Impact of naturally occurring variation in the human papillomavirus (HPV) 33 capsid proteins on recognition by vaccine-induced cross-neutralizing antibodies

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Abstract
We investigated naturally occurring variation within the major (L1) and minor (L2) capsid proteins of human papillomavirus (HPV) genotype 33. Pseudoviruses (PsV) representing HPV33 lineages A1, A2, A3, B and C exhibited comparable particle-to-infectivity ratios and morphology but demonstrated a decreased sensitivity (A2, A3, B and C) to cross-neutralization by HPV vaccine antibodies compared to the A1 sublineage. Chimeric PsVs demonstrated that these differences in sensitivity were due to polymorphisms in the L1 protein, with little or no influence from variation within the L2 protein. Site-directed mutagensis of the L1 gene identified the DE loop residue 133 and the FG residue 266 as being critical for conferring this differential sensitivity. The use of HPV33 homology models based upon the HPV16 crystal structure suggested that they are likely to act independently on more than one antibody epitope. These data improve our understanding of the potential impact of natural capsid variation on recognition by vaccine antibodies.

INTRODUCTION
Human papillomavirus (HPV) is a small, circular double-stranded DNA virus associated with the development of cervical cancer and other types of anogenital and head and neck cancers [1]. HPV replicates via host cell polymerases with an error rate of ca. 2 × 10⁻⁸ base substitutions per site per year, 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, distinct genotypes and intra-genotype variant lineages have arisen over time [3]. Only about a dozen or so genotypes from the Alpha-papillomavirus genera are associated with the development of cervical cancer [4], although the contribution of each genotype is different with HPV16 and HPV18 alone accounting for ca. 70% of cervical cancer cases [5].

HPV33 (related to HPV33 within the Alpha-9 species group) is associated with ca. 0.5% of women with normal cytology and accounts for ca. 4% of cervical cancer cases worldwide with some geographical bias in the attributable proportion [5]. Whole-genome sequence analysis of HPV33 strains has led to the delineation of three distinct variant lineages (A, B and C) and three sublineages (A1, which includes the prototype sequence M12732, A2, and A3) [6, 7]. Sublineage A3 and lineage C appear to be quite rare and have thus far been found only in Asia-Oceania and Africa, respectively, while sublineages A1 and A2 appear to be distributed widely although there may be some geographical bias (Fig. 1a). Although sublineage A1 appears to be over-represented in cervical cancer compared to the other lineages [7], data on their contribution to the risk of cervical cancer risk progression are somewhat lacking due to the relatively low prevalence of individual lineage and sublineages in the population [8, 9].

The HPV capsid consists of 360 copies of the major capsid protein L1, arranged into 72 pentamers with an upper estimate of one molecule per pentamer of the minor capsid protein, L2 [10, 11], although the distribution of the L2 protein may be asymmetrical and/or stochastic [12]. The L1 protein facilitates virus attachment to host cells, whilst the L2 protein is essential for subsequent virus infectivity. The L1 protein can self-assemble into virus-like particles (VLPs), which are the basis of the current bivalent (Cervarix) and quadrivalent (Gardasil) prophylactic HPV vaccines [13]. The HPV vaccines have demonstrated remarkable efficacy against vaccine-incorporated genotypes [14], supported by pre-clinical evidence that neutralizing antibodies are likely to play a role in mediating type-
Fig. 1. HPV33 L1 and L2 variation. (a) Geographical distribution of HPV33 variants [7]. (b) Neighbour-joining tree reconstructed using concatenated L2 and L1 sequences derived from published full-length genome sequences (GenBank accession numbers M12732, EU918766, EU918766, KU298891, KU298892 and HQ537688–HQ537707) [6, 45, 46]. Sublineage attribution (A1, A2, A3, B and C) is based upon whole-genome sequences. A further sublineage, A1b, was designated for the purpose of this study. (c) Inter- and intra-lineage sequence diversity. (d) PsV preparations characterized for particle dimension in nanometres [nm; median, inter-quartile range (IQR)], infectivity, L1 concentration and resulting particle-to-infectivity ratio. TCIDso, 50 % tissue culture infectious dose.
specific protection [15–17]. The HPV vaccines also demonstrate efficacy against some genetically related non-vaccine-incorporated genotypes (HPV31, HPV33 and HPV45) [18], supported by the detection of cross-neutralizing antibodies in the serum and genital secretions of vaccinated individuals [19–22].

