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ORIGINAL INVESTIGATION

Genetic variability of human papillomavirus type 66 L1 gene among women presenting for cervical cancer screening in Chile

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Abstract

The high-risk human papillomaviruses (HR-HPVs) are involved in the development of cervical cancer. Nevertheless, there are differences in the oncogenic potential among them. HPV-16 and HPV-18 are associated with approximately 70% of cancer worldwide, and both types are the most extensively studied HR-HPV. Great variations in the prevalence of HR-HPV have been described in different countries. The impact of these variations on the epidemiology of lesions and cervical cancer is currently unknown. A high prevalence of HPV-66 has been detected in Chile. Here, we have analyzed the genetic variability of the L1 gene from HPV-66-infected Chilean women. Higher order interactions between identified mutations were analyzed by co-variation and cluster analyses. Antigenic-index alterations following L1 mutations and B-cell epitopes were predicted by BcePred algorithm. HPV-66 L1 sequences clustered phylogenetically into two main clades. The genetic variability in the HPV-66 L1 gene involved thirty nucleotide changes. Four of these were for the first time identified in this study. Some of these variants are embedded in the B-cell epitope regions. Amino acid homology in the immunodominant epitopes of HPV-66 L1 protein (DE, FG and H1 loops) was 42.9–59.1% and 28.6–68.9% compared with HPV-16 and HPV-18, respectively. The results of this research suggest that the neutralizing epitopes of HPV-66 are antigenically different compared to HPV-16 and HPV-18. Our findings show the need to perform new structural and immunological studies on HPV-66 L1 protein to evaluate the cross-protection conferred by current HPV vaccines.

Keywords Human papillomavirus · Genetic variability · HPV-66 · L1 gene

Introduction

The *Papillomaviridae* family comprises five genera (Alpha, Beta, Gamma, Mu, and Nu) according to the phylogenetic relationships of their complete L1 gene sequences [1, 2]. As of February 28th, 2019, 226 HPV types were officially recognized by the International HPV Reference Center at the Karolinska Institute in Sweden [3]. More than 40 human papillomavirus (HPV) types have been identified infecting the genital tract and they have been grouped into the Alpha genus [1]. Among these, 18 types have been associated with anogenital cancer and called high-risk (HR) HPV [4, 5]. Other types, called low-risk (LR) HPV, have been associated with the development of benign lesions (condylomas). Among HR-HPV types, HPV-16 and HPV-18 are the most prevalent and account for approximately 70% of all cervical cancers in the world [6]. HPV-16 and HPV-18 are taxonomically grouped into two different branches, each together with six different HPV types, called α-9 and α-7 species, respectively [1]. A third branch, called α-6, includes HPV-30, 53, 56, and 66 types. The genetic and biologic characteristics of α-9 and α-7 species, especially HPV-16 and HPV-18, have been extensively studied. However, little is known about the genetic features of the α-6 species. Previous studies have shown intratypic genetic variants in HPV-66 [7–12].

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Nevertheless, no phenotypic or biological characteristic has been associated with these HPV-66 variants until now.

The prevalence of HPV types may vary in different parts of the world [13–16]. The worldwide studies show that the prevalence of HPV-66 has great geographical variations, which do not necessarily coincide with the distribution of other HR-HPVs, such as HPV-16 or HPV-18. Different studies conducted in Europe, America, Africa and Asia have shown HPV-66 prevalence from 1.3 to 12.9% among women attending cervical screening (Table 1) [12, 17–23]. Also, HPV-66 was the most prevalent (30%) HPV type among Croatian women with abnormal cervical cytology [24].

The introduction of HPV vaccine has been a fundamental strategy within national cancer control programs. The HPV vaccination program with the quadrivalent vaccine, which uses HPV-6, 11, 16 and 18 antigens, has been implemented in Chilean girls aged 9–11 years since 2014. Although HPV-16 and HPV-18 vaccines would confer cross-protection against HPV-31, HPV-33 and HPV-45 infections, it has not shown immunity against other non-vaccine HPV types, i.e., HPV-39, HPV-51, HPV-56, HPV-58, HPV-59, and HPV-66 [25]. Previous studies have shown a significant prevalence of HPV-66 among Chilean women attending cervical screening programs [26, 27]. Thus, it is necessary to characterize the genomic diversity of the HPV-66 because this knowledge could be useful to evaluate the efficacy of HPV vaccination program in populations with a high prevalence of HR-HPV non-vaccine strains. Furthermore, these studies would be desirable to evaluate public health improvements and cost savings that can be achieved by introducing and switching to new HPV vaccine in Chile. Consequently, the aim of this study was to describe the genetic variability of the L1 gene of HPV-66, and to evaluate the immunogenic impact of L1 protein variability in relation to HPV-16 and HPV-18.

Materials and methods

Sample collection

Cervical cells were collected from women attending four and two primary care centers in the northern and central metropolitan areas of Santiago city, from March 2014 to April 2016, respectively. Healthy women aged 18–64 years were asked to sign an informed consent to participate in this study, excluding those who were pregnant, hysterectomized or virgins. Primary health care centers participated in this study using infrastructure, personnel and protocols according to the national cervical cancer prevention program. The study protocol was approved by the Ethics Committee of the Servicio de Salud Metropolitano Central.

Samples were collected, according to routine procedures used in the primary care centers. Cervical exfoliated cell samples were independently processed for cytology diagnosis and molecular HPV detection. The Pap analysis was carried out by experienced cytopathologists according to Bethesda classification (Normal: negative for intraepithelial lesion; ASC-US: atypical squamous cells of undetermined significance; L-SIL: low-grade squamous intraepithelial lesion; H-SIL: high-grade squamous intraepithelial lesion; ICC: invasive cervical cancer). Cervical samples were collected in 3.0 ml of phosphate-buffered saline (PBS) + 1.0% FBS and kept refrigerated for 4–6 h before being processed in the laboratory. Cervical samples (1.0 ml) were treated with 1.0 ml of lysis buffer (NucliSENS®, cat 200292, bioMérieux, France) and frozen at – 20 °C until purification of the nucleic acids.

