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Naturally Occurring Capsid Protein Variants of Human Papillomavirus Genotype 31 Represent a Single L1 Serotype

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ABSTRACT

We investigated naturally occurring variation within the major (L1) and minor (L2) capsid proteins of oncogenic human papillomavirus (HPV) genotype 31 (HPV31) to determine the impact on capsid antigenicity. L1L2 pseudoviruses (PsVs) representing the three HPV31 variant lineages, variant lineages A, B, and C, exhibited comparable particle-to-infectivity ratios and morphologies. Lineage-specific L1L2 PsVs demonstrated subtle differences in susceptibility to neutralization by antibodies elicited following vaccination or preclinical L1 virus-like particle (VLP) immunization or by monoclonal antibodies; however, these differences were generally of a low magnitude. These data indicate that the diagnostic lineage-specific single nucleotide polymorphisms within the HPV31 capsid genes have a limited effect on L1 antibody-mediated neutralization and that the three HPV31 variant lineages belong to a single L1 serotype. These data contribute to our understanding of HPV L1 variant antigenicity.

IMPORTANCE

The virus coat (capsid) of the human papillomavirus contains major (L1) and minor (L2) capsid proteins. These proteins facilitate host cell attachment and viral infectivity and are the targets for antibodies which interfere with these events. In this study, we investigated the impact of naturally occurring variation within these proteins upon susceptibility to viral neutralization by antibodies induced by L1 VLP immunization. We demonstrate that HPV31 L1 and L2 variants exhibit similar susceptibility to antibody-mediated neutralization and that for the purposes of L1 VLP-based vaccines, these variant lineages represent a single serotype.

Human papillomaviruses (HPVs) have a double-stranded DNA genome of approximately 8 kb which is replicated via host cell polymerases with an error rate of ca. 2×10^{-8} base substitutions per site per year (1), substantially lower than that found in the majority of single-stranded RNA viruses (ca. 1×10^{-3} base substitutions per site per year) (2). Despite the low evolutionary rate of the HPV genome, variants have arisen over time, leading to the generation of distinct intragenotype lineages classified by a sequence difference of 1 to 10% across the whole genome (3). The single nucleotide polymorphisms (SNPs) that allow segregation of these variants into distinct lineages can be found in each gene/region, with the highest number accumulating in the noncoding regions (NCR1, NCR2, and URR) and the lowest number accumulating in structural (L1 and L2) genes (4).

The HPV structural genes encode the major (L1) and minor (L2) proteins that form the nonenveloped icosahedral viral capsid, which comprises 72 pentameric L1 capsomers, and each capsomer has an upper estimate of one L2 protein (5). The L1 protein mediates attachment to host cells (6), while the L2 protein is essential for subsequent viral infectivity (7).

The humoral immune response following natural HPV infection predominately targets conformational epitopes on the surface-exposed loop regions of the L1 protein (8, 9). Seroconversion generally occurs 6 to 18 months after infection, with low levels of L1 antibodies being detected in 50 to 70% of individuals (10, 11). It is not clear whether antibodies induced by natural infection protect against subsequent reinfection by the same HPV genotype, but increasing evidence indicates that high antibody titers can be associated with a reduced risk of reinfection (12–15).

The L1 protein can self-assemble into virus-like particles (VLPs), which are the basis of the current prophylactic HPV vaccines, Cervarix and Gardasil (16). Clinical trials have demonstrated the high degrees of efficacy of both vaccines against infection and cervical disease associated with vaccine genotypes HPV genotype 16 (HPV16) and HPV18. A degree of vaccine-induced cross-protection against closely related genotypes, particular HPV31, HPV33, and HPV45, has also been demonstrated (16–18). HPV vaccine type-specific protection is assumed to be mediated by L1-neutralizing antibodies, which can be detected in the serum and cervicovaginal secretions of vaccinees (16, 19–22). The role of L1-neutralizing antibodies in mediating cross-protection is less clear, although a recent study reported an association between the presence of HPV31 cross-neutralizing antibodies and a reduced risk of HPV31 infection (23). Next-generation L1 VLP-based vaccines aim to extend the breadth of coverage by incorporating an increased number of L1 VLPs (24, 25).

Intragenotype variation within the L1 protein is generally localized to the surface-exposed loop domains (26), akin to the majority of intergenotype variation (27, 28). Data informing the po-

Received 30 March 2015 **Accepted** 6 May 2015

Accepted manuscript posted online 20 May 2015

Citation Bissett SL, Godi A, Fleury MJJ, Touze A, Cocuzza C, Beddows S. 2015. Naturally occurring capsid protein variants of human papillomavirus genotype 31 represent a single L1 serotype. J Virol 89:7748–7757. doi:10.1128/JVI.00842-15.

Editor: S. R. Ross

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doi:10.1128/JVI.00842-15

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tential impact of such variation on L1 antigenicity are limited to HPV16, where L1L2 pseudoviruses (PsVs) representing lineage-specific L1 variants were neutralized to a similar extent by antibodies elicited against a single L1 VLP (29). However, the impact of a common Thr-to-Ala switch at amino acid residue position 266 within the FG loop of HPV16 was not evaluated, nor was variation within the corresponding L2. Another study found that an FG loop-specific neutralizing monoclonal antibody (MAb), H16.E70, had reduced recognition for HPV16 L1 VLP bearing a Thr at amino acid residue 266, indicating that variation within this region can impact antigenicity (30).

HPV31 is closely related to HPV16 within the alpha 9 species group and is associated with ca. 3.8% of cervical cancer cases worldwide (31). The full-genome sequence analysis of HPV31 has led to the delineation of three distinct variant lineages: A, B, and C (4). Infections due to HPV31 lineage variant A or B have been associated with an increased risk of development of cervical intraepithelial neoplasia grades 2 and 3 (CIN2/3), yet, somewhat paradoxically, infections with lineage variant C appear to persist for longer periods (32, 33). Differences in the natural history between variants of other HPV genotypes have also been observed (34, 35). The relative infectivity of variants or the ability of variants to differentially disrupt cellular differentiation is a possible virological factor which can contribute to the disparities in variant pathology (3). One study suggested that the genetic background of the host may also play a role, since African-American women were found to be less likely to clear a HPV31 lineage variant C infection than a lineage variant A infection, yet there was no difference in the likelihood of clearing a lineage variant A over C infection in Caucasian women (33).

