

Naturally Occurring Major and Minor Capsid Protein Variants of Human Papillomavirus 45 (HPV45): Differential Recognition by Cross-Neutralizing Antibodies Generated by HPV Vaccines

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We investigated naturally occurring variation within the major (L1) and minor (L2) capsid proteins of human papillomavirus genotype 45 (HPV45). Pseudoviruses (PsVs) representing HPV45 sublineages A1, A2, A3, B1, and B2 exhibited comparable particle-to-infectivity ratios and morphologies but demonstrated both increased (A2, A3, and B1) and decreased (B2) sensitivities to cross-neutralization by HPV vaccine antibodies compared to that of the A1 sublineage. Mutant PsVs identified HI loop residue 357 as being critical for conferring this differential sensitivity.

The evolutionary mutation rate of the human papillomavirus (HPV) double-stranded DNA genome is low at ca. 2×10^{-8} base substitutions per site per year (1, 2), yet distinct genotypes and intragenotype variant lineages have arisen over time (3). HPV genotype 45 (HPV45) is closely related to HPV18 within the Alpha-7 species group and is associated with ca. 5% of cervical cancer cases worldwide (4, 5). Whole-genome sequence analysis of HPV45 strains has led to the delineation of distinct variant lineages (A and B) and sublineages (A1, A2, A3, B1, and B2) (3, 6, 7), with the possibility of a lineage C suggested from subgenomic sequences (8). Although firm data on their contribution to the risk of cervical disease progression are lacking, in part due to the low relative prevalences of individual lineages and sublineages in the population, current evidence does support some lineage-specific bias such that sublineage variant B2 (and possibly A3) appears to be overrepresented in patients with high-grade disease compared to controls (8–10). There may also be some geographical bias to the distribution of HPV45 sublineages (9). Intragenotypic variation occurs throughout the HPV genome, but the consequences of these polymorphisms on the functions of the resulting gene products are uncertain.

The HPV structural genes encode the major (L1) and minor (L2) capsid proteins. The L1 protein multimerizes to form the nonenveloped icosahedral viral capsid (comprising 72 L1 pentameric capsomers) that mediates attachment to host cells (11), while the L2 protein is essential for viral infectivity (12). Structural alterations of the external surface topography of L1 can be conferred by minor sequence differences between genotypes (13), supporting observations that almost all neutralizing monoclonal antibodies (MAbs) that target these external surfaces are type specific (14–17). Nevertheless, functional antibody cross-reactivity is a common feature of sera from recipients of the Cervarix (bivalent) and Gardasil (quadrivalent) vaccines (18–22) and may be responsible for conferring HPV vaccine-induced cross-protection (23).

It is reasonable to consider that lineage-specific variation in surface-exposed domains (7, 24, 25) may influence capsid recognition by HPV vaccine-derived antibodies. Single-cycle replication-incompetent pseudoviruses (PsVs) representing HPV16 L1, but not L2, variants (26) appear to exhibit little difference in their

susceptibilities to type-specific antibodies elicited by HPV16 virus-like particles (VLP). We recently demonstrated that although PsVs incorporating HPV31 L1 and L2 lineage variants (A, B, and C) were susceptible to cross-neutralizing antibodies elicited by the Cervarix and Gardasil HPV vaccines, there were lineage-specific differences in sensitivity (27). Here we examine the potential impact of lineage-specific L1 and L2 HPV45 variation on sensitivity to cross-neutralizing antibodies elicited by the Cervarix and Gardasil HPV vaccines.

To improve estimates of the sublineage consensus sequences, we first generated 35 L1 (bp 5608 to 7149, numbered according to the HPV45 reference sequence [X74479]) and L2 (bp 4236 to 5627) sequences from samples collected from 16- to 24-year-old females previously confirmed as HPV45 DNA positive by the Linear Array HPV genotyping test (Roche) (28). Additional HPV45 L1 and L2 sequences were downloaded from the National Center for Biotechnology Information (NCBI [<http://www.ncbi.nlm.nih.gov/>] accession numbers X74479 [29], DQ080002 [30], EF202156 to EF202167 [6], and KC470250 to KC470260 [7]). X74479 is considered to be the reference sequence for the HPV45 genotype (3), while DQ080002 (30) was used as the basis of the HPV45 pseudovirus. These sequences are not identical, so for clarity, we refer to X74479 for sequence-based comparisons and to DQ080002 for comparison of biological data. The concatenated L1 L2 (2.9-kb) fragment contained sufficient numbers of diagnostic motifs to allow segregation of sequences into the sublineages A1, A2, A3, B1, and B2 defined (3, 6, 7) by whole-genome sequence analysis (Fig. 1A). Mean intralinear