Table 1 HPV-66 prevalence among women in different geographic regions, 2006–2018

Country	City/region	Population	*n*	HPV-66 positive %	References
Croatia	Osijek-Baranja	Women with abnormal cervical cytology	100	30.0	[23]
Italy	Abruzzo, Campania, Lazio, Tuscany, Emilia-Romagna and Piedmont	Women attending gynecological control	2289	12.9	[18]
Argentine	Buenos Aires, Santa Fe	Women attending gynecological control	962	10.8	[12]
Wales	South Wales	Women attending gynecological control	1911	9.2	[17]
South Africa	Soweto and Cape Town	Sexually active women aged 16–22 years	291	8.6	[20]
Italy	Naples city and Apulia region	Women attending gynecological control	654	7.4[a]–8.2[b]	[19]
Denmark	Copenhagen	Women attending gynecological control	5068	3.1	[22]
Canada	Nunavut, NWT, Labrador, and Yukon	Women attending gynecological control	14,598	1.8	[20]
China	Jiangsu Province	Chinese women enrolled in A clinical trial of an HPV vaccine	6051	1.3	[21]

[a]Single infections

[b]Multiple infections

DNA purification and HPV detection/typing

DNA was extracted using an automated commercial method (NucliSENS® easy MAG®, cat 280140, bioMérieux, France). We amplified the following genomic regions: 450 bp of HPV L1 gene with PGMY 09/11 primers [28], and 141 bp of albumin gene with ALB-Fw and ALB-Rv primers for internal calibration [29]. PCR conditions have been previously described [26, 27].

Genotyping of HPV was performed by the conventional PCR method followed by reverse line blot, according to the previously described procedures [29]. PCR conditions have been previously described [27]. PGMY-PCR-positive samples were typed by reverse line blot hybridization using 6, 11, 16, 18, 26, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 55, 56, 57, 58, 59, 69, 66, 68, 70, 73, 82, 83, and 84 type-specific oligoprobes [30]. Positive reactions were reported by chemiluminescence using AmershamTM ECLTM Detection Reagents according to the manufacturer's recommendations (GE Healthcare, Little Chalfont, UK).

Amplification and sequencing of L1 gene

A fragment of 1727 bp containing the complete L1 gene was amplified using primers HPV66 FW frag1 (5´-TCCTGATAT AGTTTTACCTACAG-3´) and HPV66 RV frag3 (5´-AAC ATACAGTACAAGCACAACC-3´). Faint amplicons were re-amplified by semi-nested PCR with HPV66 RV frag1 (5´-CTGCGTAAGTAAAACCACATAG-3´) and HPV66 FW frag2 (5´-ATTACAGGAATCAAAGGCTGAG-3. The PCRs were carried out in a final 25 µl Go Taq Master mix (Promega). The thermal conditions were 95 °C for 5 min, 35 cycles of 95 °C for 1 min, 53 °C for 1 min, 72 °C for 2 min and a final extension to 72 °C for 10 min. Sequencing of L1 gene was performed with the Big Dye Terminator V.3.1 cycle sequencing kit (Applied Biosystem) in a 3500 Genetic Analyzer (Applied Biosystem). The 5´ ends were completed with an extra primer HPV rev sec (5´-AGGATTATAGAA AGATGGATCAG-3´).

HPV-66 genetic variability and phylogenetic analysis

Genetic variability was analyzed by the d*N*/d*S* value. The d*N*/d*S* is the ratio between the proportion of non-synonymous substitutions (d*N*) and the proportion of synonymous substitutions (d*S*). d*N*/d*S* = 1: all sites are neutral; d*N* /d*S* < 1: most of amino acid changes are deleterious and negatively selected; d*N*/d*S* > 1: positive selection on the gene.

Chilean HPV-66 L1 sequences were compared to those available in Genbank, EMBL (http://www.ebi.ac.uk). The complete Chilean sequences of L1 gene were aligned with sequences from reference HPV types using the CLUSTAL X program [31]. The phylogenetic tree was constructed using the neighbor-joining method by the mega6 software. The accuracy of the resulting tree was measured by bootstrap resampling of 1000 replicates. Nine nucleotides sequences for L1 gene generated in this study were deposited in the GenBank database with accession numbers MH607467–MH607475.

B-cell epitope prediction

The putative impact of variability in L1 gene of HPV-66 was estimated in silico by predicting the B-cell epitopes. The B-cell epitope of prototype sequences was predicted using the BcePred server, which is available from URL: http://www.imtech.res.in/raghava/bcepred/. The prediction is based on physico-chemical properties of the amino acids, such as hydrophilicity, flexibility/mobility, accessibility, polarity, exposed surface, turns, and antigenic propensity [32].

Statistical analysis

The association of L1 mutations with different grades of cytological cervical lesions was evaluated by Fisher's exact test. Additionally, Fisher's exact test was performed to evaluate the simultaneous or independent presence of each pair of mutations in L1 gene.

Results

Study population

We previously carried out an HPV epidemiological surveillance among 3235 women attending six primary care health centers from Santiago city for 2 years [27]. The presence of any HR-HPV was detected in 334 (10.3%) women. HPV-16 and HPV-66 were the most prevalent HR-HPVs with 97 (3.0%) and 51(1.6%) infected women, respectively (Fig. 1). Multiple and single infections were determined among 29 and 22 women, respectively (Fig. 1). In this research, the complete sequence of the HPV L1 gene was determined in nine single HPV-infected patients. Eight and one from these patients showed normal cytology and low-grade lesion, respectively.

HPV-66 genomic diversity and phylogenetic analysis

Although it was detected a genetic variability among nine Chilean HPV-66 strains, the L1 showed a low mean number of nucleotide substitutions per site [mean (SE) = 0.009 (0.008)]. The d*N*/d*S* value for L1 sequences [mean (95% CI)