Two nonsynonymous, lineage-specific SNPs within the L1 of HPV31 are located within the FG loop at positions 267 and 274 (26). The FG loop of HPV31 has been shown to be an important antigenic domain targeted by both type-specific and cross-reactive L1 MAbs (36, 37). In the present study, we generated additional HPV31 L1 and L2 sequences and synthesized representative antigens in order to investigate the potential impact of this variation. Such data should improve our understanding of the potential biological impact of naturally occurring HPV31 variation.

MATERIALS AND METHODS

Study samples. Residual vulva-vaginal samples that had been collected from 16- to 24-year-old females who were undergoing chlamydia testing in England and that had previously been confirmed to be HPV31 DNA positive using the Hybrid Capture 2 HPV DNA test (Qiagen) and the Linear Array HPV genotyping test (Roche) (38) were selected for L1 and L2 sequencing. Cervical cell samples from HPV31 DNA-positive women (ages, 19 to 76 years) attending gynecological care at the San Gerardo Hospital (Milan, Italy; ethical approval study code 08/UNIMIB-HPA/HPV1) following a cytological diagnosis of atypical squamous cells of undetermined significance (ASCUS) or low-grade squamous intraepithelial lesions (LSILs) were available for L1 and L2 sequencing. Serum samples were available from HPV31 DNA-positive women within this cohort. Serum samples were available from 12- to 15-year-old girls 1 month after receiving three doses of Cervarix or Gardasil HPV vaccine (20). A panel of HPV31 MAbs was available as either ascitic fluid or tissue culture supernatant (39).

Sequencing of HPV31 capsid genes. The L1 gene (bp 5443 to 7119 [40] or the FG loop region from bp 6141 to 6476; numbered according to the HPV31 reference sequence with GenBank accession number J04353) and the L2 gene (bp 3921 to 5725) were amplified with Platinum *Taq* high-fidelity DNA polymerase (Life Technologies) and sequenced using an ABI 3730 genetic analyzer. Sequence data were collated using DNASTAR Lasergene (v9.0) software (DNASTAR, Inc.). Additional HPV31 L1 and L2 sequences were downloaded from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/; GenBank accession numbers HQ537666 to HQ537687 [4], U37410 [41], and J04353 [42]) and analyzed using the neighbor-joining tree algorithm, with bootstrap values ($n = 500$ iterations) being generated using the MEGA (v6) program (43). HPV31 L1 variant residues were mapped to the surface of the HPV16 capsomer crystal structure (PDB accession number 2R5H) and analyzed using the Swiss-PDP viewer algorithm (v4.0; Deep View) (44).

L1 VLPs. HPV31 L1 VLPs were expressed using a Bac-to-Bac baculovirus system (Life Technologies) and purified on an iodixanol (Sigma-Aldrich) gradient as previously described (45). The L1 protein was visualized by SDS-PAGE, the gel was stained with SimplyBlue SafeStain (Life Technologies), and the L1 protein concentration was determined by comparison with a standard curve derived from known input concentrations of bovine serum albumin. Gel analysis was carried out using ImageJ software (U.S. National Institutes of Health; http://imagej.nih.gov/ij) to determine the L1 concentration of the gradient fractions. VLP formation was confirmed by electron microscopic analysis of negatively stained particles. The HPV31 L1 VLPs shared a 100% amino acid sequence identity with the amino acid sequence of the L1 protein of the HPV31 reference sequence (GenBank accession number J04353) of lineage variant A. Site-directed mutagenesis with a QuikChange kit (Stratagene) was employed to generate L1 sequences representing lineage variants HPV31 B and HPV31 C. The L1 VLPs were used as target antigens in an enzyme-linked immunosorbent assay (ELISA), as previously described (20, 45). The panel of HPV31 MAbs was tested at a standardized input concentration of 250 μg/ml of mouse IgG for all MAbs except 31.D24, for which the starting input concentration was 20 μg/ml. The MAbs were subjected to serial dilutions, the IgG concentration which resulted in a 50% maximal binding optimal density (OD) was estimated by interpolation, and the results are presented as the 50% binding concentration.

Mouse immunizations. VLPs were adsorbed onto aluminum hydroxide (Alhydrogel; Brenntag Biosector) before addition of the monophosphoryl lipid A (MPL)-based Sigma adjuvant system (Sigma-Aldrich). BALB/c mice were injected intramuscularly with 2 μg of VLPs on day 0 and day 14, before a terminal blood sample was taken at day 21. Pretreatment blood samples were taken from all mice prior to the initial immunization. A total of 10 mice were immunized with either HPV31 A VLPs, HPV31 B VLPs, or HPV31 C VLPs over three separate immunization schedules. All animal husbandry and procedures were carried out in strict accordance with United Kingdom Home Office guidelines, were governed by the Animals (Scientific Procedures) Act of 1986, and were performed under licenses PPL 70/7412 and 70/7414.

L1L2 pseudoviruses. A bicistronic psheLL vector (46) containing codon-optimized HPV31 L1 and L2 genes from the HPV31 reference sequence (GenBank accession number J04353) of lineage variant A was expressed and purified on an iodixanol (Sigma-Aldrich) gradient as previously described (47). The L1 and L2 genes from lineage variants HPV31 B and HPV31 C were either synthesized by GeneArt (Life Technologies) or generated by site-direct mutagenesis with a QuikChange kit (Stratagene). Particle formation and particle size were determined by electron microscopic analysis of negatively stained particles. The L1 concentrations of PsV stocks were estimated by semiquantitative L1 Western blot analysis using CamVir-1 antibody (Abcam, United Kingdom), and the 50% tissue culture infective dose ($TCID_{50}$) was estimated using the Spearman-Karber equation as previously described (47). Particle-to-infectivity (PI) ratios were determined on the basis of an estimated particle amount of 3×10^7 particles per ng L1 protein (http://home.ccr.cancer.gov/lco/production.asp), with the ratio being normalized for the input volume and the $TCID_{50}$. The presence of the L2 protein and the reporter gene (luciferase) in purified PsV stocks was confirmed by qualitative L2 Western blot analysis using HPV16 L2 antipeptide-containing sera (amino

