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Genetic characterization and clinical implications of human papillomavirus type 16 (HPV16) variants from northeastern Argentina

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ABSTRACT

Background: Human papillomavirus type 16 (HPV16) plays a central role in the development of cervical cancer. Worldwide studies indicate the existence of HPV16 variants that show different geographic distributions and oncogenic potential.
Objective: Our goal was to describe the genetic variation of HPV16 isolates identified in urban women with different grades of cervical lesions living in northeastern Argentina.
Study design: We analyzed 116 HPV16-positive cervical samples (16 NLIM, 62 L-SIL, 16 H-SIL and 22 cervical cancer) from patients attending health centers in Misiones (Argentina) during 2006–13. HPV16 isolates were genetically characterized through PCR amplification and direct sequencing of 364 bp within the long control region, and the resulting sequences classified into variants based on phylogenetic analysis (lineages A, B, C and D). A potential association between HPV16 variants and lesion grade was evaluated through an odds ratio (OR) test. A temporal framework for the origin of HPV16 variants was assessed through coalescence analysis (BEAST v 1.7.5).
Results: Phylogenetic analysis of HPV16 sequences showed that 92.1% of the samples clustered with lineage A, and 6.9% to lineage D. HPV16 variants from lineage D were more frequently associated with high-grade lesions and cancer (HSIL+) than lineage A variants at an OR of 13.8 (1.6–117.0). The time to most common recent ancestor (t_{MCRA}) of all variants was 119,103 years before present (HPD 95% = 48,486–197,239), a date consistent with the time frame for modern human evolution.
Conclusion: Our results suggest that HPV16 variants from lineage D may represent an additional risk factor for the development of cervical cancer in women living in northeastern Argentina. This study provides new information about viral isolates present in Argentina that will contribute to the monitoring of HPV16 infection in the vaccine era.

1. Introduction

Cervical cancer is the second most common cancer among women worldwide, with an estimated 527,624 new cases and 265,653 deaths occurring in 2013 (Bruni et al., 2014). Worldwide epidemiological and laboratory studies have revealed that several different concomitant factors and a progressive process are necessary for cervical cancer to develop, and have established a central role for human papillomavirus (HPV) genital infection (zur Hausen, 2009). Certain oncogenic viral types such as HPV16 and HPV18, which cause the majority of cases of cervical cancer, have been categorized as human carcinogens by the International Agency for Research on Cancer (IARC, 1995, 2007). The magnitude of the association between HPV16 infection and cervical squamous cell carcinoma is very high, with an OR of 434.5 (CI 95% = 278.2–678.7) (Muñoz et al., 2003).

HPV16 is a member of the *Papillomavirus* Family (genus *Alpha-papillomavirus*, species A9), and its genome consists of a

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circular, double-stranded 8-kb DNA molecule. The analysis of partial and complete viral sequences has allowed the identification of "viral variants" which differ from the original isolate by about 2% (de Villiers et al., 2004). Early studies of HPV variants indicated that their distribution varied considerably in different geographical regions, suggesting that the virus and its hosts have co-evolved over time (Ho et al., 1991, 1993; Chan et al., 1992; Yamada et al., 1997). For HPV16, five phylogenetic lineages have been defined according to their suspected origin: European (E), Asian (As), Asian-American (AA), and African (Af1 and Af2). This phylogenetic classification was determined based on the analysis of the nucleotide sequence of the long control region (LCR) of the virus (Ho et al., 1993). Subsequent studies of other viral genes such as E6 and L1 and whole viral genomes have expanded this phylogenetic classification up to nine lineages: European (E), European-Asian (EAs), Asian-American types 1 and 2 (AA1, AA2), North American type 1 (NA1), and African types 1a, 1b, 2a and 2b (Af1a, Af1b, Af2a, Af2b) (Cornet et al., 2012).

Although the geographical designation of viral lineages has been widely used by the papillomavirus community, an alphanumeric classification system has recently been developed (Burk et al., 2013). Because this nomenclature provides a standardized taxonomy that facilitates the comparison of variants across geographic regions and among different populations, it will be adopted in the manuscript. According to this system, the E and EAS variants are identified as lineage A, Afr1 variants as lineage B, Afr2 as lineage C, and NA/AA as lineage D. Furthermore, the nine sublineages have been described as A1, A2, A3 (European, E), A4 (Asian, As), B1 (Afr1a), B2 (Afr1b), D1 (NA), D2 (AA1), and D3 (AA2) (Burk et al., 2013). From a clinical point of view, several studies have provided evidence that HPV16 variants from lineage D are associated with an increased risk of persistent infection and development of cervical lesions in Latin American women (Hildesheim et al., 2001; Berumen et al., 2001; Sichero et al., 2007; Smith et al., 2011), hence, may be related to a different pathogenic potential (Sichero et al., 2012).