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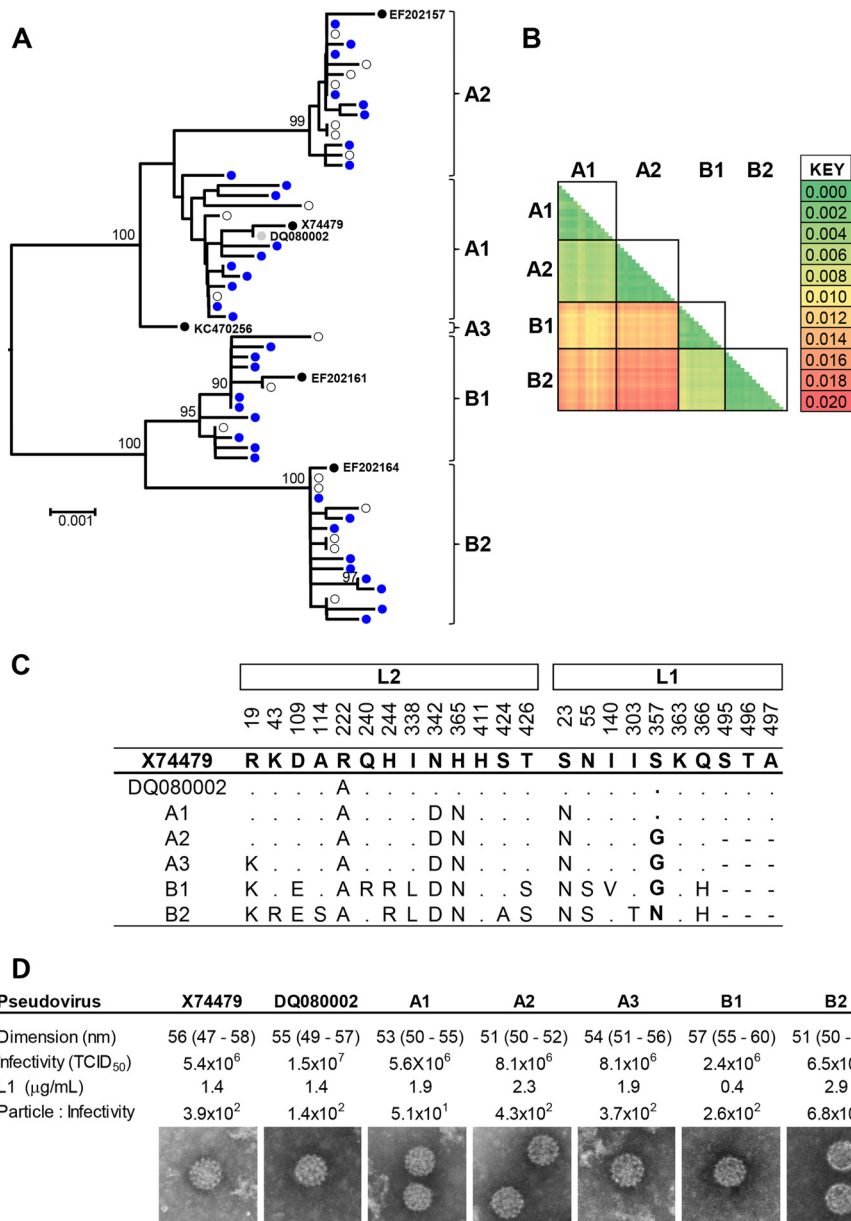