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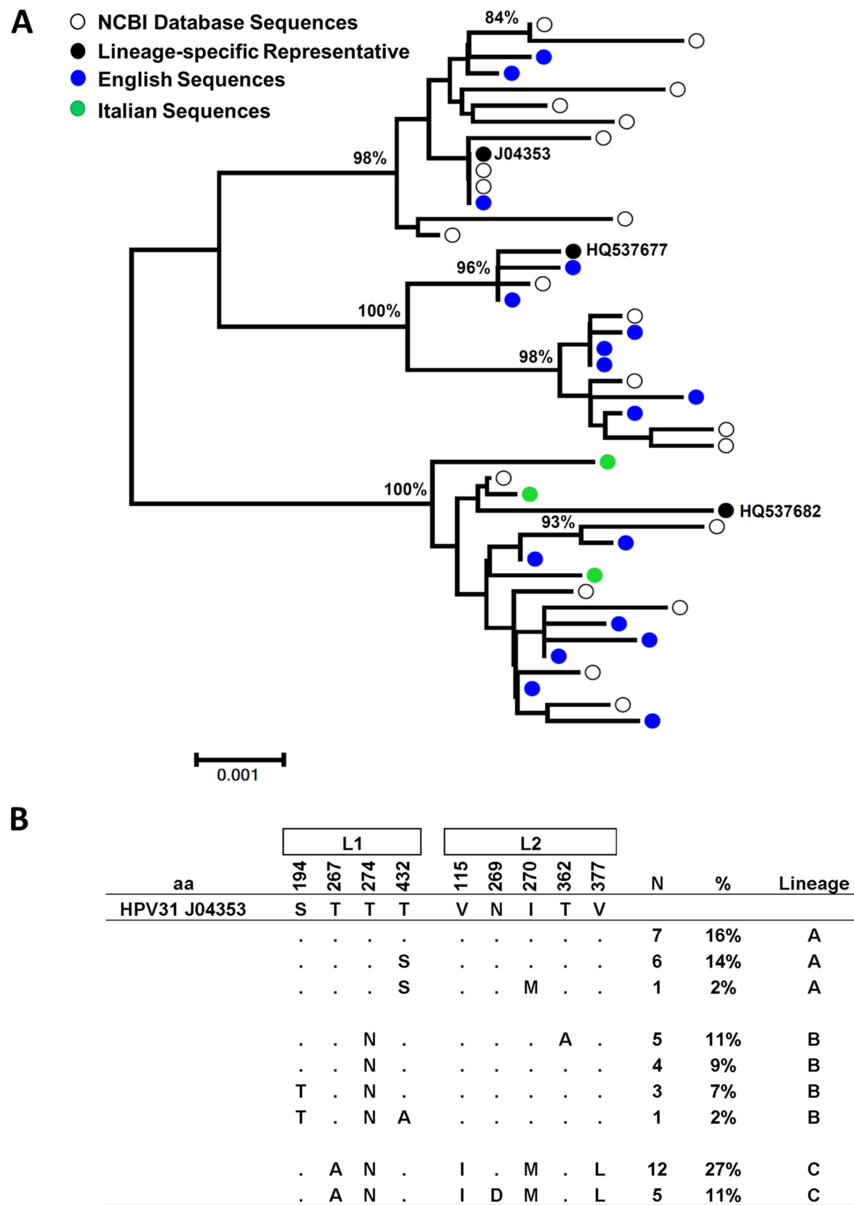

aa	L1 194	L1 267	L1 274	L1 432	L2 115	L2 269	L2 270	L2 362	L2 377	N	%	Lineage
HPV31 J04353	S	T	T	T	V	N	I	T	V			
	.	.	.	.	.	.	.	.	.	7	16%	A
	.	.	.	S	.	.	.	.	.	6	14%	A
	.	.	.	S	.	.	M	.	.	1	2%	A
	.	.	N	.	.	.	.	A	.	5	11%	B
	.	.	N	.	.	.	.	.	.	4	9%	B
	T	.	N	.	.	.	.	.	.	3	7%	B
	T	.	N	A	.	.	.	.	.	1	2%	B
	.	A	N	.	I	.	M	.	L	12	27%	C
	.	A	N	.	I	D	M	.	L	5	11%	C

FIG 1 HPV31 L1 and L2 variation. (A) Phylogenetic tree constructed from concatenated L1 and L2 nucleotide sequences, including representative sequences from lineages A (J04353), B (HQ537677), and C (HQ537682). (51) and bootstrap values of >95%. (B) Site-specific amino acid (aa) covariation within the L1 and L2 proteins. N, number of sequences in the phylogenetic tree represented by each L1 and L2 combination.

acids 17 to 36) and qualitative PCR (bp 1222 to 1641; pGL4.51; Promega) following DNA extraction (QIAamp DNA blood minikit; Qiagen), respectively. The PsV neutralization assay was performed as previously described (48) with minor modifications (47). A standardized input of 100 $TCID_{50}$s was used for all PsVs, and samples were subjected to serial dilutions, with the antibody titer or concentration resulting in an 80% reduction of the luciferase signal (in relative light units) produced by the control wells containing PsV only being estimated by interpolation. HPV antibody control reagents were included in each assay run (49) alongside heparin (H-4784; Sigma-Aldrich), which was used as a positive inhibitor control. The median neutralization titer and interquartile range (IQR) for the positive-antibody-control reagent (high-titer HPV16/18) were as follows: for HPV31 A PsVs, 231 (IQR, 173 to 337; $n = 14$); for HPV31 B PsVs, 462 (IQR, 387 to 671; $n = 10$); and for HPV31 C PsVs, 500 (IQR, 354 to 589; $n = 12$). The negative-antibody-control reagent (HPV negative) had a titer of <40 in all assays ($n = 42$).

Statistical analysis. The Wilcoxon paired signed-rank test was used to compare neutralization titers using Stata (v12.1) software (StataCorp, College Station, TX).