The aim of this study was to describe the genetic variation at the LCR of HPV16 isolates among urban women with normal cytology and different grades of cervical lesions, living in northeastern Argentina (Posadas, Misiones Province). Misiones Province is considered a region with high mortality rates of cervical carcinoma (15.5/100,000) compared to other urban areas of the country, such as Buenos Aires (6.3/100,000) (Arrossi, 2008). Previous research has also shown that HPV16 prevalence is 6% in urban women with normal cytology and 51% in individuals with cervical cancer (Badano et al., 2011, 2012), although the genetic variation of the associated isolates is still unknown. Consequently, an expanded analysis of HPV16 sequence variation at the LCR will provide new information about viral isolates present in the Misiones population, and also contribute to the monitoring of HPV16 infection in the region.

2. Methods

2.1. Study population

This study was conducted in the city of Posadas, the capital of Misiones Province. The city is located at the south of the province, at the margins of the Paraná River (27°21′59″S; 55°53′39″W). Study subjects included women attending several private practices in the city and the public Hospital (*Escuela de Agudos "Dr. Ramón Madariaga"*). Information about their ethnicity was not collected. However, judging from the usual demographics for patients at the hospital and medical centers, we estimated that the sample is broadly representative of the population of Posadas (i.e., white-admixed of Amerindian-European descent). Unpublished data from our research group has indicated a European maternal contribution (mitochondrial DNA) of roughly 50%. Because of the existence of reported gender bias in this population, the extent of European admixture is probably higher (Corach et al., 2010).

2.2. Nomenclature

The cytological classifications used in this study are as follows: NILM = negative for intraepithelial lesion and malignancy; L-SIL = low-grade squamous intraepithelial lesion, including human papillomavirus/mild dysplasia/cervical intraepithelial neoplasia (Solomon et al., 2002) and women with discordant papanicolaou-colposcopy results, who, after being tested positive for HPV16, were referred for biopsy/treatment by the gynecologist; H-SIL = High-grade squamous intraepithelial lesion; CIS = carcinoma in situ; and ISCC = invasive squamous cell carcinoma. The denotation H-SIL+ is used to cluster H-SIL + CIS + ISCC patients.

2.3. Biological samples

The samples used in this study came from previous epidemiological studies described in Badano et al. (2011) (n = 139, controls) and Badano et al. (2012) (n = 56 H-SIL+). These studies did not describe the genetic variation of HPV16 isolates. In addition, an additional 657 samples (cervical scrapes) were obtained by trained gynecologists working in different private centers in Posadas from 2006–13. These samples came from women suspected to have abnormal cytological changes (either by colposcopy and/or pap cytology) and, thus, were referred to the Laboratorio de Biología Molecular Aplicada (Universidad Nacional de Misiones) for HPV typing. At the laboratory, all patients signed an informed consent, and their samples were incorporated into this study.

2.4. Ethical permission

All participants gave their informed consent, and data confidentiality was maintained throughout the study. This study was conducted with the approval of the Ethics Committee of the "Departamento de Docencia e Investigación, Comité de Bioética, Hospital Dr. Ramón Madariaga, Posadas, Misiones".

2.5. HPV16 sequencing

A total of 852 samples, including 139 NLIM, 654 L-SIL, 16 H-SIL, 14 CIS and 29 ISCC, were screened for HPV infection with L1 consensus primers MY09–MY11 (Bernard et al., 1994). The typing of HPV DNA positive samples was performed by E6-Nested Multiplex PCR (E6-NMPX) with cocktails of primers C-1 (HPV-High Risk 16, 18, 31, 45, and 59) and C-2 (HPV-High Risk 33, 56, 52, 58 and HPV-Low Risk 6 and 11) (Sotlar et al., 2004). Among these samples, 123 of them, including 16 NLIM, 68 L-SIL, 16 H-SIL, 10 CIS and 13 ISCC, were positive for HPV16 (52.6% single HPV16 infection and 47.4% with co-infections with other high-risk types) and available for sequencing analysis of the LCR. The occurrence of co-infections did not affect the ability to recover LCR-HPV16 type specific sequences.