The HPV L1 external loops are the target for the majority of type-specific neutralizing antibodies [23, 24], so it is reasonable to consider that lineage-specific variation in these surface-exposed loop domains may impact antibody recognition [25]. However, data informing the potential impact of such variation on L1 antigenicity are limited to the examination of HPV16, HPV31 and HPV45 using lineage-specific variants. PsVs representing HPV16 L1, but not L2, variants exhibit little difference in their susceptibility to type-specific antibodies elicited by HPV16 VLP [26]. PsVs incorporating HPV31 L1 and L2 lineage variants (A, B and C) exhibited low-magnitude lineage-specific differences in sensitivity [27], while HPV45 sublineages (A1, A2, A3, B1 and B2) exhibited differential sensitivity to vaccine antibodies, mediated by a single residue in the HI loop of HPV45 L1 [28]. Here we examine the potential impact of lineage and sublineage L1 and L2 HPV33 variation on sensitivity to cross-neutralizing antibodies elicited by the bivalent and quadrivalent HPV vaccines.

RESULTS

HPV33 amino acid variation and pseudovirus generation

The concatenated L2L1 (2.9 kb) DNA fragment alone contained sufficient numbers of diagnostic motifs to segregate sequences into the sublineages A1, A2, A3, B and C previously defined [3, 6] by whole-genome sequence analysis (Fig. 1b). A cluster of three distinct A1 sequences was considered as a separate sublineage (A1b) for the purpose of this study. A consensus sequence for each sublineage was determined (Fig. 1c), and bicistronic psheLL vectors containing codon-optimized HPV33 L1 and L2 genes representing these variants were generated. HPV33 variant PsVs displayed similar particle sizes (median 53 nm; IQR 51–56) and particle-to-infectivity ratios (median 3.5/C2102; IQR 2.5/C2102–6.9/C2102) (Fig. 1d).

Sensitivity of variant HPV33 PsVs to antibody-mediated neutralization

Sera from 12- to 15-year-old girls collected following three doses of bivalent or quadrivalent HPV vaccine were used to assess HPV33 lineage and sublineage variant sensitivity to cross-neutralizing antibodies using a PsV-based neutralization assay. Neutralizing antibody titres against the A1 and A1b sublineages were similar: median (IQR) 48 (39–88) and 44 (39–83; n=52), respectively. Conversely, PsV variants A2, A3 and B and C exhibited ca. twofold decreased sensitivity to cross-neutralizing antibodies compared to the A1 PsV (P<0.001; Wilcoxon Signed-Rank test) (Fig. 2a). This decreased sensitivity was more marked [median (IQR) fold decrease of 5.5 (2.4–17.9); n=12] when considering those individual sera that had titres greater than 100 against the A1 sublineage [median (IQR) titre 260 (181–578)]. No difference in the magnitude of these differences was observed between the two vaccines (Fig. 2b, c) (Mann–Whitney U test, P=0.827).
Identification of key residues on the L1 loops for cross-neutralizing antibodies

Variable residues in the BC (residue 56), DE (residue 133) and FG (residue 266) loops of the L1 protein and two residues (residues 350 and 372) in the L2 protein (Fig. 1c) appeared to track with these differences in neutralizing antibody sensitivity (Fig. 2a).