Nucleotide sequence accession numbers. The HPV31 L1 and L2 sequences generated in this study were assigned the following GenBank accession numbers: KJ754561 to KJ754580.

RESULTS

HPV31 L1 and L2 amino acid variation. Full-length HPV31 L1 and L2 sequences were represented by contemporary English ($n = 17$) and Italian ($n = 3$) sequences, in addition to available NCBI database sequences ($n = 24$). Analysis of the aligned, concatenated L1 and L2 sequences demonstrated three distinct clusters consistent with the lineages HPV31 A, HPV31 B, and HPV31 C (Fig. 1A). Lineage A contained 14 sequences, including the

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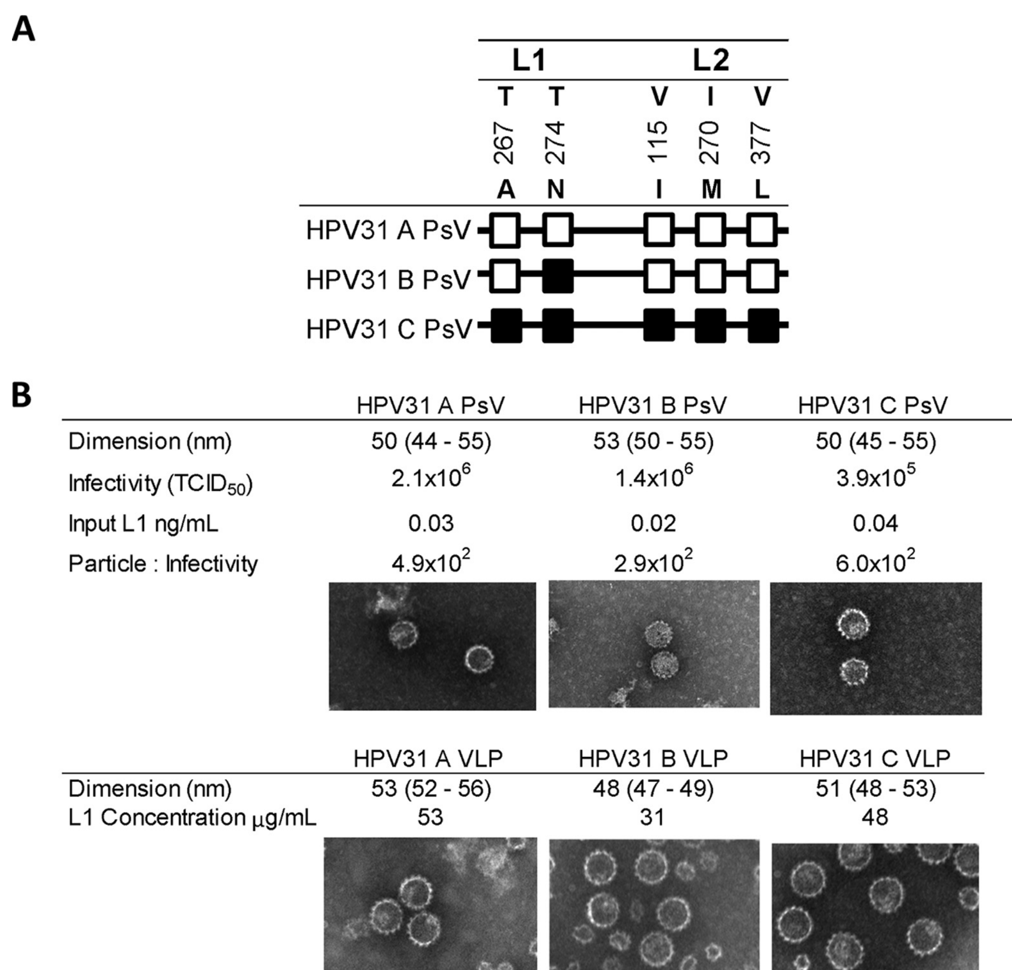

FIG 2 HPV31 L1 and L2 variants. (A) Graphical representation of L1 and L2 variant protein combinations. Site-specific amino acid alterations from the reference (HPV31 A; top sequence, open squares) are indicated using the residue position and resulting amino acid sequence code (filled squares). (B) L1L2 pseudovirus preparation characterized for the median (IQR) particle dimension, infectivity, and L1 concentration. (C) Median (IQR) L1 VLP dimensions (for particles >40 nm in diameter) and L1 concentrations.

HPV31 reference sequence (GenBank accession number J04353), and 7 of these demonstrated variation from the reference sequence in L1 (T432S) and a single sequence demonstrated variation within L2 (I270M) (Fig. 1B). All 13 sequences in lineage B demonstrated variation from the reference sequence at L1 amino acid position 274 (T274N) within the FG loop, while 4 sequences also varied at position 194 (S194T) and 1 varied at position 432 (T432A). Five sequences exhibited variation from the reference sequence at L2 position 362 (T362A). Lineage C contained 17 sequences, all of which demonstrated variation from the reference sequence at L1 amino acid positions 267 (T267A) and 274 (T274N) within the FG loop and L2 positions 115 (V115I), 270 (I270M), and 377 (V377L); 5 sequences also varied from the reference sequence at L2 amino acid position 269 (N269D).

Sensitivity of variant HPV31 L1L2 PsVs to antibody-mediated neutralization. L1L2 PsVs representing lineage variants HPV31 A (GenBank accession number J04353), HPV31 B, and HPV31 C were generated from the consensus L1 and L2 sequences representing each lineage and bore the major L1 variant residues (at positions 267 and 274) and L2 variant residues at positions 115, 270, and 377 (Fig. 2A). All three lineage variant PsVs, here referred to as HPV31 A PsVs, HPV31 B PsVs, and HPV31 C PsVs, generated similarly sized PsV particles of about 50 nm and produced comparable PI ratios of ca. 10^2 (Fig. 2B). The PsV preparations also contained the L2 protein and the luciferase reporter plasmid (data not shown).

