Estimates
E6	M0	−974.780024	$d_n/d_s = 0.5622$
	M1	−961.838218	$p_0 = 0.7312$
	M2	−950.996257	$p_0 = 0.6422$; $p_1 = 0.3279$; $d_n/d_s = 11.6461$
	M3 (K = 3)	−950.405998	$p_0 = 0.5989$; $p_1 = 0.3689$; $d_n/d_{s(0)} = 0.4295$; $d_n/d_{s(1)} = 0.001$; $d_n/d_{s(2)} = 9.8994$
	M5	−960.435008	$\alpha = 0.0574$; $\beta = 0.0133$
	M7	−962.219003	$p = 0.0178$; $q = 0.0376$
	M8	−950.425030	$p_{beta} = 0.9678$; $p = 0.6583$; $q = 1.6973$; $d_n/d_s = 9.8858$
E7	M0	−457.651984	$d_n/d_s = 0.2755$
	M1	−457.866279	$p_0 = 0.6788$
	M2	−457.648022	$p_0 = 0.0000$; $p_1 = 0.0000$; $\omega = 0.4011$
	M3 (K = 3)	−457.648066	$p_0 = 0.3296$; $p_1 = 0.3329$;. $d_n/d_{s(0)} = 0.2755$; $d_n/d_{s(1)} = 0.2755$; $d_n/d_{s(2)} = 0.2755$
	M5	−457.651936	$\alpha = 115.3644$; $\beta = 416.1659$
	M7	−457.653412	$p = 121.3345$; $q = 289.0995$
	M8	−457.649207	$p_{beta} = 0.9999$; $p = 47.9240$; $q = 125.6978$; $d_n/d_s = 0.2900$

Table 3. Results of likelihood ratio tests of positive selection

Gene	Model 1	Model 2	2 (Lik. Diff.)	*p* value	$d_n/d_s > 1$ Detected?	Positively Selected Codons
E6 (150 codons)	M1	M2	21.683922	0.00001956	Yes	17 [$P(d_n/d_s > 1) = 0.9997$] 21 [$P(d_n/d_s > 1) = 0.9969$] 34 [$P(d_n/d_s > 1) = 0.9341$] 90 [$P(d_n/d_s > 1) = 0.9380$]
	M1	M3	22.86444	0.00013477	Yes	17 [$P(d_n/d_s > 1) = 1.0000$] 21 [$P(d_n/d_s > 1) = 0.9998$] 34 [$P(d_n/d_s > 1) = 0.9921$] 90 [$P(d_n/d_s > 1) = 0.9854$]
	M7	M8	23.587946	0.00003045	Yes	17 [$P(d_n/d_s > 1) = 1.0000$] 21 [$P(d_n/d_s > 1) = 0.9997$] 34 [$P(d_n/d_s > 1) = 0.9890$] 90 [$P(d_n/d_s > 1) = 0.9831$]
E7 (97 codons)	M1	M2	0.436514	0.80391881	No	None
	M1	M3	0.436426	0.97938744	No	None
	M7	M8	0.008410	0.99979539	No	None

with respect to charge, polarity, or other user-specified property. It allows numerical comparison of frequency of substitutions that result in ''radical'' versus ''conservative'' changes in amino acids with respect to the property of interest. Diversification analysis of E6 codons revealed no significant excesses of radical amino acid substitutions (data not shown). In other words, radical amino acid substitutions were not more likely to occur than conservative substitutions at these sites. This applies to changes in amino acid charge, polarity, and hydrophobicity. Our analysis was performed on all positively selected sites using all taxa, unique positively selected site haplotypes, site 17 only (see below), and site 90 only (see below).