To investigate the influence of the L2 site variations (residues 350 and 372) on the decreased sensitivity to neutralizing antibodies, two chimeric PsVs based upon the sublineages A1 and A2, A11,A212 and A211,A112, were generated (Fig. 3a). These PsVs displayed particle-to-infectivity ratios and dimensions similar to those of the variant PsVs (data not shown). The chimeric PsVs were tested alongside the consensus A1 and A2 PsV using a subset of vaccine sera (Fig. 3b). Median (IQR) neutralizing antibody titres against the A11,A212 chimera and the consensus A1 PsV were similar: 84 (43–256) and 101 (45–253), respectively. The A211,A112 chimera exhibited a median 4.5 (3.5–7.8)-fold reduction in sensitivity, which was similar to the 4.7 (3.5–9.7)-fold reduction exhibited by the consensus A2 PsV. These data indicate that substitutions in the L1 sequence of A2 (Fig. 1c) were primarily responsible for the noted decreased antibody sensitivity and that there was little or no influence of the L2 protein in this respect.

We next generated three mutant HPV33 A1 PsVs to examine the impact of individual substitutions in the BC (T56N), DE (G133S) and FG (T266K) loops on sensitivity to cross-neutralizing antibodies (Fig. 3a). Relative to a median (IQR) titre of 92 (46–211) for the HPV33 A1 PsV, substitutions G133S and T266K exhibited median (IQR) reductions in neutralizing antibody sensitivity of 1.9 (1.6–3.4) and 1.4 (1.2–4.0)-fold, respectively, while substitution T56N [1.0 (1.0–1.4)-fold] had little or no impact. Substitution T266K is also present in the A1b variant, suggesting that K135R and/or G268E substitutions (Fig. 1c) act in a compensatory manner to rescue the A1 phenotype (Fig. 2a).

A mutant A1 PsV containing both G133S and T266K substitutions displayed similarly reduced sensitivity to neutralizing antibodies as PsV A2 (Fig. 3c). Furthermore, a PsV in which the reciprocal DE (S133G) and FG (K266T) residue sequences were created in the context of the A2 sublineage variant demonstrated similar sensitivity to the consensus A1 PsV (Fig. 3c). Taken together, these data demonstrate that two L1 residues in the DE (residue 133) and FG (residue 266) loops are sufficient to confer the differential sensitivity of sublineages A1 and A2 PsV to cross-neutralizing antibodies elicited by the HPV vaccines.

Prediction of HPV33 L1 loop topography

We next used the crystal structure of the HPV16 L1 pentamer to create homology models of HPV33 A1 and A2 L1 variants (Fig. 4). The quality of the predicted models was demonstrated by their Qualitative Model Energy Analysis (QMEAN4) Z-scores, which were −2.66 and −2.89 for the A1 and A2 models, respectively, and by their Global Model Quality Estimation (GMQE) score, which was 0.98 for the two models. Pairwise model comparison of HPV33 A1 with A2 was performed by superimposition (root mean square deviations of 0.12 Å). The model predicted no difference in the DE (G133S) and FG (T266K) loop structures (Fig. 4c), while the BC (T56N) loop exhibited a projected shift of a mean ± standard error (± s.e.) of 3.02±0.74 Å. This shift appeared to have no impact on recognition by cross-neutralizing antibodies (Fig. 3c). Notably, residue 133 on the DE loop is located a mean 22.3 (±0.5) Å distance from residue 266 on the FG loop.

DISCUSSION

This study evaluated the potential impact of naturally occurring polymorphisms within the HPV33 L1 and L2 genes on capsid protein antigenicity. We generated L1L2 PsVs representing lineage and sublineage variants A1, A1b, A2, A3, B and C and evaluated their susceptibility to cross-neutralization by HPV vaccine-induced antibodies. While no difference was observed between the A1 and A1b variants, lineage variants A2, A3, B and C demonstrated a reduced sensitivity to cross-neutralization compared to the A1 sublineage. Residues within the DE loop (residue 133) and the N-terminal portion of the FG loop (residue 266) appeared to be necessary and sufficient to confer this differential neutralization phenotype.