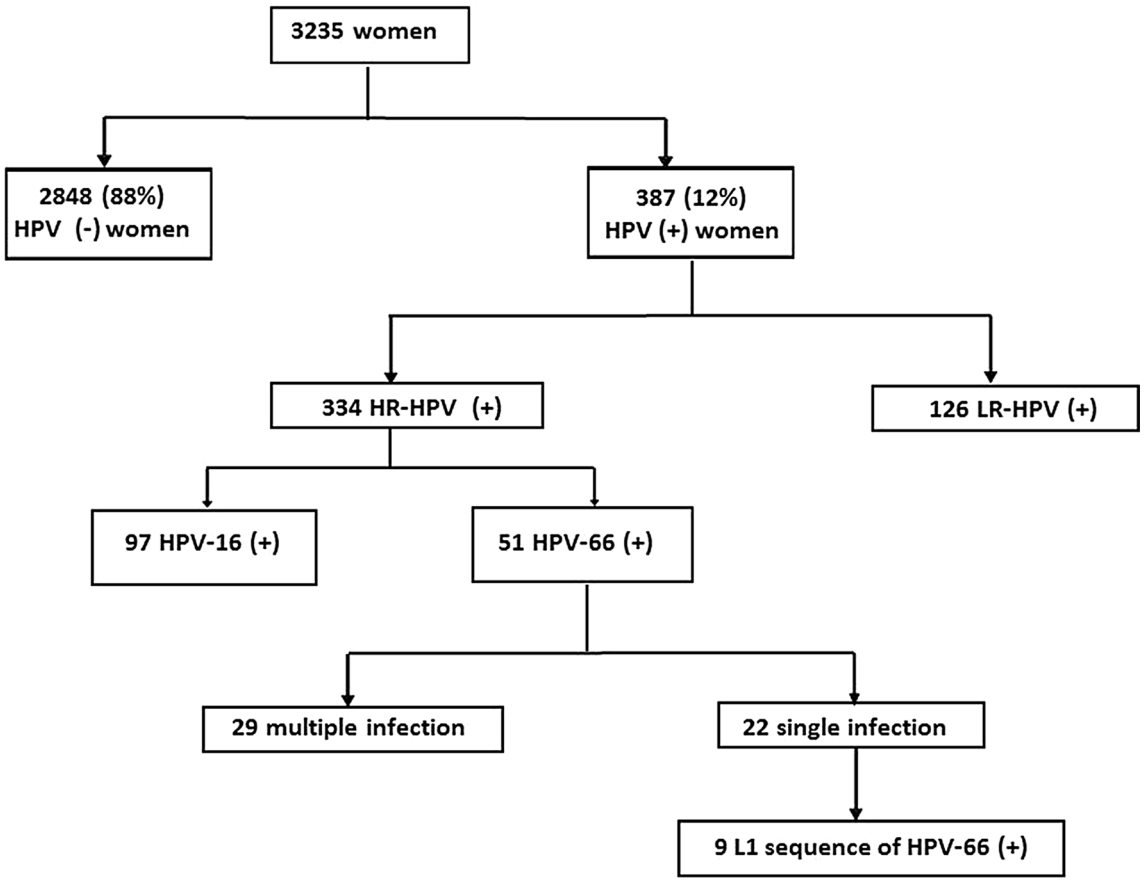

Fig. 1 Enrollment of participants, molecular diagnosis of HPV infection and studied cases

d*N*/d*S* = 0.140 (0.003–0.277)] was low. In addition, no site was identified under positive selective pressure.

L1 sequences obtained from this study along with other reference sequences obtained from GenBank were used to construct the phylogenetic tree for L1 gene of HPV-66 (Fig. 2). Two main clusters or clades of phylogenetically related strains were identified (Fig. 2). However, no specific association with Chilean strains from different geographical areas or cytological cervical lesion was observed.

HPV-66 L1 sequence variations

Nucleotide analysis of the 1516 bp fragment of the L1 gene from nine patients infected with HPV-66 revealed the presence of 30 nucleotide changes (Table 2). The nucleotide mutations at positions 5956 (TTG to CTG), 6043 (TTA to CTA), 6213 (TTA to TTG), and 6681 (GCA to GCT) were identified for the first time in the present study. Besides, a new deletion of nine nucleotides was detected in five patients without evidence of cytological lesions. This mutation causes the loss of three amino acids (proline–arginine–proline) at position 472 of L1 protein. Overall, at least one nucleotide change was detected in 8/9 (88.9%) patients analyzed, 6/9 (66.7%) presented more than one mutation, and 5/9 (55.6%) presented twenty or more mutations.

By co-variation analysis, a positive correlation was identified among the nucleotide mutations at positions 5805 (TCC to TCT), 6049 (GGT to AGT), 6141 (GGG to GGT), 6278 (CTA to CAA), 6660 (AGC to AGT), 6711 (GCC to GCA), 6849 (AAT to AAC), 6927 (CAG to CAA), 6984 (GAA to GAG), 7017 (CTG to CTA), and 7101 (AGG to AGG), and a deletion at positions 7059–7066. All these variations, with the exception of a viral strain, were found exclusively in sequences belonging to group A.

By comparing amino acid sequences of the Chilean HPV-66 L1 proteins and reference HPV-66 (U31794.1) strain, four mutations (G135S, G179 V, L211Q and R486 K) were identified in 5/9 patients each (55.6% prevalence). G135S, G179 V and L211Q mutations resulted located in the β-D–β-E, β-E–α1, β-E–α1-connecting domains in L1 protein secondary structure, respectively (Fig. 3). R486 K mutation was located in the α5–C-terminal region-connecting domain of the L1 protein secondary structure (Fig. 3). Three mutations (I139A, S142 N and S496L) were identified in 4/9 patients each (44.4% prevalence). I139A, S142 N and S496L mutations were located in the β-D–β-E, β-D–β-E and