FIG 1 HPV45 L1 and L2 variation. (A) Neighbor-joining tree constructed (MEGA v6 [42]) from concatenated L1 and L2 nucleotide sequences. Sublineage (A1, A2, A3, B1, and B2) attribution is based upon whole-genome sequencing (representative sequences are included [3, 6, 7]) and is supported by bootstrap values of $\geq 90\%$. (B) Inter- and intralineage sequence diversity. A3 was omitted from this analysis due to a low representation of sequences. (C) Site-specific amino acids within the consensus L1 and L2 protein sequences used to generate PsV. (D) PsV preparations were characterized for particle dimension in nanometers (median [IQR]), infectivity, L1 concentration, and the resultant particle-to-infectivity ratio. TCID₅₀, 50% tissue culture infective dose.

sequence diversity was 0.14% (standard deviation [SD], 0.09%), while mean interlineage sequence diversity was 1.27% (SD, 0.14%) (Fig. 1B). A consensus sequence for each sublineage was determined (Fig. 1C), and bicistronic psheLL vectors (31) containing codon-optimized HPV45 L1 and L2 genes representing these consensus sublineage variants were generated (20) (Fig. 1D). All HPV45 variant PsVs displayed similar particle sizes (median, 55 nm; interquartile range [IQR], 53 to 57 nm) and particle-to-infectivity ratios (27) (median, 2.9×10^2 ; IQR, 2.0×10^2 to 4.7×10^2).

Sera from 12- to 15-year-old girls, collected following three

doses of Cervarix or Gardasil HPV vaccine (22), were used to assess HPV45 sublineage variant sensitivity to cross-neutralizing antibodies (Fig. 2A) in a PsV-based neutralization assay (20, 32). PsVs based upon the reference sequence (A1, X74479) and a consensus A1 sequence displayed neutralization sensitivities similar to that of the commonly used HPV45 PsV (A1, DQ080002 [30]). These data suggest that L2 (R222A, N342D, and H365N) and L1 (S23N) variant residues (Fig. 1C) have no discernible impact on sensitivity to cross-neutralizing antibodies elicited by the HPV vaccines. PsV variants A2, A3, and B1 exhibited ca. 3-fold-increased sensitivity ($P < 0.001$; Wilcoxon signed-rank test) to