Genetic variation in the HPV16 samples was determined through PCR amplification and direct sequencing of 364 bp within the non-coding LCR (Ho et al., 1991, 1993; Chan et al., 1992), which has been extensive used for taxonomic purposes (Smith et al., 2011; Cornet et al., 2012). Briefly, PCR products were visualized by agarose gel electrophoresis, and the amplicons were purified with the ADN PuriPrep-GP extraction kit (INBIO, Argentina). The purified amplicons were sequenced using the original forward primer through sequencing services (Cromatida, Argentina), while 42

sequences were obtained using both Forward and Reverse primers. Twenty HPV16 LCR sequences were already available because they were part of a multicenter study being conducted across Argentina (Basiletti et al., 2012).

The HPV16 LCR sequences were read and analyzed using Codon Code aligner software v. 3.0.1 (CodonCode Corporation). The first 20 nucleotides of each strand were trimmed in order to exclude illegible regions, seven sequences (5.7%) were unreadable and excluded from the study. The remaining sequences ($n = 116$; 322 bp) were unequivocally aligned and the positions of the single nucleotide polymorphisms (SNPs) designed according to the European Reference genome (NCBI# NC 001526). Variants that were found at least twice were counted as "natural" variants, and those found only once were repeated in order to exclude them as potential PCR mutation artifacts. To identify a novel variant, the sequences were compared with those previously published at GenBank by using the BLAST program (Altschul et al., 1990).

2.6. Phylogenetic analysis

The sequences obtained were classified based on phylogenetic analysis as HPV16 lineages A, B, C and D. Phylogenetic trees were constructed using all sequences from this study ($n = 116$) and published sequences belonging to the following lineages: A (NC001526, KF466832, KF466824, KF466819, KF466817, KF466812, KF466800, KF466797, KF466789, KF466769, KF466736, KF466704, KF466693, KF466679), B (KF466577, KF466540), C (KF466653, KF466635, KF466594), and D (KF466527, KF466523, KF466510, KF466851, KF466844) (Cornet et al., 2012). The phylogenetic tree was obtained using the Bayesian method implemented in BEAST v 1.7.5 software (see Section 2.8).

2.7. Statistical analysis

The distribution of HPV16 variants according to the lesion grade was compared by χ^2 or two-tailed Fisher exact test. Logistic regression was used to estimate the odds ratio (OR) and 95% confidence intervals (CIs) using SPSS software (SPSS, Inc., Chicago).

2.8. Coalescent analysis

Dating of the time to most recent common ancestor (t_{MRCA}) of the HPV16 sequences was carried out by Monte Carlo Markov Chain (MCMC) Bayesian coalescent analysis implemented in BEAST v 1.7.5 (Drummond and Rambaut, 2007). To select the nucleotide substitution model that best fit the sequence data, we used jModelTest v 2.1.3 (Darriba et al., 2012) and the Akaike Information Criterion (AIC). The XML file was then manually modified to change the original GTR model into the selected one (TPM1uf: Kimura 81 with unequal base frequencies). The Bayesian Skyline Plot (BSP) was selected as a model to estimate the evolutionary and coalescent parameters. BSP is considered to be more flexible than other demographic models for exploring the data as it can fit a wide range of demographic scenarios (Pybus et al., 2000; Strimmer and Pybus, 2001; Drummond et al., 2005). BSPs were run under the two molecular clock models – strict and relaxed uncorrelated lognormal. A substitution rate of 1×10^{-7} subs/site/year (s/s/y) was set according to Halpern (2000). This theoretical value comes from the bottleneck hypothesis, according to which the approximately 2% difference between major variants of mucosal types reflects some 100,000 years of human evolution.

The MCMC were run for 5×10^7 generations, sampling every 5000th generation in order to achieve an Effective Sample Size (ESS) > 200. A minimum of five independent MCMC simulations were performed for each clock-model combination. The fiftieth run of each model was set to include the estimation of the marginal likelihood by Stepping Stone method (Baele and Lemey, 2013). Those two runs were used to compute the log10 Bayes Factor of the strict clock versus relaxed clock and the best clock model was selected for the estimation of t_{MRCA}. Values greater than 1 log10 Bayes Factor were taken as significant difference in the performance of the models. All BEAST run logs were analyzed with TRACER program version 1.5 (Available from http://beast.bio.ed.ac.uk/Tracer) after discarding 2% of the run length as burn-in. The maximum clade credibility tree (MCCT) was constructed with the TreeAnnotator tool after discarding 2% of the sampling. The MCCT summarizing the posterior information of topologies and the median branch lengths from the trees sampled was then visualized with FigTree V1.4.0 software (http://tree.bio.ed.ac.uk/software/figtree/).

2.9. GenBank accession number

All sequences described in this study were deposited in GenBank under the accession numbers: KM094931 – KM095046.