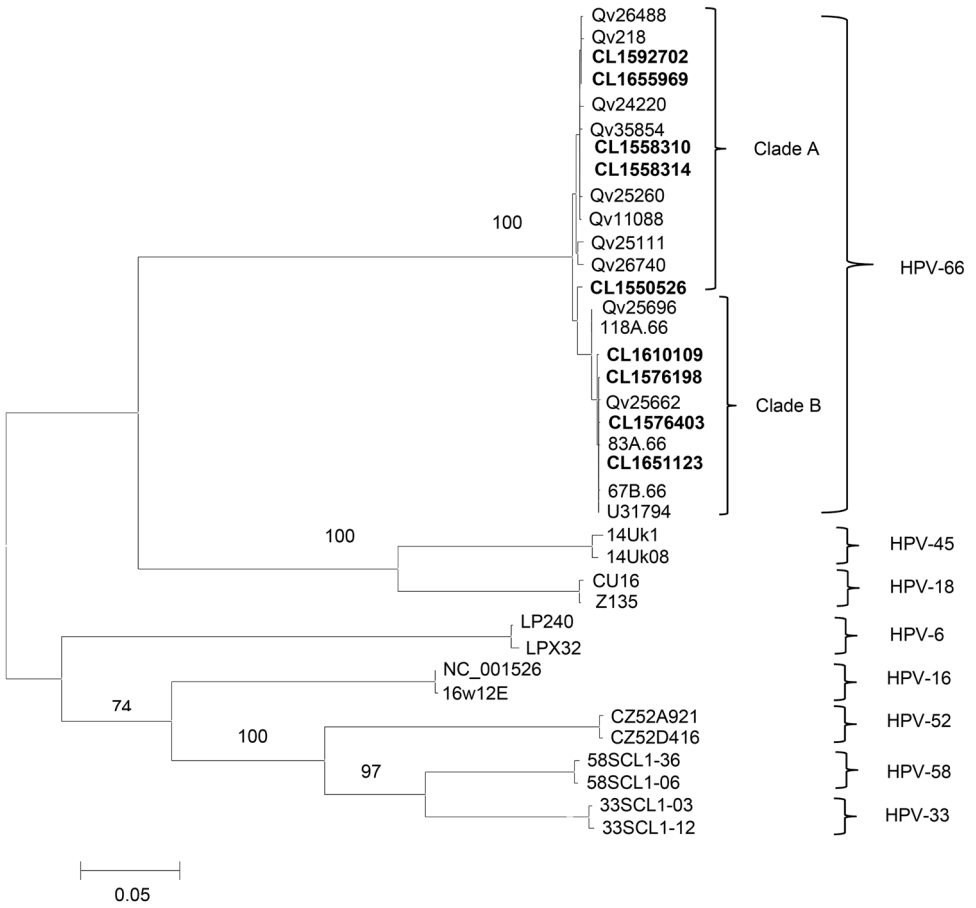

Fig. 2 Phylogenetic tree of HPV-66 L1 gene. The tree was built with MEGA6 software. Branch lengths were estimated with the best fitting nucleotide neighbor-joining method. According to a hierarchical likelihood ratio test and were drawn to scale with the bar at the bottom indicating 0.05 nucleotide substitutions. The tree was rooted using the midpoint rooting method. Chilean HPV-66 strains are reported in bold. Accession number of HPV-66 sequences (EF177184, EF177191, EF177189, EF177182, EF177186, EF177190, EF177188, EF177183, EF177185, KU298928, EF177186, KU298927, KU298926, U31794, this study MH607467- MH607475); HPV-45 sequences (KU049733, KU049730); HPV-18 sequences (GQ180792, KC470213); HPV-6 sequences (HE599240, HE962028); HPV-16 sequences (NC_001526, AF125673); HPV-52 sequences (JN874436, JN874434); HPV-58 sequences (KU550637, KU550607); and HPV-33 sequences (KU550665, KU550674)

α5–C-terminal region-connecting domains of the L1 protein secondary structure, respectively (Fig. 3). Finally, one mutation (I139T) was identified in one patient (11.1% prevalence). This mutation was located in the β-D–β-E-connecting domain of the L1 protein secondary structure (Fig. 3). All of these mutations were previously described. The association between amino acid mutations and cytological abnormalities was not statistically significant.

Otherwise, the G135S, I139A and S142 N mutations had different effect on the estimated hydrophilicity, accessibility and antigenic propensity for amino acids from 132 to 145 of the L1 protein (Table 3). The accessibility was slightly increased for amino acids 132–145 (mean change = + 0.2–0.3), while the hydrophilicity showed a 0.7 increase for amino acids from 136 to 142. The antigenic propensity showed a 0.2 increase for amino acids from 132 to 135, a 1.1–1.6 decrease for amino acids from 136 to 142, and a 0.3 decrease for amino acids from 143 to 145. Additionally, the G179 V mutation had effect on the estimated hydrophilicity, accessibility and antigenic propensity of the L1 protein (Table 3). The hydrophilicity and accessibility were decreased for amino acids 176–182, while the antigenic propensity was increased for these amino acids. The substitution of a glycine residue with a valine caused a 0.6 and 0.1 decrease in the calculated hydrophilicity and accessibility for amino acids from 176 to 182, respectively. At the same positions also the antigenic propensity had a 0.6 increase. The L211Q mutation had effect on the estimated hydrophilicity, accessibility and antigenic propensity of the L1 protein (Table 3). The hydrophilicity and accessibility were increased for amino acids 208–214, while the antigenic propensity was

Table 2 Genetic variability of L1 HPV-66 sequences

WT codon	TCC	TTG	GAG	TTA	GGT	ATA	ATA		AGC	GCA	GGG	GGT		GGT	CTT	CTT		TTA	CCG
Nucleotide position interested[3]	5805	5956	6033	6043	6049	6061	6062	6062	6071	6129	6141	6182	6182	6183	6205	6207	6207	6213	6225
Mutated codon	TCT	CTG	GAA	CTA	AGT	GCA	GCA	ACA	AAC	GCG	GGT	GTC	GTT	GTC	TTG	TTG	CTG	TTG	CCA
WT amino acid	S	L	E	L	G	I	I	I	S	A	G	G	G	G	L	L	L	L	P
Amino acid position[b]	53	104	129	133	135	139	139	139	142	161	165	179	179	179	187	187	187	189	193
Mutated amino acid[c]					S	A	A	T	N			V	V	V					
Cytology, *n* (%)																			
Negative, *n* = 8	4 (50.0)	1 (12.5)	3 (37.5)	1 (12.5)	4 (50.0)	3 (37.5)	3 (37.5)	1 (12.5)	3 (37.5)	3 (37.5)	4 (50.0)	3 (37.5)	1 (12.5)	3 (37.5)	3 (37.5)	3 (37.5)	1 (12.5)	1 (12.5)	5 (62.5)
L-SIL, *n* = 1	1 (100)	0 (0.0)	1 (100)	0 (0.0)	1 (100)	1 (100)	1 (100)	0 (0.0)	1 (100)	1 (100)	1 (100)	1 (100)	0 (0.0)	1 (100)	1 (100)	1 (100)	0 (0.0)	0 (0.0)	1 (100)
Overall, *n* = 9	5 (55.6)	1 (11.1)	4 (44.4)	1 (11.1)	5 (55.6)	4 (44.4)	4 (44.4)	1 (11.1)	4 (44.4)	4 (44.4)	5 (55.6)	4 (44.4)	1 (11.1)	4 (44.4)	4 (44.4)	4 (44.4)	1 (11.1)	1 (11.1)	6 (66.7)
WT codon	CTA	TTA	AAA	AGC	GCA	GCC	AAT	GGC	TTA	CAG	GAA	CTG	GGC	CCT	AGA	CCC	AGG	TCA	
Nucleotide position interested[a]	6278	6346	6351	6660	6681	6711	6849	6855	6858	6927	6984	7017	7059	7060	7063	7066	7101	7131	
Mutated codon	CAA	CTA	AAG	AGT	GCT	GCA	AAC	GGA	TTG	CAA	GAG	CTA	GGC	(—)	(—)	(—)	AAG	TTA	
WT amino acid	L	L	K	S	A	A	N	G	L	Q	E	L	G	P	R	P	R	S	
Amino acid position[b]	211	234	235	338	345	355	401	403	404	427	446	457	471	472	473	474	486	496	
Mutated amino acid[c]	Q	–	–	–	–	–	–	–	–	–	–	–	–	Del	Del	Del	K	L	
Cytology, *n* (%)																			
Negative, *n* = 8	4 (50.0)	3 (37.5)	1 (12.5)	4 (50.0)	1 (12.5)	4 (50.0)	4 (50.0)	5 (62.5)	5 (62.5)	4 (50.0)	4 (50.0)	4 (50.0)	4 (50.0)	4 (50.0)	4 (50.0)	4 (50.0)	4 (50.0)	3 (37.5)	
L-SIL, *n* = 1	1 (100)	1 (100)	1 (100)	1 (100)	0 (0.0)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	
Overall, *n* = 9	5 (55.6)	4 (44.4)	2 (22.2)	5 (55.6)	1 (11.1)	5 (55.6)	5 (55.6)	6 (66.7)	6 (66.7)	5 (55.6)	5 (55.6)	5 (55.6)	5 (55.6)	5 (55.6)	5 (55.6)	5 (55.6)	5 (55.6)	4 (44.4)	