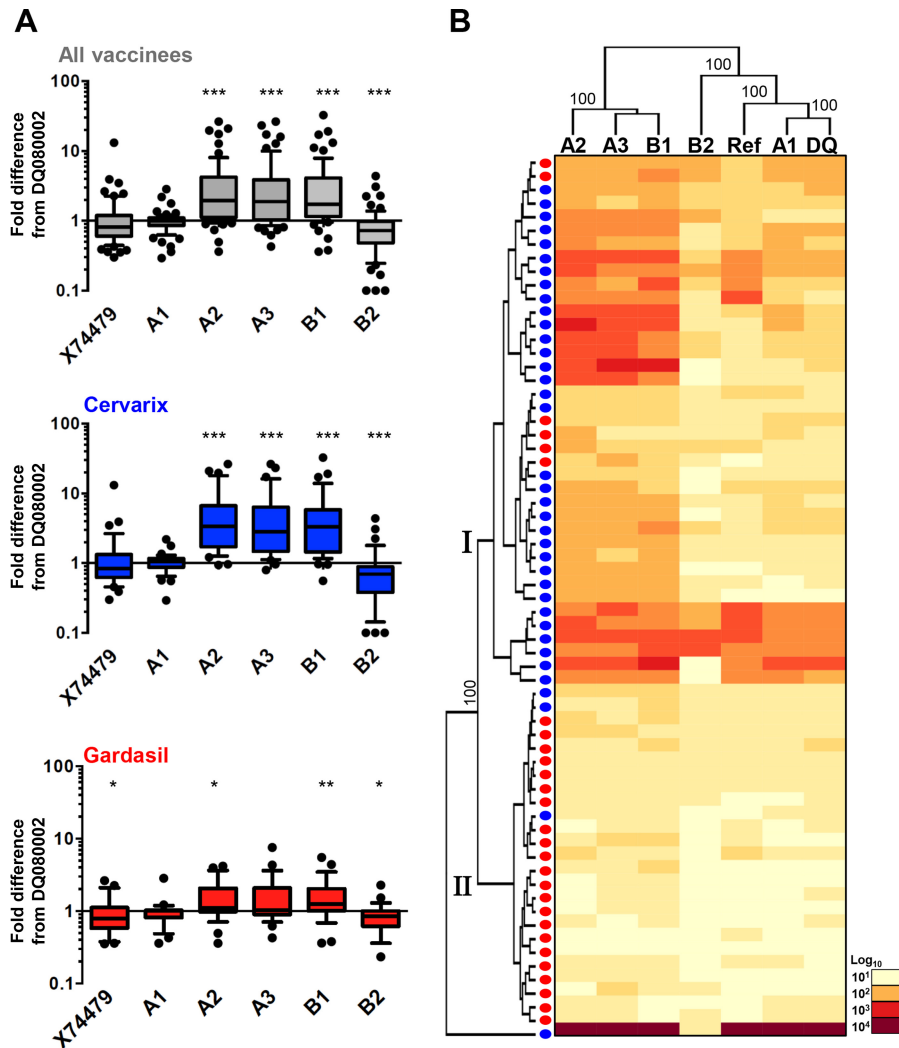


FIG 2 Neutralization sensitivities of sublineage HPV45 L1 and L2 pseudoviruses. (A) Box (median, IQR) and whisker (10th and 90th percentiles) plots of the fold difference in neutralization titer from that of the DQ080002 PsV for each PsV (reference sequence A1 [X74479] and consensus constructs for A1, A2, A3, B1, and B2) for all ($n = 65$) vaccine sera tested and separately for Cervarix ($n = 37$) and Gardasil ($n = 28$) sera. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (Wilcoxon paired signed-rank test). (B) Hierarchical clustering of \log_{10} PsV neutralization titers (center, heat map) reordered according to serological (left) and PsV (top) dendrograms constructed from the resulting Euclidean distance matrices, with clusters supported by bootstrap values as indicated. Blue dots, Cervarix sera; red dots, Gardasil sera.

cross-neutralizing antibodies, while variant B2 displayed slightly decreased sensitivity ($P < 0.001$), compared to that of the DQ080002 HPV45 PsV.

The source of the antibodies appeared to influence the magnitude of these sensitivity differences for some sublineage variants. For example, sublineage variant A2 was 3.4 (IQR, 1.8 to 6.4)-fold more sensitive than the DQ080002 PsV to sera from Cervarix recipients, compared to 1.1 (1.0 to 1.9)-fold for the sera from Gardasil recipients ($P < 0.001$) (Fig. 2A). As HPV45 antibody titers generated by the Gardasil vaccine are generally lower than those generated by the Cervarix vaccine (19, 22), we also compared the data from a subset of sera with low titers (< 50). Median antibody titers neutralizing the DQ080002 HPV45 PsV were 37 (IQR, 34 to 42; $n = 14$) and 32 (28 to 42; $n = 18$) for the sera from Cervarix and Gardasil recipients, respectively (Mann-Whitney U test, $P = 0.203$), while titers neutralizing the A2 variant PsV were 172 (IQR, 89 to 205) and 39 (IQR, 27 to 50), respectively ($P <$

0.001). Similar differences were apparent for PsV variants A3 and B1 (data not shown).

We next subjected the \log_{10} -transformed PsV neutralization assay data to hierarchical clustering (<http://www.hiv.lanl.gov/content/sequence/HEATMAP/heatmap.html>) and identified two serum clusters; cluster I contained predominantly sera from Cervarix recipients ($n = 33$; Gardasil $n = 6$), while cluster II contained mostly sera from Gardasil recipients ($n = 22$; Cervarix $n = 4$) (Fisher's exact test, $P < 0.001$) (Fig. 2B). The variant PsV formed three distinct branches: one containing the A1 PsV (including the consensus A1 sequences [DQ080002 and X74479]), another containing PsVs A2, A3, and B1, and another containing PsV B2. These data support distinct profiles for HPV45 sublineage variant sensitivity to cross-neutralizing antibodies generated by the HPV vaccines and suggest that the antibody repertoires generated by the Cervarix and Gardasil HPV vaccines are not identical.