Recent cryo-electron microscope analyses have demonstrated that the epitope footprints recognized by a number of HPV16 mAbs include amino acid residues from multiple L1 loops [29–31]. The epitope of the H16.V5 mAb, for example, includes loops from two neighbouring L1 monomers, with the majority of contact residues predicted to be in the DE and FG loops but with a small contribution from residues located in the EF and HI loops. Mapping of type-specific neutralizing mAb epitopes for HPV33 [32] has shown that several variable loops contribute to form these conformational epitopes. The binding site of mAb H33.B6 included amino acids from the DE and the C-terminal portion of the FG loop, while the binding site of mAb H33.E12 appeared to require residues in the DE loop and both the N- and C-terminal portions of the FG loop. Vaccine-induced cross-neutralizing antibodies against HPV31 appear to require residues in the DE loop and the C-terminal portion of the FG loop [33]. In the present study, the residues in the DE (residue 133) and N-terminal FG (residue 266) loops, responsible for conferring the observed phenotype, were discrete, suggesting that they act independently upon more than one antibody epitope or by subtle topographical changes not predictable by homology modelling.

HPV PsVs have been used widely to monitor antibody responses to vaccines and natural infection [20–22, 34–36], as well as to elucidate steps in the entry process [37]. Nevertheless, differences between how PsVs behave in vitro and how authentic HPV33 lineage variants behave in vivo are uncertain, although this is a limitation of all PsV-based systems.
The nonavalent Gardasil9 vaccine, comprising additional genetically related genotypes HPV31, HPV33, HPV45, HPV52 and HPV58, has demonstrated efficacy in a three-dose schedule [38], has the potential to reduce the incidence of cervical cancer by over 90% [5, 39], and will likely be adopted by many national immunization programmes in time. To date, however, tens of millions of adolescent girls have been vaccinated with the bivalent or quadrivalent vaccine [40], for which a better understanding of their immunogenic properties, including the seroconversion rates, breadth, magnitude and durability of the antibody responses against non-vaccine genotypes, is warranted.

Whether differences in PsV variant sensitivity noted here will influence the prevalence of individual variant lineages over time in countries that have introduced national vaccination programmes will require further study. These data inform our understanding of the antigenicity of the HPV structural proteins and may be useful to guide impact modelling of the current HPV vaccines and inform post-vaccine surveillance programmes.

**METHODS**

**Study samples**

Serum samples were available from 12, to 15-year-old girls randomized to receive three doses of Cervarix or Gardasil as part of a Phase IV clinical trial comparing HPV vaccine immunogenicity (www.clinicaltrials.gov: NCT00956553; REC number 09/H0720/25) [20]. A subset of samples were selected for analysis based upon HPV33 cross-neutralizing antibody titres (n=52).

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Fig. 3. Neutralization sensitivity of mutant HPV33 L1L2 pseudo viruses. (a) Graphical representation of HPV33 L1 and L2 protein combinations from A1 (white) and A2 (grey). (b, c) Box (median, IQR) and Whisker (10th and 90th percentiles) plots of fold difference in neutralization titres from the A1 lineage variant PsV for (b) chimeric (A1\_1A2\_2, A2\_1A1\_2) and (c) mutagenized PsV and A2 lineage variant PsVs for a subset of vaccine sera (n=16). *, P<0.05; **, P<0.01; ***, P<0.001 (Wilcoxon Signed-Rank test).