Discussion

It is likely that E6 contains codon sites that are evolving under the influence of diversifying selection. E7 does not appear to contain any such sites, despite the presence of amino acid variation. It is of interest to note that while genealogical techniques have uncovered numerous proteins from ribo- and retroviruses that experience diversifying selection this is the first such demonstration in a DNA virus. The processes that account for directional selection operating on E6 are difficult to identify experimentally due mostly to the complex reproductive strategy of the virus and the unknown characteristics of the individual host environments from which these haplotypes were obtained. In addition, complications associated with culturing the virus *in vitro*, as well as its low mutation rate, effectively prevent experimental examination of its evolutionary dynamics. Nevertheless, a significant amount of information is known about the functions of E6 and E7 with respect to viral replication and therefore we will focus on two aspects that may be related to external selective forces: Immunogenicity and functional interaction of viral and cellular proteins.

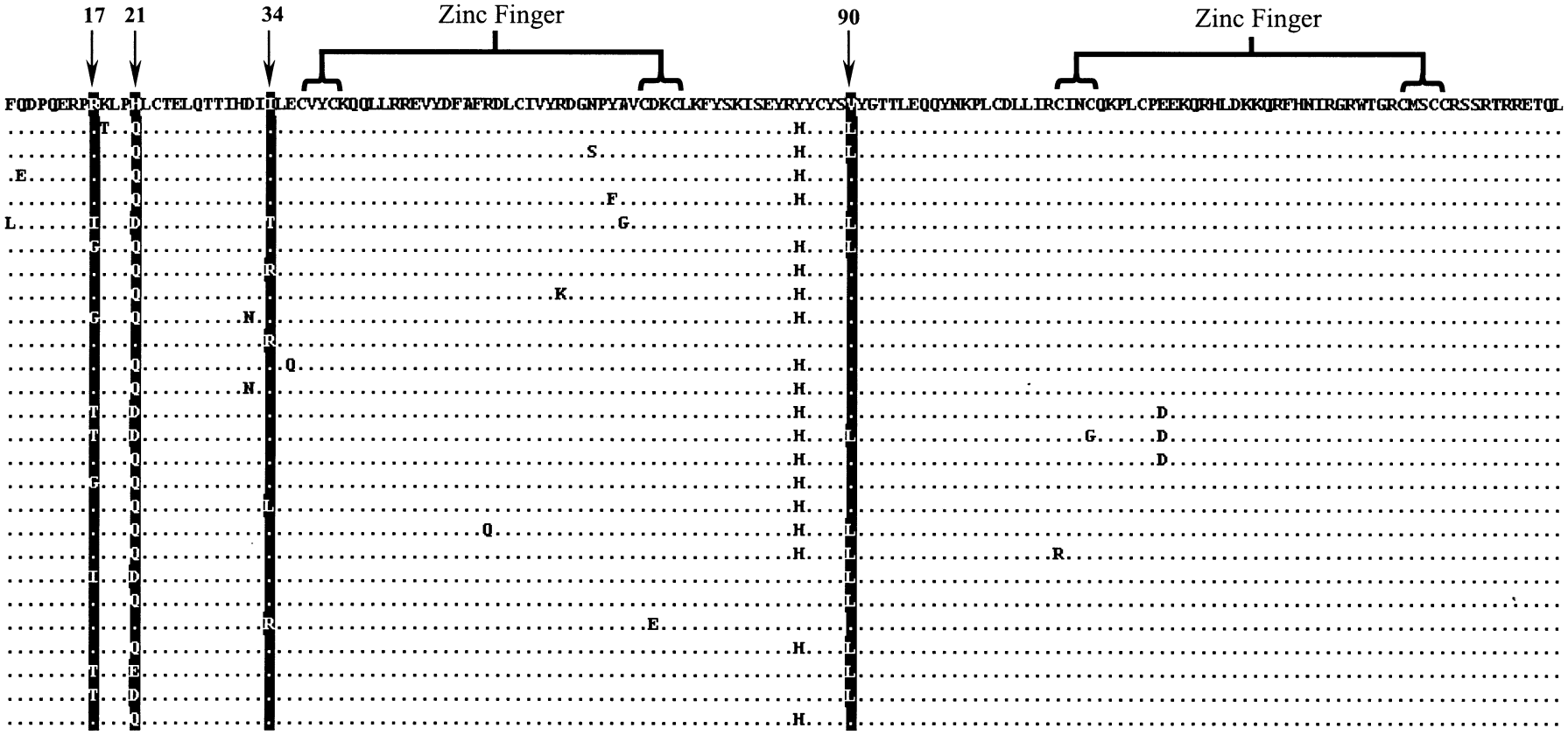

Fig. 1. Alignment of unique E6 amino acid sequences used in this study (sites 9–158). Sites identified as having $d_n/d_s > 1$ are indicated.

Table 4. Amino acids observed in positively selected E6 sites and their corresponding biochemical traits

Site	Amino Acid	Frequency	Charge	Polarity	Hydrophobicity
17	R	28	Positive	Polar	Hydrophilic
	G	7	Neutral	Polar	Hydrophilic
	T	6	Neutral	Polar	Hydrophilic
	I	2	Neutral	Non-polar	Hydrophobic
21	Q	32	Neutral	Polar	Hydrophilic
	D	7	Negative	Polar	Hydrophilic/Acidic
	H	3	Positive	Polar	Hydrophilic
	E	1	Negative	Polar	Hydrophilic/Acidic
34	I	38	Neutral	Non-polar	Hydrophobic
	R	3	Positive	Polar	Hydrophilic
	L	1	Neutral	Non-polar	Hydrophobic
	T	1	Neutral	Polar	Hydrophilic
90	V	25	Neutral	Non-polar	Hydrophobic
	L	18	Neutral	Non-polar	Hydrophobic

Immune Selection