The HPV31 variant PsVs were tested against sera from girls who received either the Cervarix or the Gardasil ($n = 46$) HPV vaccine (Table 1). Both HPV31 B and C PsVs were more sensitive to neutralization than HPV31 A PsVs. HPV31 B PsVs displayed a median 1.7-fold (IQR, 1.1- to 2.4-fold; Wilcoxon paired signed-rank test, $P < 0.001$) increased sensitivity to vaccine-induced cross-neutralizing antibodies compared to that of HPV31 A PsVs, while HPV31 C PsVs displayed a 1.4-fold (IQR, 1.1- to 1.6-fold; $P < 0.001$) increased sensitivity compared to that of HPV31 A PsVs. The increased sensitivity of HPV31 B and C PsVs to cross-neutralizing antibodies was independent of the HPV vaccine received (Table 1).

All three HPV31 variant PsVs were susceptible to neutralization by a small panel of longitudinal serum samples (collected at 0, 6, 12, and 18 months) from women naturally infected with HPV31

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TABLE 1 Neutralization sensitivity of variant HPV31 L1L2 PsVs to HPV vaccine-induced antibodies

Vaccinee group	No. of serum samples	HPV31 A PsV titer[a]	HPV31 B PsVs		HPV31 C PsVs	
			Titer	Fold difference[b]	Titer	Fold difference
Cervarix vaccinees	22	1,026 (646–1,543)	1,469 (1,260–2,582)[c]	1.8 (1.1–2.5)	1,180 (923–1,721)[d]	1.3 (1.0–1.7)
Gardasil vaccinees	24	712 (382–1,363)	1,016 (759–1,435)[c]	1.5 (1.1–2.3)	968 (659–2,249)[e]	1.4 (1.1–1.6)
All vaccinees	46	885 (499–1,435)	1,273 (973–2,253)[e]	1.7 (1.1–2.4)	1,096 (763–2,216)[e]	1.4 (1.1–1.6)

[a] Neutralization titer data are presented as the median (IQR) 80% antibody neutralization titers generated from the 2 to 5 data sets per serum sample.
[b] Median (IQR) fold difference in the neutralization titers for HPV31 B PsVs and HPV31 C PsVs compared to the neutralization titer for HPV31 A PsVs.
[c] $P < 0.01$ using the Wilcoxon paired signed-rank test.
[d] $P < 0.05$ using the Wilcoxon paired signed-rank test.
[e] $P < 0.001$ using the Wilcoxon paired signed-rank test.

(Table 2). Median antibody neutralization titers against HPV31 A, B, and C PsVs were 576 (IQR, 391 to 1,144), 839 (IQR, 587 to 1,899), and 882 (IQR, 337 to 1,895), respectively.

Immunogenicity of variant HPV31 L1 VLPs. HPV31 L1 VLPs bearing L1 variant residues T267A and T274N (Fig. 2A), here referred to as HPV31 A, HPV31 B, and HPV31 C VLPs, were expressed and used to immunize BALB/c mice. All three preparations contained VLPs of various sizes, ranging from ca. 20 nm to up to 60 nm in diameter, with VLPs of >40 nm in diameter constituting 30% of the HPV31 B VLP preparations, 37% of the HPV31 A VLP preparations, and 57% of the HPV31 C VLP preparations (Fig. 2C).

The three variant PsVs demonstrated differential susceptibility to neutralization by mouse polyclonal serum containing antibodies to HPV31 VLPs (Fig. 3). Both HPV31 B PsVs (median $\log_{10}$ neutralization titer, 4.27; IQR, 3.75 to 4.55; Wilcoxon paired signed-rank test, $P = 0.008$) and HPV31 C PsVs (median $\log_{10}$ neutralization titer, 4.18; IQR, 3.86 to 4.49; $P = 0.007$) were more sensitive to neutralization by sera containing antibodies to HPV31 A VLPs than the homologous HPV31 A PsVs were (median $\log_{10}$ neutralization titer, 4.09; IQR, 3.54 to 4.26). HPV31 A PsVs were less sensitive to neutralization by sera containing antibodies to HPV31 B VLPs (median $\log_{10}$ neutralization titer, 3.74; IQR, 3.53 to 4.07; $P = 0.031$), while HPV31 C PsVs demonstrated increased sensitivity (median $\log_{10}$ neutralization titer, 4.12; IQR, 3.96 to 4.53; $P = 0.028$) compared to that of HPV31 B PsVs (median $\log_{10}$ neutralization titer, 3.98; IQR, 3.69 to 4.22). Both HPV31 B and C PsVs had similar sensitivities to neutralization by sera containing antibodies to HPV31 C VLPs (for HPV31 B PsVs, median $\log_{10}$ neutralization titer, 4.42 [IQR, 4.04 to 4.35]; for HPV31 C PsVs, median $\log_{10}$ neutralization titer, 4.34 [IQR, 4.08 to 4.55]), while HPV31 A PsVs demonstrated a reduced sensitivity (median $\log_{10}$ neutralization titer, 4.01; IQR, 3.84 to 4.14; $P = 0.021$). There were also differences in the magnitude of the antibody response on the basis of reactivity against homologous PsVs, with HPV31 C VLPs (median $\log_{10}$ neutralization titer, 4.34; IQR, 4.08 to 4.55) being slightly more immunogenic than HPV31 A VLPs (median $\log_{10}$ neutralization titer, 4.09; IQR, 3.54 to 4.26; $P = 0.018$) and HPV31 B VLPs (median $\log_{10}$ neutralization titer, 3.98; IQR, 3.69 to 4.22; $P = 0.006$).

TABLE 2 Neutralization sensitivity of variant HPV31 L1L2 PsVs to serum antibodies induced by natural infection

Sample	Detected variant	Time point (mo)	Neutralization titer[a]		
			HPV31 A PsVs	HPV31 B PsVs	HPV31 C PsVs
P1	HPV31 A	0	3,987	11,084	5,591
		6	1,363	2,284	2,870
		12	1,144	2,057	1,895
		18	391	2,616	401
P2	HPV31 C	0	—	—	—
		6	148	113	104
		12	68	54	58
		18	106	106	93
P3	HPV31 C	0	—	—	—
		6	1,890	661	882
		12	637	587	542
		18	337	377	232
P4	HPV31 C	0	486	1,357	337
		6	549	727	893
		12	1,497	620	2,378
		18	NA	NA	NA
P5	HPV31 C	0	638	1,899	679
		6	837	667	2,193
		12	511	839	1,208
		18	576	982	1,235

[a] —, neutralization titers of <50 were assigned a value of 25 for calculation purposes; NA, not available.