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Fig. 4. Homology modelling of HPV33 sublineage variants. HPV33 sublineage variant homology models based upon HPV16 L1 pentamer crystal (PDB code: 2R5H) with top (a) and side surface-filled views (b) shown for HPV33 A1, with external loops indicated by dark grey shading. All five L1 monomers of the capsid are pictured, with external loops adjacent to sites of interest highlighted as indicated (yellow BC\_5; pink DE\_1; red EF\_1; orange FG\_1 and green HI\_5). (c) Superposition model for HPV33 A2 (blue) relative to HPV33 A1 (gold) used to indicate relative shifts in the position of the BC, DE and FG loops. BC loop residue 56, DE loop residue 133 and FG loop residue 266 are indicated.
L1L2 pseudovirus

Bicistronic psheLL vectors containing codon-optimized HPV 33 L1 and L2 genes representing consensus lineage variant sequences (GeneArt; Thermo Fisher Scientific) were generated and used to transfect 293TT cells. Additional chimeric or mutagenized PsVs were made by standard restriction enzyme digestion and site-directed mutagenesis (QuickChange Site-Directed Mutagenesis Kit; Agilent Technologies) techniques. All inserts were confirmed by Sanger sequencing. PsVs were purified by ultracentrifugation on an iodixanol (Sigma-Aldrich) gradient following the alternative protocol as previously described [19]. Particle formation and size were determined by electron microscopic analysis of negatively stained particles. Ten PsV particles from each preparation were measured in nanometres (nm) and the median diameter and inter-quartile range (IQR) were calculated. The equivalent of a 50 % tissue culture infectious dose (TCID$_{50}$) was estimated for each PsV preparation using the Spearman-Karber method. The L1 concentrations of PsV stocks were determined by semi-quantitative Western blot using the CamVir-1 antibody (Abcam). Particle-to-infectivity ratios were determined on the basis of an estimated particle amount of $3 \times 10^7$ particles per ng L1 protein (http://home.ccr.cancer.gov/lco/production.asp), with the ratio normalized for the input volume and the TCID$_{50}$.

Neutralization assay

The PsV neutralization assay [35] was performed as previously described [19]. A standardized input of 300 TCID$_{50}$ was used for all PsVs. At this input level, the median output RLU was more than three Log$_{10}$ (median 3.37, IQR 3.26–3.56)-fold over background RLU and well within the linear dynamic range of the assay ($r^2=0.987$). The median amount of L1 protein at this level of input was 0.1 (IQR 0.06–1.3) ng ml$^{-1}$. These data are consistent with our previous report [19]. Serum samples were subjected to serial dilutions, with the antibody titre, resulting from an 80 % reduction in the luciferase signal produced by control wells containing PsV and cells only, estimated by interpolation. For analysis purposes, serum samples with titres lower than the lower limit of detection (LOD, 20) were assigned a censored value of 10. The median neutralization titre of a high HPV16/18 antibody plasma pool [41] against HPV33 A1 was 51 (IQR 49–54; n=8), while the HPV-negative plasma pool was negative (titre <20; n=8) in all tests.

Homology modelling

HPV33 L1 homology models were created from the L1 amino acid sequences of A1 and A2 variants using the SWISS MODEL (http://swissmodel.expasy.org/) [42, 43]. The crystal structure of the HPV16 L1 capsomer (PDB code: 2RSH) was used as template [24]. The quality of a predicted model was assessed by their Qualitative Model Energy Analysis (QMEAN4) and Global Model Quality Estimation (GMQE) scores [44]. DeepView Swiss-Pdb viewer v4.0 was used to perform pairwise L1 model comparisons by superimposition, and predicted structural differences between models were measured in Å. The superimposition of L1 homology models was supported by a RMS value, which represents the average Å distance between corresponding atoms in the two models.

Statistical analysis

The Wilcoxon Signed-Rank test was used to compare neutralization titres between two HPV33 variants, and the Wilcoxon Rank-Sum (Mann–Whitney U) to test for differences in the magnitude of responses between vaccines. All tests were performed using the statistical package Stata 13.1 (StataCorp, College Station, TX).

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

Samples were available from a Phase IV clinical trial comparing HPV vaccine immunogenicity (www.clinicaltrials.gov: NCT00956553; REC number 09/H0720/25).

References