Although there were multiple amino acid residue differences

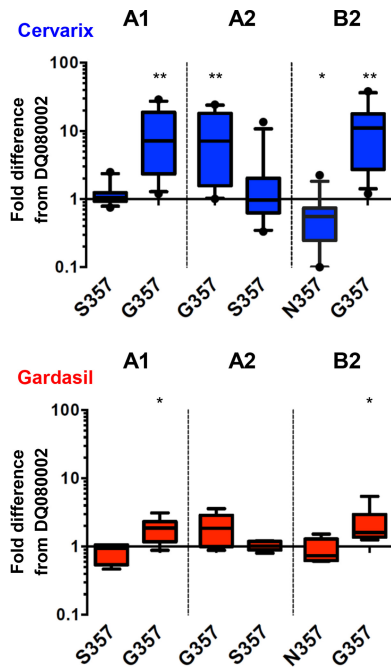


FIG 3 Neutralization sensitivities of mutant HPV45 L1 and L2 PsVs. Box (median, IQR) and whisker (10th and 90th percentiles) plots of fold differences in neutralization titer from that of the DQ080002 PsV for PsVs A1, A2, and B2 and their indicated mutants in Cervarix ($n = 12$) and Gardasil ($n = 6$) sera. *, $P < 0.05$; **, $P < 0.01$ (Wilcoxon paired sign-rank test).

between the L1 and L2 variant lineage sequences (Fig. 1C), residue 357 in the HI loop appeared to track differences in neutralizing antibody sensitivities displayed by the variant PsVs (Fig. 2). We next constructed three mutant HPV45 PsVs (A1 S357G, A2 G357S, and B2 N357G) to examine the potential impact of this HI loop residue on sensitivity to cross-neutralizing antibodies (Fig. 3). These mutant PsVs displayed particle-to-infectivity ratios similar to those of the variant PsVs (data not shown). These contextual substitutions support a strong influence of residue 357 in the HI loop on PsV sensitivity to cross-neutralizing antibodies elicited by the current HPV vaccines; specifically, a glycine at position 357 renders the PsV more sensitive to such antibodies, followed in decreasing order by serine and asparagine. For example, the S357G substitution in the context of PsV A1 increased sensitivity to sera from Cervarix recipients from a median of 182 (IQR, 94 to 288) to 1,018 (IQR, 774 to 1,155) ($P < 0.001$), while replacement of the glycine with serine in the context of PsV A2 (G357S) reduced sensitivity from 906 (IQR, 796 to 1,138) to 174 (IQR, 73 to 295) ($P < 0.001$).

We next used the crystal structure of the HPV18 L1 pentamer (13) to create homology models (33, 34) of HPV45 A1, A2, and B2 L1 variants (<http://swissmodel.expasy.org/>), which were visualized using Swiss-PDP viewer v4.0 software (Fig. 4A and B). Pairwise model comparisons of HPV45 A2 and B2 with A1 were performed by superimposition (root mean square deviations of 0.20 Å and 0.16 Å, respectively) (Fig. 4C and D). The models predicted that substitution at HI residue 357 may influence local topography by shifting the adjacent FG loop by a mean \pm standard