Antigenicity of variant HPV31 L1 VLPs and L1L2 PsVs. HPV31 L1 MAbs against immunogens representing the HPV31 reference sequence (GenBank accession number J04353) were previously generated (39). The majority of MAbs were raised against L1L2 VLPs; the exceptions were MAbs 31.D24 and 31.A19, where L1 VLP immunogens were used. Generally, all the type-specific MAbs bound L1 VLPs and L1L2 PsVs representing the lineage variants A, B, and C at similar 50% binding concentrations (in micrograms per milliliter) by ELISA; the exceptions to this were MAb 31.F16, which demonstrated a higher binding concentration against the HPV31 B PsVs than the HPV31 A PsVs, while both MAb 31.H12 and MAb 31.H17 bound L1 VLPs representing HPV31 lineage variants B and C at lower concentrations than L1 VLPs representing HPV31 lineage variant A. Although the cross-reactive MAbs (MAbs 31.D24, 31.B5, 31.C19, and 31.E22) bound the L1 VLPs, they did not bind or neutralize the L1L2 PsVs (Table 3). All type-specific MAbs were able to neutralize the three variant PsVs to similar orders of magnitude (Table 3). HPV31 C PsVs demonstrated an increased sensitivity, ca. 3.5-fold, to neutralization by FG loop MAb 31.F16 and a ca. 4.0-fold increase in sensitivity to neutralization by MAb 31.H17 (epitope unknown) compared to the sensitivity of HPV31 A and B PsVs (Table 3).

The three neutralizing FG loop MAbs (MAbs 31.B1, 31.F16,

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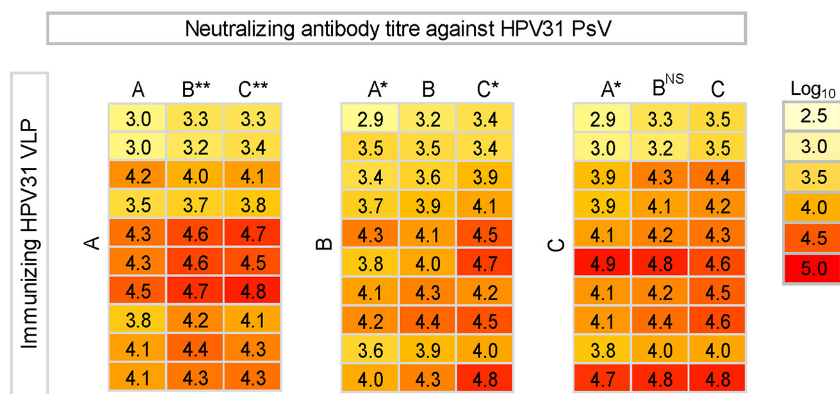

FIG 3 Heat maps representing the potential of serum from mice immunized with variant HPV31 L1 VLPs to neutralize variant HPV31 L1L2 PsVs. The $\log_{10}$ neutralization titers of sera from BALB/c mice ($n = 10$) following variant HPV31 VLP immunization carried out over three separate schedules are presented as the averages for two data sets per sample. The key on the right indicates the $\log_{10}$ heat map gradient. P values, obtained using the Wilcoxon paired signed-rank test, represent differences in median neutralization titers from homologous variant VLP and PsV pairs. *, $P < 0.05$; **, $P < 0.01$; NS, no significant difference ($P > 0.05$).

and 31.H12) recognize conformational epitopes which encompass variant amino acid position 267 and are adjacent to amino acid 274 (Fig. 4A). The FG loop of monomer 1 (FG_1) is adjacent to the BC_5, DE_1, DE_5, EF_1, HI_4, and HI_5 loops within the capsomer (Fig. 4B), and residues 267 and 274 are within close proximity (within 10 Å) to residue positions predominantly within the adjacent BC_5, FG_1, and HI_5 loops (Fig. 4C and D). These include lysine residues at position 279 within the FG loop and position 362 within the HI loop.

DISCUSSION

This study attempted to evaluate the potential impact of nonsynonymous SNPs within the HPV31 L1 and L2 genes on capsid protein antigenicity and immunogenicity. We generated additional HPV31 L1 and L2 sequences to supplement those already available and created L1L2 PsVs and L1 VLPs representing lineage variants A, B, and C to evaluate lineage-specific immunogenicity and antigenicity, including susceptibility to antibody-mediated neutralization.

All three variant L1L2 PsVs were susceptible to neutralization by vaccine-induced cross-neutralizing antibodies. HPV31 B and C PsVs demonstrated an increased sensitivity to neutralization compared to that of HPV31 A PsVs, but the difference was of a low magnitude. The cross-protection afforded by the current prophylactic vaccines is an unexpected additional benefit, although no correlate of protection has been defined (10). If cross-neutralizing antibodies are determined to be the immune effectors of vaccine-induced cross-protection, it is important to demonstrate that the contemporary circulating HPV31 variants, represented by L1L2 PsVs, do not exhibit resistance to cross-neutralization by such antibody specificities.