WT wild type, *L-SIL* low-grade squamous intraepithelial lesions

[a]Nucleotides were numbered according to the HPV-66 reference sequence U31794, considering the whole HPV-66 genome

[b]Amino acids were numbered starting from the first amino acid of the specific protein

[c]Synonymous changes are reported with a dash "–"

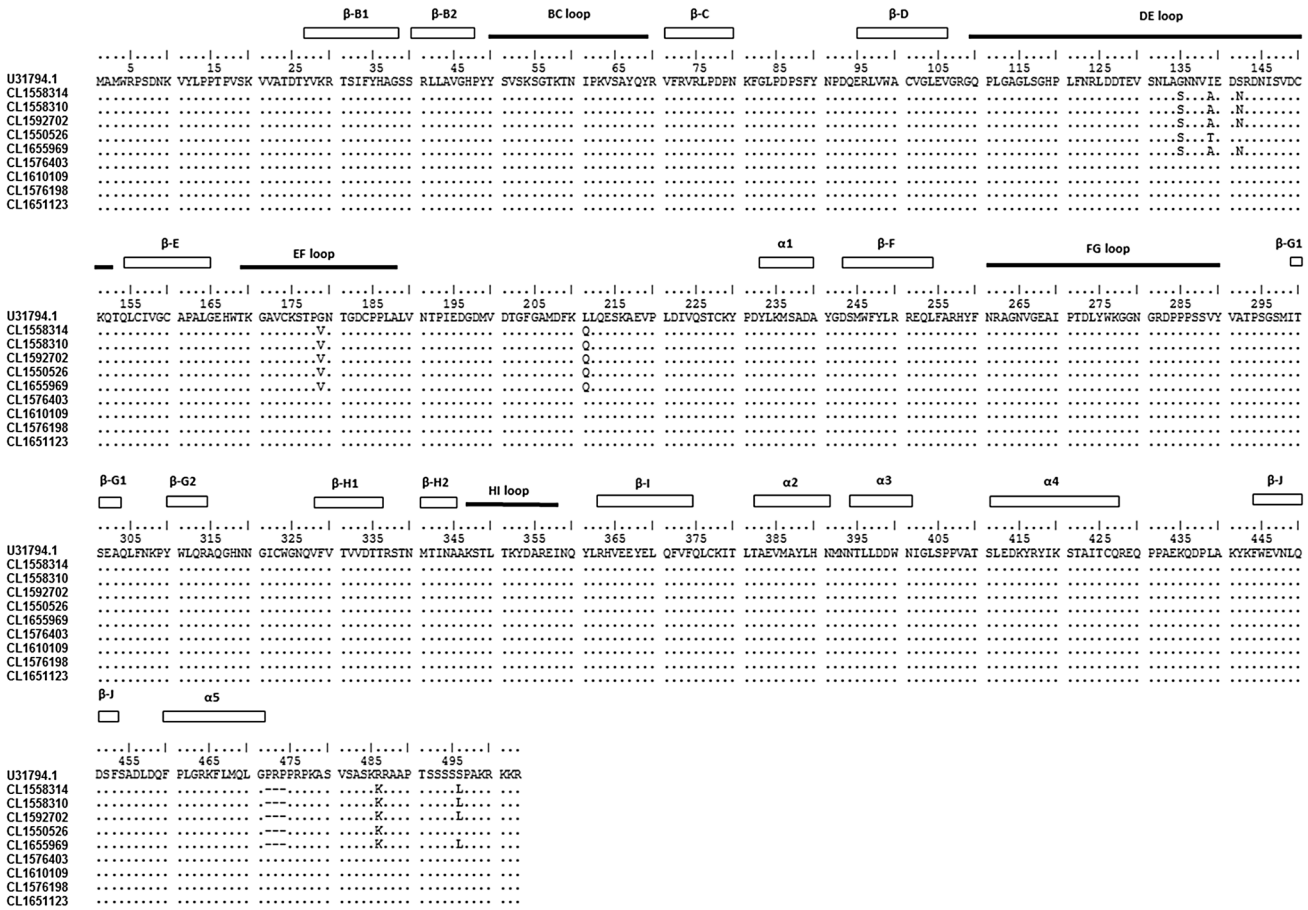

Fig. 3 Amino acid sequence alignment of HPV-66 L1 protein. The secondary structural elements identified (α-sheets and β-sheets) from the HPV-16 crystal structures are reported above the sequences (□). The five loops displayed on the surface of L1 protein are marked and labeled. The accession numbers of HPV-66 are reported. Numbers refer to each HPV sequence. HPV-66 sequence variations are aligned to the reference sequence of the corresponding genotype. Secondary structural elements are indicated as reported in Bishop et al. (2007). Identical amino acids are indicated with dots "·". Gaps in the alignment are indicated with dashes "–". Clade A is formed by CL1558314, CL1558310, CL1592702, CL1550526 and CL1655969 Chilean HPV-66 sequences. Clade B is formed by CL1576403, CL1610109, CL1576198 and CL1651123 Chilean HPV-66 sequences

decreased for these amino acids. The substitution of a neutral hydrophobic residue (leucine) with a neutral hydrophilic one (glutamine) produced a 1.0 and 0.4 increase in the calculated hydrophilicity and accessibility for amino acids from 208 to 214, respectively. At the same positions also the antigenic propensity had a 0.3 decrease.

The deletion of amino acids from 472 to 474 had effect on the estimated hydrophilicity, accessibility and antigenic propensity of the L1 protein (Table 3). Indeed, the hydrophilicity and accessibility were decreased for amino acids 472 and 473, while the antigenic propensity was increased for both amino acids. The 472 and 473 deletions caused a 0.4–0.6 and 0.7–0.8 decrease in the calculated hydrophilicity and accessibility, respectively. At the same positions also the antigenic propensity had a 0.2 increase. On the contrary, at position 474, the deletion caused a 0.3 and 0.2 decrease in the calculated accessibility and antigenic propensity, respectively. At the same position, the hydrophilicity had a 0.3 increase.

The R486 K mutation had no effect on the estimated hydrophilicity, accessibility and antigenic propensity for amino acids from 483 to 489 of the L1 protein (Table 3). Finally, the S496L mutation had different effect on the estimated hydrophilicity, accessibility and antigenic propensity of the L1 protein (Table 3). The accessibility and antigenic propensity were slightly decreased (mean change = −0.2) and slightly increased (mean change = + 0.3) for amino acids 493–499, respectively. On the contrary, the hydrophilicity showed a 0.9 increase for amino acids from 493 to 499.