Immunogenic peptides [specifically cytotoxic T-lymphocye (CTL) epitopes] that exist in proteins of intracellular parasites may be subjected to diversifying selection imposed by cell-mediated immune responses. Protein variants containing epitopes against which a host can react will experience a smaller probability of surviving T-cell attack than a coexisting protein variant that does not (see Gould and Bangham 1998 for review). Selective pressure can thus direct amino acid evolution in viral proteins away from sequences capable of being recognized by a particular host or host population. This has been demonstrated mostly in viruses with RNA genomes that are capable of rapid, intrahost evolutionary change (see Phillips et al. 1991, Nowak et al. 1995, Wolinsky et al. 1996, McMichael and Phillips 1997, da Silva and Hughes 1999, Kuiken et al. 1999, Haydon et al. 2001).

Intrahost variation is essentially nonexistent in DNA viruses such as HPVs that use host enzymes for genome replication. Yet, while HPVs evolve slowly compared to ribo- and retroviruses, they evolve much more rapidly than humans. An "equilibrium" distribution of amino acid sequences that maximizes the survival of a virus population in the context of a particular host MHC gene pool could potentially arise. Evidence suggests that Epstein-Barr virus (EBV), a DNA virus, experiences local allele frequency alteration in response to host population MHC makeup (de Campos-Lima et al. 1993). The long-term effect of MHC makeup on d_n/d_s ratios within EBV CTL epitopes may not be driving amino acid change, however (Khanna et al. 1997).

If the observed diversification of the E6 gene is driven by immune selection, it might be expected that the E6 protein would be highly immunogenic relative to E7. While a number of studies have identified CTL epitopes within both E6 and E7 proteins (in fact, few amino acids in either protein fall outside known CTL epitopes), it has not been demonstrated that either protein is more or less immunogenic than the other (see Dillner 1990, Strang et al. 1990, Comerford et al. 1991, Altmann et al. 1992, Kast et al. 1994, Sarkar et al. 1995, Bauer et al. 1995, Ressing et al. 1995, Dunn et al. 1997).