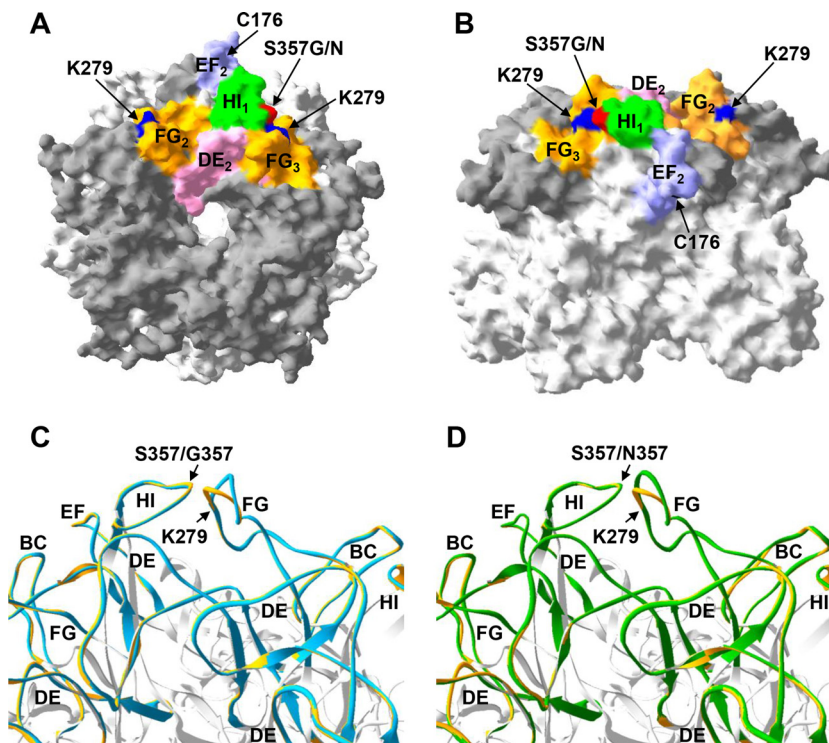


FIG 4 Homology modeling of HPV45 sublineage variants. HPV45 sublineage variant homology models are based upon the HPV18 L1 pentamer crystal (Protein Databank [PDB] accession number 2R5I.1), with top (A) and side surface-filled (B) views shown for HPV45 A1 and with external loops indicated by dark-gray shading. External loops adjacent to the indicated HI loop (in this case, the HI loop of monomer 1 [HI₁]) are indicated. Residues C176, K279, and S357 are also indicated for context. Superimposition models for HPV45 A2 (blue) (C) and HPV45 B2 (green) (D) relative to HPV45 A1 (gold) are used to indicate relative shifts in the position of the FG loop. HI loop residue 357 and FG loop residue K279 are indicated.

error [SE] of $3.0 \pm 1.2 \text{ \AA}$ (HPV45 A2) or $1.5 \pm 0.8 \text{ \AA}$ (HPV45 B2) relative to its position in the HPV45 A1 model.

Notably, K279 in the FG loop is within 3.5 \AA of residue 357, and the corresponding residue in HPV16 and HPV18 is involved in HPV binding to heparin sulfate (35). Recent cryo-electron microscopy studies have significantly improved the resolution of the antigenic domains of two classes of HPV16-neutralizing MAbs, exemplified by H16.U4 (36) and H16.V5 (15, 37). The H16.U4 epitope encompasses residues in the C-terminal portion of L1, which is involved in forming intercapsomer contacts via the “invading arm” disulfide bridge between Cys 428 and Cys 175 on adjacent capsomers of HPV16 L1. The epitopes of H16.V5-like MAbs include residues primarily within the DE and FG loops of the external surface of the capsomer, with contribution from residues within the BC, EF, and HI loops. The model predictions made herein for HPV45 suggest that the subtle structural alterations conferred by substitution of HI 357 occur within a domain proximal to the type-specific immunogenic domain on the external surface of HPV16 L1. These data suggest that variation within this region may influence the presentation of cross-neutralizing antibody epitopes in a way similar to the observation that subtle structural differences between genotypes in this region may bestow type-specific susceptibility to neutralizing MAbs (13).

The quality of the predicted models was demonstrated by their Qualitative Model Energy Analysis (QMEAN4) (38) Z-scores, which were -2.81 , -2.91 , and -2.93 for the A1, A2, and B2 models, respectively, and by their global model quality estimation (GMQE) score, which was 0.99 for all three models. HPV45 contains an insertion (S282, A283) within the FG loop relative to the HPV18 template, and although this is common to all HPV45 sequence variants examined here, it may nevertheless introduce a certain degree of measurement error into these model predictions.

Taken together, these data suggest that HPV45 lineage variants differ in their sensitivities to cross-neutralizing antibodies induced by the HPV vaccines through subtle alteration of L1's topography. HPV PsVs have been used widely to monitor antibody responses to vaccines and natural infection (18, 22, 32, 39, 40), as well as to elucidate steps in the entry process (41). Nevertheless differences between how PsVs behave *in vitro* and how authentic HPV45 lineage variants behave *in vivo* are uncertain, although this is a limitation of all PsV-based systems. Whether differences in PsV variant sensitivities noted here will influence the prevalence of individual variant lineages over time in countries that have introduced national vaccination programs will require further study. These data inform our understanding of the antigenicity of the HPV structural proteins and may be useful in guiding impact modeling of the current HPV vaccines and informing postvaccine surveillance programs.

Nucleotide sequence accession numbers. The L1 and L2 sequences (KU049723 to KU049757) generated in this report have been deposited in GenBank under the indicated accession numbers.

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