Table 3 Physico-chemical properties of HPV-66 L1 protein. Hydrophilicity, accessibility and antigenic propensity were predicted using the BcePred server (http://www.imtwch.res.in/raghava/bcepred/)

Position	Wild-type amino acid residue	Hydrophilicity (Parker)	Accessibility (Emini)	Antigenic propensity (Kolaskar)	Chilean strains amino acid residue	Hydrophilicity (Parker)	Accessibility (Emini)	Antigenic propensity (Kolaskar)
132	N	0.945	1.206	0.216	N	0.996	1.365	0.376
133	L	0.895	1.178	0.130	L	0.945	1.337	0.289
134	A	1.571	1.599	− 0.572	A	1.622	1.758	− 0.413
135	**G**	**0.926**	**1.328**	**− 0.143**	**S**	**0.977**	**1.487**	**0.016**
136	N	− 0.022	0.889	0.291	N	0.667	1.188	− 0.881
137	N	1.053	1.300	− 0.170	N	1.742	1.599	− 1.342
138	V	1.552	1.571	0.831	V	2.241	1.870	− 0.341
139	**I**	**1.603**	**1.730**	**0.991**	**A**	**2.273**	**2.019**	**− 0.614**
140	E	1.426	1.860	1.103	E	2.096	2.150	− 0.502
141	D	1.616	1.832	1.207	D	2.286	2.122	− 0.398
142	**S**	**2.292**	**2.253**	**0.505**	N	**2.962**	**2.543**	**− 1.099**
143	R	2.292	2.253	0.505	R	2.324	2.403	0.232
144	D	2.210	2.075	0.691	D	2.241	2.225	0.419
145	N	1.344	1.683	1.289	N	1.375	1.832	1.016
176	S	1.369	1.337	1.879	S	0.775	1.225	2.467
177	T	2.045	1.758	1.177	T	1.451	1.646	1.765
178	P	2.286	2.169	0.595	P	1.691	2.057	1.184
179	**G**	**2.286**	**1.711**	**0.531**	**V**	**1.691**	**1.599**	**1.119**
180	N	2.507	1.832	0.362	N	1.913	1.720	0.950
181	T	2.267	1.421	0.943	T	1.672	1.309	1.532
182	G	2.267	1.421	0.943	G	1.672	1.309	1.532
208	D	− 0.022	1.197	− 0.141	D	0.939	1.608	− 0.413
209	F	− 0.964	1.122	0.294	F	− 0.003	1.533	0.022
210	K	**− 0.717**	1.449	1.467	K	0.244	1.860	1.195
211	**L**	**0.041**	**1.786**	**1.496**	**Q**	**1.002**	**2.197**	**1.224**
212	L	− 0.180	1.664	1.665	L	0.781	2.075	1.393
213	Q	0.762	2.178	1.479	Q	1.723	2.589	1.207
214	E	0.534	1.730	0.403	E	1.495	2.141	0.132
471	G	0.345	1.786	1.164	G	0.345	1.786	1.164
472	**P**	**0.743**	**2.038**	**1.439**	**Deletion**			
473	**R**	**0.629**	**2.141**	**1.275**	**Deletion**			
474	**P**	**1.344**	**2.468**	**1.060**	**Deletion**			
475	P	1.344	2.926	1.125	P	0.971	2.244	1.284
476	R	1.344	2.683	− 0.105	R	0.724	1.917	0.111

Table 3 (continued)

Position	Wild-type amino acid residue	Hydrophilicity (Parker)	Accessibility (Emini)	Antigenic propensity (Kolaskar)	Chilean strains amino acid residue	Hydrophilicity (Parker)	Accessibility (Emini)	Antigenic propensity (Kolaskar)
477	P	1.489	2.403	0.056	P	1.717	2.150	− 0.164
483	A	1.679	1.851	0.304	A	1.774	1.870	0.370
484	S	1.533	2.132	0.143	S	1.628	2.150	0.209
485	K	1.900	2.253	− 1.455	K	1.995	2.272	− 1.390
486	**R**	**1.622**	**2.103**	**− 2.625**	**K**	**1.717**	**2.122**	**− 2.560**
487	R	1.622	2.346	− 1.395	R	1.717	2.365	− 1.329
488	A	1.540	2.393	− 1.514	A	1.634	2.412	− 1.449
489	A	1.590	2.094	− 1.420	A	1.685	2.113	− 1.354
493	S	2.437	1.832	1.242	S	1.445	1.599	1.517
494	S	2.437	1.832	1.242	S	1.445	1.599	1.517
495	s	2.241	1.636	0.191	S	1.249	1.403	0.466
496	s	**2.191**	**1.935**	**0.096**	L	**1.198**	**1.702**	**0.371**
497	p	2.045	2.216	− 0.065	P	1.053	1.982	0.210
498	A	1.995	2.515	− 0.160	A	1.002	2.281	0.116
499	K	1.944	2.814	− 0.254	K	0.952	2.580	

Positions of mutated amino acids are reported in bold

B-cell and neutralizing epitope prediction of HPV-66 L1 protein

Immunodominant epitopes of HPV, which generate the neutralizing antibodies, are located on the FG, H1 and DE loops of the L1 protein [33, 34]. By comparing amino acid sequences of the L1 protein of Chilean HPV-66 strains and HPV-16, it was possible to detect 56.8–59.1, 53.3 and 42.9% of homology in the DE, FG and H1 loops, respectively (Table 4a). Similarly, the homology between L1 protein from Chilean HPV-66 and HPV-18 was 68.2, 50.0 and 28.6% in DE, FG and H1 loops, respectively (Table 4a). In addition, many amino acid mutations were detected in the B-cell epitopes predicted of the L1 of HPV-66, when compared with the reference sequences of the HPV-16 and HPV-18 (Table 4b). Two exclusive HPV-66 epitopes were detected on the EF loop and the β-I region, whose amino acid sequences are not observed in the L1 protein of HPV-16 and HPV-18. Furthermore, the exclusive epitope on the EF loop of the L1 of five Chilean HPV-66 strains showed the G179 V mutation, when compared with the HPV-66 reference sequence (Table 4b).