One of the E6 amino acid sites identified as evolving under positive selection (site 17) is potentially very informative. We have found four amino acids in this position (R = 64.3%, G = 16.7%, T = 14.3%, and I = 4.8%). Under M3, the maximum likelihood d_n/d_s estimate for this site is ~9.9. Ellis and colleagues (1995) found a small but significant association between HLA-B7 positive individuals and an E6 mutant at this amino acid position (R → G). This site is located within a HLA-B7 CTL epitope, and while the mutation does not affect HLA binding it drastically alters the epitope's exposure to $CD8^+$ T-cell receptors (Ellis et al. 1995). This may result in different survival probabilities of strains related to (1) host HLA-B makeup and (2) amino acid composition of HLA-B viral epitopes. In other words, certain E6 site 17 variants may be better adapted for replication within hosts of a specific HLA makeup. Bontkes and colleagues (1998) failed to detect a significant association of any E6 or E7 variants with specific HLA types in a sample of 40 cancer patients. Thus it is possible that immune selection is only a localized phenomenon, if it exists at all.

Protein-protein interactions, such as those between pathogen epitopes and MHC proteins are influenced by amino acid charge (Monos et al. 1984). Hughes and Hughes (1995) present evidence of greater than expected amino acid charge variation in the peptide binding regions of human class I HLA molecules. In order to explore further this possibility we examined the biochemical extremism of specific amino acid

changes that took place in E6 codons identified as diversifying. The results indicate that diversification of these sites is essentially random with respect to charge, polarity, and hydrophobicity. While this in no way proves immune selection is not influencing evolution at these sites, when coupled with the results of Bontkes et al. (1998), it downplays the significance of avoidance of epitope binding in the viral life cycle.

Viral-Cellular Protein Functional Interaction

E6 and E7 are responsible for direct interaction with cellular proteins p53 and p105RB respectively (Scheffner et al. 1990, Werness et al. 1990, Huibregtse et al. 1991, Scheffner et al. 1993, Dyson et al. 1989, Jones et al. 1997). The specific mechanisms of E6 and E7 interaction with other cellular proteins are not well researched. Naturally occurring polymorphism in these host proteins may promote polymorphism in viral proteins to allow for successful binding. In contrast with selection to discourage epitope-MHC binding, the function of this variation is to *facilitate* protein–protein binding. As such, we might expect less radical diversification of amino acids with respect to biochemical traits such as charge.

p53 is in fact known to be polymorphic in human populations, and to vary according to latitude and ethnicity (Beckman et al. 1994), but p105Rb is nearly monomorphic (Yandell et al. 1989, Schubert and Hansen 1996). One particular p53 substitution (codon 72 R $\rightarrow$ P) is the subject of most polymorphism analyses. Van Duin and colleagues (2000) uncovered a significant association between cervical cancer patients homozygous for the p53 codon 72 R variant and the HPV16 E6 codon 90 L variant. This E6 amino acid site appears in our study to be evolving under diversifying selection but the two amino acids observed here (V and L) are not radically different in charge, polarity, or hydrophobicity (these amino acids are also similar in weight and surface area). Interestingly, p53 protein variants have been shown to be differentially susceptible to E6-induced degradation based on amino acid site 72 composition (Storey et al. 1998). Therefore, selective pressure driven by ability to interact with p53 is likely to exist.

Recently, E6 and E7 proteins have been the foci of efforts to develop therapeutic vaccines for cervical cancer, since they are overexpressed in tumor cells (see Murakami et al. 1999 for review). In addition, E6 and E7 have been considered potential molecular targets for HPV antiviral treatments (see Phelps et al. 1998 for review). The evolutionary potential of E6 and E7 could have serious implications for the efficacy and development of epitope-based vaccination strategies and of antiviral medications. Therefore, such topics should be of great importance to researchers investigating these genes for therapeutic reasons. Site-directed mutagenesis studies targeting positively selected sites could provide invaluable information regarding virus-host interactions and the usefulness of targeting this protein in anti-HPV therapy.

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