TABLE 3 Sensitivities of variant HPV31 L1 VLPs and L1L2 PsVs to MAbs[a]

					MAb IgG concn[b] (μg/ml)								
					L1 VLP ELISA			L1L2 PsV ELISA			L1L2 PsV neutralization		
Epitope	MAb	Structure	Specificity	Neutralizing	A	B	C	A	B	C	A	B	C
FG loop	31.B1	C	TS	Yes	1.01	0.49	0.53	4.48	4.85	3.78	9.48	7.17	6.34
	31.D24	L	XR	No	0.041	0.016	**0.013**	—	—	—	—	—	—
	31.F16	C	TS	Yes	0.050	0.038	0.035	0.0075	**0.11**	0.0061	0.31	0.35	**0.090**
	31.H12	C	TS	Yes	0.93	**0.28**	**0.29**	1.09	1.78	1.37	0.25	0.52	0.58
Unknown	31.A19	C	TS	Yes	1.63	1.89	1.57	1.65	4.27	3.28	3.71	3.59	1.44
	31.B5	L	XR	No	28	26	21	—	—	—	—	—	—
	31.C19	C	XR	No	25	15	25	—	—	—	—	—	—
	31.C24	C	TS	Yes	19	18	16	23	37	38	31	38	11
	31.E16	C	TS	Yes	12.00	5.99	7.28	7.70	8.16	12.00	79	104	66
	31.E22	L	XR	No	207	167	153	—	—	—	—	—	—
	31.H17	C	TS	Yes	0.26	**0.068**	**0.089**	0.37	0.72	0.41	0.204	0.156	**0.051**

[a] Epitope location, structure (C, conformational; L, linear), specificity (TS, type specific; XR, cross-reactive), and MAb neutralizing potential were taken from the work of Fleury et al. (39). —, MAbs for which binding or neutralization concentrations could not be determined at the highest input concentrations (by ELISA, 250 μg/ml for all MAbs except 31.D24, for which the highest input concentration was 20 μg/ml, and by neutralization assay, 125 μg/ml for all MAbs except 31.D24, for which the highest input concentration was 10 μg/ml). Concentration values in bold indicate a ≥3-fold difference from the concentration obtained for the antigen representing HPV31 variant A for a single MAb within an assay format.

[b] For the L1 VLP ELISA and L1L2 PsV VLP ELISA, the 50% binding concentration; for L1L2 PsV neutralization, the average 80% neutralization concentration. All results are averages from 2 to 3 experiments per assay format.

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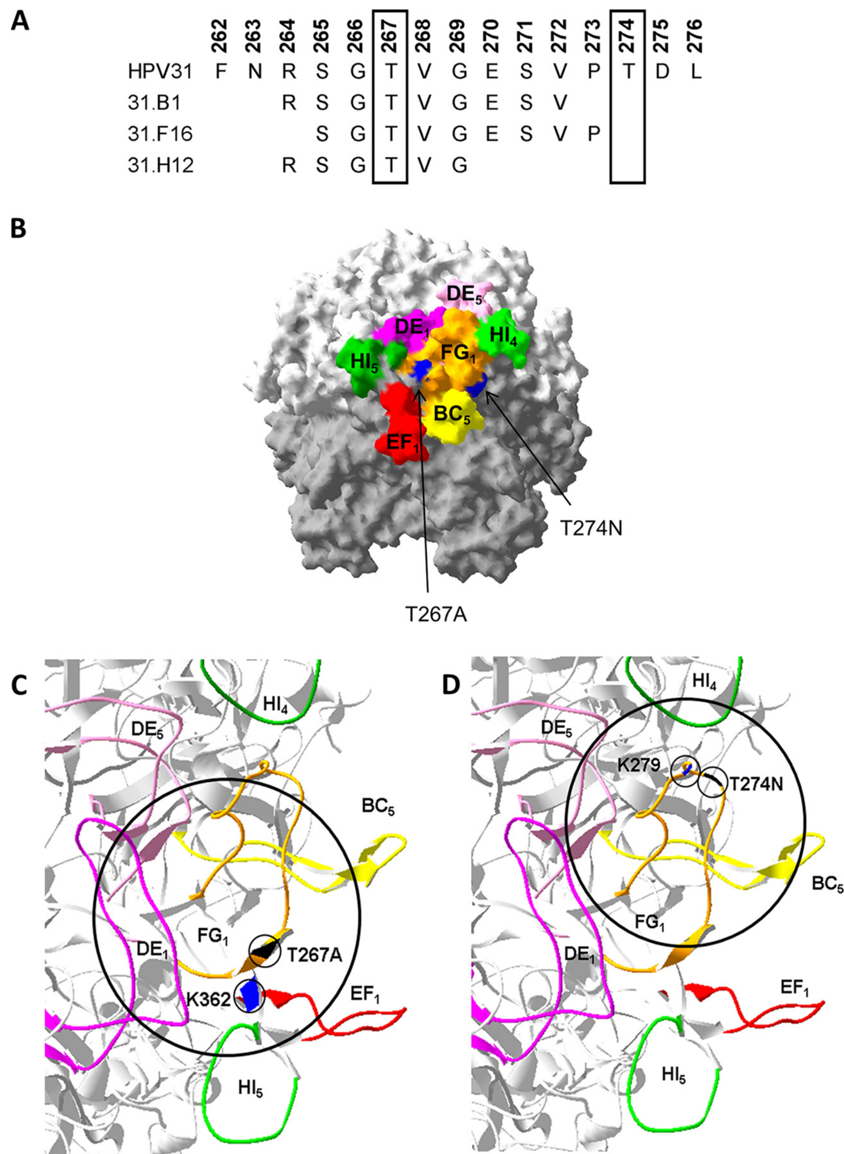

FIG 4 Crystal model surface highlighting HPV31 FG loop variant residue locations. (A) Linear amino acid epitope footprint of HPV31 FG loop MAbs. (B) Side view highlighting loops in close proximity to FG loop variant residues 267 and 274. (C and D) Top view of loop ribbons. Circled areas indicate regions within a 10-Å radius of residues 267 (C) and 274 (D), as determined by the Swiss-PDP viewer algorithm. Blue, lysine residues at positions 279 and 362; orange, FG loop of monomer 1 (FG$_1$); black, residues 267 and 274. Neighboring loops on the same monomer (dark pink, DE$_1$; red, EF$_1$) or adjacent monomers (dark green, HI$_4$; yellow, BC$_5$; light pink, DE$_5$; light green, HI$_5$) are indicated. The remaining surface-exposed regions of the capsomer are colored in light gray, and core regions are colored in dark gray.

The increased sensitivity of HPV31 B and C PsVs to cross-neutralization suggests that the Asn residue at position 274, which is common to both variants, enhances the recognition of HPV31 L1 epitopes by cross-neutralizing antibodies produced against vaccine HPV16 L1 VLPs. It is unlikely that the Asn residue in itself has a critical role within a cross-neutralizing epitope, since the switch from Thr and Asn is a relatively subtle change, as both amino acids have polar uncharged side chains. However, the change of residue at position 274, near the tip of the FG loop, may result in local structural changes which increase the level of recognition of more distal epitope residues.