L1-neutralizing antibodies are directed to non-contiguous conformational epitopes on the exposed surface of the protein on the hypervariable FG, H1 and DE loops. In this study, we detected many amino acid mutations in the L1 protein of HPV-66, when compared with HPV-16 reference sequence. Several mutations of amino acids were detected in three B-cell epitopes on two exposed hypervariable exposed loops (Fig. 4). These epitopes were associated

Table 4 Homology of amino acid sequences in DE, FG and H1 loops of L1 HPV-66 with HPV-16 and HPV-18 and B-cell epitopes of L1 HPV-66

(A) Homology of amino acid sequences in DE, FG and H1 loops of L1 proteins of HPV-66 compared with HPV-16 (NC_0015264) and HPV-18 (spP06794) references

Chilean HPV-66 strain	HPV-16 homology (%)			HPV-18 homology (%)		
	DE loop	FG loop	H1 loop	DE loop	FG loop	H1 loo
CL1655969	59.1	53.3	42.9	68.2	50.0	28.6
CL1558310	59.1	53.3	42.9	68.2	50.0	28.6
CL1558314	59.1	53.3	42.9	68.2	50.0	28.6
CL1592702	59.1	53.3	42.9	68.2	50.0	28.6
CJ1550526	56.8	53.3	42.9	68.2	50.0	28.6
CL1576403	56.8	53.3	42.9	68.2	50.0	28.6
CL1610109	56.8	53.3	42.9	68.2	50.0	28.6
CL1651123	56.8	53.3	42.9	68.2	50.0	28.6
CL1576198	56.8	53.3	42.9	68.2	50.0	28.6

(B) Amino acid residue changes mapped into B-cell epitopes

L1 epitope prediction for B cell[a]	L1 region	Amino acid changes inserted in epitope sequence[b]	HPV type[c]
^{9}***NK*** VYLPPTPVSKVV^{22}			66, 16, 18
^{45}*VGHPY**YSVSKS***^{55}	β-B2/BCIoop		66, 16
^{68}QYRVFRVRLP^{77}	β-C/CDIoop		66, 16, 18
^{101}CVGLEVG^{107}	β-D		66, 16
^{116}***L*** SGHPL**F**^{122}	DE loop		66, 16
^{lA6}***ISVDC*** KQTQLCI**V** GC^{160}	DE loop/β-E		66, 16, 18
^{173}***VCKSTPV***^{179}	EF loop	G179 V	66
^{218}EVPLDI**VQ ST** CKYPDY**L**^{234}	EF loop/α1		66, 16, 18
^{283}***PPPSSVYV***^{290}	FG loop		66, 18
^{326}NQ**V** FVTVVDTT^{336}	β-Hl		66, 16, 18
^{360}***QYLRHVE***^{366}	β-l		66
^{368}Y**E** LQF ***V*** FQLCKITLT^{382}	β-l/α2		66, 16, 18
^{402}***IGLSPPV***^{408}	α3		66, 18

[a]The amino acids in bold and italic are the positions of change in the predicted B-cell epitopes with respect to HPV-16 reference (NC_0015264)

[b]Amino acid changed inserted in epitope sequence with respect to HPV-66 reference (U31794.1)

[c]HPV type with predicted epitope

with the neutralizing conformational epitope of L1 protein. They were located in three different regions: two on DE loop (aa 116–160) and one on FG loop (aa 283–290), according to the structure of L1 reported by Bishop et al. [35]. Both variable regions on DE loop were designated in this paper as DE1 (aa 116–122) and DE2 (aa 146–160) (Fig. 4).

Discussion

HPV-66 has been described in different geographical locations of the world although with heterogeneous prevalences. HPV-66 has been determined in low frequency among women attending cervical screening control in Denmark (3.0%), China (1.3%), and Canada (1.8%) [20, 22, 23]. Also, HPV-66 infection was reported in 4.7% of Chinese women with HSIL and invasive cervical carcinoma [36]. However, high frequency of HPV-66 has been found among women with precancerous lesions. Thus, HPV-66 was the most frequent HPV found in Croatian women [24]. This genotype was detected in 30% of women with cervical intraepithelial neoplasia [24]. In addition, HPV-66 was detected in 14.5% CIN II/III precancerous lesions in Kenia [37]. HPV-66 was detected in women presenting for cervical screening programs in Italy (8.2% and 12.9%), South Wales (9.2%), South Africa (8.6%), and Kenya (9.0%) [18, 19, 21, 37]. These results confirm the heterogeneity of HPV-66 prevalence in different geographic regions.

HPV-66 was the HPV type with the highest prevalence (range between 8.5 and 17%) among women attending routine gynecological screening in Argentina [12]. Additionally, HPV-66 was reported to be one of the most frequent HPV genotypes (ranging from 1.4 to 1.6%) among women attending health care centers in Chile [26, 27]. Also, the frequency of HPV-66 has been described in precancerous lesions and cervical cancer in Chile [27]. It has also been reported that