Subtle differences in variant antigenicity were identified when the activities of a panel of HPV31 MAbs against the HPV31 lineage variants was tested in a neutralization assay. HPV31 C PsVs demonstrated increased sensitivity to neutralization by the FG loop MAb 31.F16 in comparison to that of both HPV31 A and B PsVs, indicating that the double residue switch at positions 267 (T267A) and 274 (T274N) impacts MAb 31.F16 epitope recognition. It has previously been demonstrated by comparison of L1 pentamer crystal structures from different genotypes (HPV11, HPV16, HPV18, and HPV35) that L1 antigenic determinants can be altered by a shift of a few angstroms within the loop as a result of a single residue substitution (28).

In contrast, the other two FG loop MAbs (MAbs 31.B1 and 31.H12) neutralized all variants to a similar extent. These data corroborate previous data from a bacterial cell surface display

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model which demonstrated that these three MAbs recognize overlapping, yet distinct, FG loop epitopes (36). Residues 267 and 274 are in close proximity to the Lys279 and Lys362 residues or near the Lys54, Asn57, Lys60, and Lys367 residues. The corresponding residues of HPV16 are involved in HPV binding to heparin sulfate (50), which is an essential step for a successful HPV infection, and the FG loop MAbs may neutralize by abrogating this virus-host interaction.

The panel of HPV31 MAbs bound all three variant L1 VLPs, when used as target antigens in an ELISA format, indicating that the residues at positions 267 and 274 were not critical in the epitope footprints recognized by this panel of MAbs. However, when variant L1L2 PsVs were used as the target antigens, the 50% binding concentration of the four cross-reactive MAbs (MAbs 31.D24, 31.B5, 31.C19, and 31.E22) could not be determined due to the reduction in epitope recognition. This observation implies that inclusion of the L2 protein within the PsV capsid alters L1 epitope exposure and therefore impacts L1 protein antigenicity. It has been reported that a subset of HPV16 MAbs demonstrated reduced binding to L1L2 PsVs compared to L1 VLPs, with the differential binding being thought to be as a result of L2 altering the conformation or availability of L1 epitopes (51). These findings imply that the capsids of native HPV virions, represented by L1L2 PsVs and the L1-only VLPs within the prophylactic vaccine preparations, differ in their L1 antigenicity.

All three variant L1L2 PsVs were susceptible to neutralization by the HPV31 VLP antibodies generated in mice; however, differences in neutralization sensitivity were evident. Both HPV31 B and C PsVs demonstrated increased sensitivity to antibody-mediated neutralization in comparison to HPV31 A PsVs, irrespective of the variant L1 VLPs used as the immunogen. These findings are in line with those of a previous study of HPV16 variants which demonstrated that sera containing antibodies raised against an HPV16 European variant were able to neutralize pseudoviruses representing a range of geographical variants of HPV16, with a $\leq$4-fold difference in neutralization titer between the homologous and heterologous types being detected, leading to the conclusion that HPV16 variants belong to a single serotype (29).

The criterion used to designate serotypes is generally based upon a fold difference in antibody-mediated neutralization titers between viral types, which differ in magnitude and range between virus families: for adenovirus, 8- to 16-fold (52); for rotavirus, $\geq$20-fold (53); and for polyomavirus, 4- to 100-fold (54). For HPV there are no currently defined criteria with which to designate L1 serotypes. It is reasonably clear that HPV genotypes induce high-titer, type-specific neutralizing antibody responses which represent different serotypes (55–57). However, for lineage variants, the relationship between L1 sequence and antigenicity is less clear (29, 58).

Although HPV31 lineage variants demonstrated differences in susceptibility to neutralization by antibodies elicited by vaccination or preclinical L1 VLP immunization and MAbs, the difference was $<$4-fold, and under this criterion, as defined for HPV16 (29), HPV31 variants should be considered to belong to a single serotype. This implies that the choice of a representative HPV31 L1 sequence for VLP-based vaccines is not critical.

Given the relatively low prevalence of HPV31 (59), only a small panel of serum samples from HPV31 DNA-positive women was available. These data suggest that all three HPV31 lineage variants were susceptible to neutralization by antibodies derived from natural infection. However, further work will be required to address this issue appropriately by utilizing a larger panel of samples with an equal representation of variant lineages.

There are potential shortcomings to this work. L1 VLP immunizations were carried out using a relatively small number of animals, and while all three constructs induced neutralizing antibodies against all three variant PsVs, the variability inherent in using small groups of animals may have concealed subtle differences in immunogenicity. Although HPV L1L2 PsVs have been used widely to monitor antibody responses to vaccines and natural infection (22, 47, 48, 60), as well as elucidate steps in the entry process (61–64), there are likely to be some differences between how these behave *in vitro* and how authentic HPV31 lineage variants behave *in vivo*, although this is a limitation of most PsV-based systems.

Despite these caveats, these data suggest that HPV31 lineage variant PsVs display similar sensitivities to recognition by antibodies elicited following vaccination with the current HPV vaccines and after preclinical HPV31 L1 VLP immunization, indicating that HPV31 variants belong to a single L1 serotype. Such data may be useful to guide modeling of the impact of the current L1 VLP vaccines and informing postvaccination surveillance programs. These data also inform our understanding of the antigenicity of the HPV structural proteins.

ACKNOWLEDGMENTS

The sera from young girls who received Gardasil or Cervarix came from a study funded by the Department of Health Policy Research Programme (National Vaccine Evaluation Consortium, 039/0031).

The views expressed in this publication are those of the authors and not necessarily those of the Department of Health.

We are indebted to John T. Schiller and Chris Buck (National Cancer Institute, Bethesda, MD) for access to the p31sheLL clone. We thank Ilaria Epifano from the University of Milano-Bicocca, Monza, Italy, for the natural infection HPV31 DNA and antibody status data.

We declare no conflicts of interest.

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