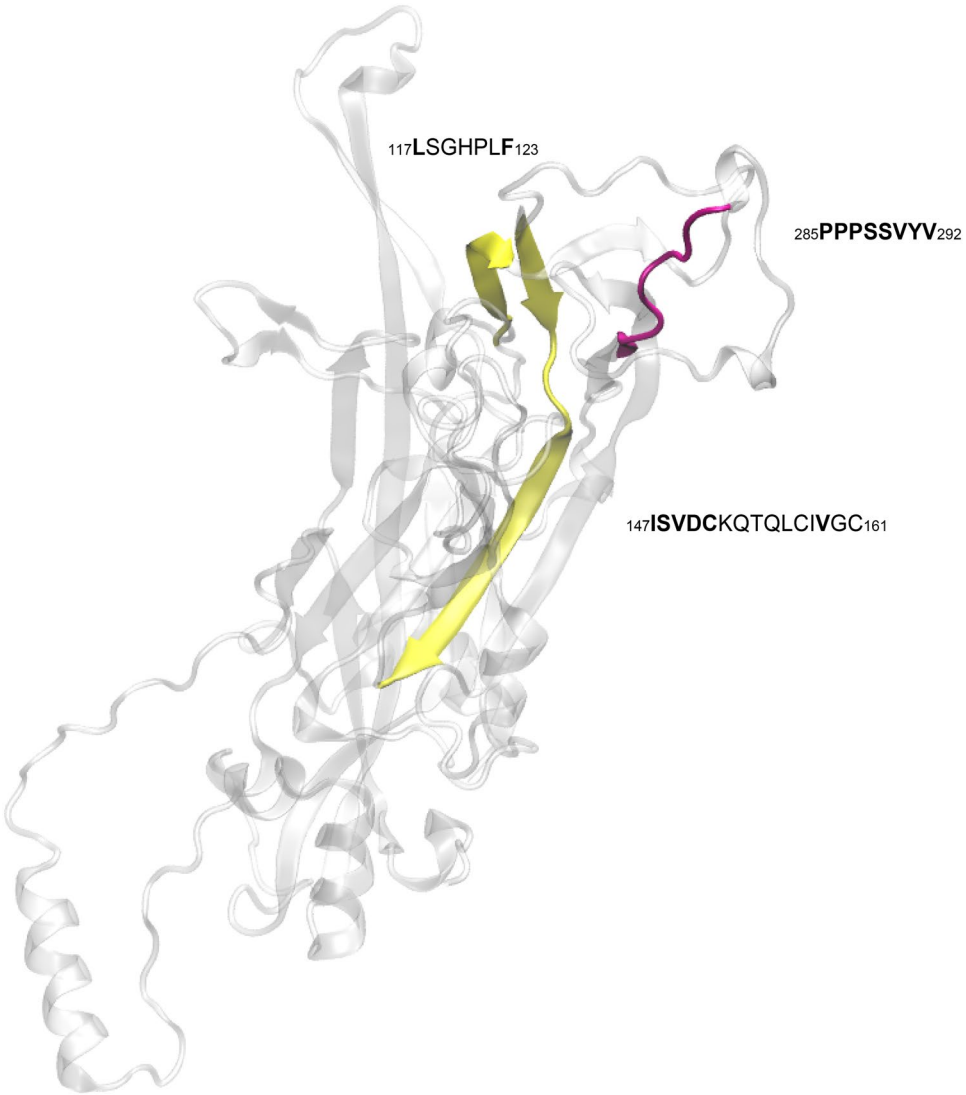

Fig. 4 HPV-66 L1 monomer. The 3D model was built by comparative modeling based on high-resolution crystal structure of the HPV-16 L1 monomer (PDB: 1DZL). Using MODELLER 9v9 software (http://salilab.org/modeller/) comparative models were generated and saved for further refinement and validations. L1 monomer, viewed roughly normal to the five axes of a pentamer. The structure includes HPV-66 B-cell epitopes on DE and FG loop. Two DE and one FG epitopes are yellow and red labeled, respectively. Specific HPV-66 amino acids are reported in bold

5.3% (6/113) of women with HSIL seen in gynecological centers (tertiary care) in Santiago, Chile, are infected with HPV-66 [27]. Consequently, these studies show a high prevalence of HPV-66 in countries from South America. The present report described the genomic diversity of HPV-66 variants from Chilean women attending public health care in Santiago city.

Previous reports have described the association of HPV-66 infection with different grades of cervical lesions or cancer [5, 37, 38]. Here, we describe HPV-66 strains from eight normal cytological cervical samples and one L-SIL. Nucleotide and amino acid variability were low in HPV-66 sequences. Based on the phylogenetic analysis of L1 of HPV-66, the Chilean HPV variants identified in this study were distributed into two main clades defined as A and B. Dichotomy was already observed in genetic sequence of L1 HPV-66 gene [11]. Mutations in L1 gene could be explained by genetic drift and some positive selection among closely related variants [8].

A positive correlation was identified among the nucleotide mutations at positions 5805, 6049, 6141, 6278, 6660, 6711, 6849, 6927, 6984, 7017, and 7101, and one deletion at position 7059–7066. Of these, three mutations resulted in the amino acid change: 6049 (G135S), 6278 (L211Q), and 7101 (R486 K). The G135S, L211Q and R486 K mutations resulted located in the β-D–β-E, β-E–α1, and α5–C-terminal region connecting domain in L1 protein secondary structure, respectively. The G135S mutation, which is possibly associated with I139A and S142 N mutations, was found to cause a marked decrease in the calculated antigenic index. Also, the L211Q mutations slightly decreased the antigenic propensity. None of the HPV-66 mutations identified have been previously associated with different pathogenic potential in other high-risk types, neither corresponded to known variants of HPV-16. Nevertheless, some HPV-66 L1 protein mutations might alter viral antigenicity, with possible implications in vaccine design [39]. Undoubtedly, other studies in patients with different cervical lesions are necessary to evaluate this hypothesis.

Among HPV types, the highest variability of the L1 protein is located on the outer loops of the protein. This variability contributes to the antigenicity differences among HPV variants [35, 40]. Two small non-contiguous and hypervariable regions constitute the main conformational epitope of L1 [41, 42]. These regions are located on FG and H1 loops of L1 protein [33, 43]. Both regions contribute significantly to the specificity of antibodies during natural infection [43, 44]. Also, it has been suggested that BC and DE loops would be important in the binding of antibodies from human sera to the L1 protein [45]. Consequently, it is currently accepted that FG, H1 and DE loops participate in the immunodominant epitopes of HPV-16; and these epitopes generate neutralizing antibodies [33, 34, 45]. In addition, it has recently been described that Tyr^{135} and Val^{141} amino acids on the DE loop are critical residues for binding of HPV-16-neutralizing antibodies [34]. All Chilean HPV-66 have amino acid mutation at the Tyr^{135} position: five and four strains have serine and glycine mutation, respectively. Likewise, the aspartic acid mutation was detected at the Val^{141} position from all Chilean HPV-66 strains. Here, it was detected that L1 proteins of Chilean HPV-66 compared with HPV-16 had 53.7-56.1, 68.4 and 43.7% homology on the DE, FG and H1 loops, respectively. Thus, these results suggest a great structural and antigenic difference between L1 HPV-66 and HPV-16. This amino acid variability should affect the specificity of neutralizing antibodies against both proteins.

On the other hand, it has been described that vaccines elaborated with HPV-16 and HPV-18 L1 antigens would only generate cross-protection against HPV-31, HPV-33 and HPV-45 infections [25]. According to this, the tetravalent vaccine would not confer immunity against infection with other HR-HPVs, e.g., HPV-66. We predicted that tetravalent vaccine would not produce cross-protection in 29.5 and 33.3% of women with pre-neoplastic lesions and squamous cervical carcinoma in Chile, respectively [27]. Here, we have described a great antigenic variability between L1 HPV-66 and HPV-16. These results strongly suggest that HPV-16/18-based vaccines would not produce cross-protection against HPV-66 infections.

HPV-66, 26, 53, 67, 68, 70, 73 and 82 have been linked to 2.6–3.0% incidence of cervical cancer [46–48]. Considering the limited impact of these HPV types on cervical cancer worldwide, it has not been recommended to include them in new HPV vaccines [46]. However, it has recently been reported that HPV-66 was the most prevalent and the second most prevalent HPV type in Croatian and Brazilian women with abnormal cervical cytology, respectively [24, 49]. In addition, the molecular signature of HPV-induced carcinogenesis (presence of type-specific spliced E6*|mRNA; increased expression of p16; and decreased expression of cyclin D1, p53 and Rb) in cervical cancers containing single infections with HPV-26, HPV-53, HPV-66, HPV-67, HPV-68, HPV-70, HPV-73 or HPV-82 was similar to that in cancer cases associated with established carcinogenic types [50]. Consequently, these findings together with our results launch a review into the efficacy of HPV vaccine in countries with a high prevalence of other HR-HPV than HPV-16 and HPV-18.

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Compliance with ethical standards

Conflict of interest The authors declare they have no conflict of interest.

Ethical approval Six public health care centers and their referral hospital participated in this study using the infrastructure, personnel and protocols already in place under the national cervical cancer prevention program. The study protocol was approved by the Ethics Committee of the Servicio de Salud Metropolitano Central.

Informed consent Women were invited to participate through an outreach campaign in the catchment area of each health center and, if interested, received an appointment with the health center midwife. Eligible women who agreed to participate signed an informed consent form and entered the study.

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