3. Results

3.1. Study population

A total of 116 samples, including 16 NLIM, 62 L-SIL, 16 H-SIL, 10 CIS and 12 ISCC, were analyzed. he age distribution for each group was as follows: NLIM = mean age 28.7 years (SD = 8.8; range 19–52; median 27); L-SIL = mean age 27.1 years (SD = 7.0; range 17–54; median 26); H-SIL+ = mean age 39.3 years (SD = 11.6; range 20–65; median 39); and total population = mean age 31.5 years (SD = 10.7; range 17–65; median 29).

3.2. HPV16 variants and phylogenetic analysis

We identified 16 variable sites in the LCR sequence (from nt 7526 to nt 7847), which occurred in eleven unique combinations, five of which being previously unreported. A summary of the nucleotide sequence of each HPV16 variant and its frequency is shown in Table 1. Overall, 7.8% (9/116) of the circulating HPV16 variants were putatively novel.

The phylogenetic analysis of our samples showed that about 93.1% (108/116) of the samples from Misiones clustered within lineage A (formerly E branch), and 6.9% (8/116) within lineage D (formerly AA/NA branch) (Fig. 1). Among the A isolates, the frequency of sublineage A1 (E-prototypic variants) was 87.0% (94/108), whereas among the D isolates, 87.5% (7/8) belonged to the sublineage D2 (designated by a diagnostic SNPs at position 7743T/G) and 12.5% (1/8) to D1 (designated by a SNPs position at 7834G/T), respectively.

3.3. Distribution of HPV variants according to the lesion grade

The frequency of HPV16 variants according to the Pap cytology is shown in Table 1. The HPV16 variants from lineage D showed a differential distribution according to the severity of the cervical lesion (χ^2 test; p value of 0.003). The association analysis of HPV16 variants and cytological diagnosis is shown in Table 2. Our results indicate that HPV variants from lineage D were more frequently found in women with H-SIL+ than those from lineage A, at an OR of 13.8 (CI 95% = 1.6–117.0).

3.4. Coalescent analysis

The convergence of the MCMC chains was evaluated by the visual inspection of the trace file (no trend), by the value of the

Table 1
Summary of nucleotide sequence variation in the long control region (LCR) of Human papillomavirus type 16 (HPV16) isolates from Posadas city, Misiones, Argentina.

	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7					
	5	5	6	6	6	6	7	7	7	7	7	7	7	8	8	8					
	5	7	3	6	7	8	1	2	4	6	8	9	9	2	3	3					
	2	5	6	9	0	9	4	9	3	4	6	2	9	2	4	7					
Isolate/Ref	G	G	A	C	A	C	T	A	T	C	C	C	G	C	G	A	Lineage	NLIM	LSIL	HSIL	Total
KM094931	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	12	52	30	94
KM094945[a]	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	A	0	0	1	1
KM094944	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	1	4	0	5
KM094975[a]	A	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	A	1	1	0	2
KM094989[a]	.	.	C	.	.	.	G	.	.	.	.	.	.	.	.	.	A	0	4	0	4
KM094997[a]	.	.	C	.	.	.	G	.	.	.	.	T	.	.	.	.	A	1	0	0	1
KM095025	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	1	0	0	1
KM095012	.	.	.	T	.	A	.	T	G	T	T	.	.	.	.	.	D	0	1	1	2
KM094941	.	.	.	T	.	A	.	C	G	T	T	.	.	.	.	.	D	0	0	4	4
KM094960[a]	.	.	.	T	.	A	.	C	G	T	T	G	.	.	.	C	D	0	0	1	1
KM094934	.	.	.	T	G	A	.	C	.	T	T	.	.	.	T	.	D	0	0	1	1
Total																		16	62	38	116

Nucleotide positions are designed according to the European Reference genome (NCBI# NC 001526).
[a] Putative novel variant.

ESS of each parameter estimated (ESS > 200) and by comparison of the multiple independent runs. Although some parameters showed an ESS of less than 200 in some runs, all of the runs consistently showed similar results (Supplementary Materials, Table S1 and Fig. S1). Moreover, the Bayes Factor analysis indicated that both clocks performed equally well (Supplementary Materials, Table S2). Therefore, we selected the one that used the fewest parameters (strict molecular clock). The logs and trees file of the five independent runs were combined with the LogCombiner tool after discarding the initial 2% of the samples as burn-in. The molecular dating for the HPV16 phylogenetic tree is shown in Fig. 1. In our population, all variants coalesced to a t_{MCRA} of 119,103 years (HPD 95% = 48,486–197,239). The mean estimates for the t_{MCRA} of each lineage were as follows: A = 41,793 years (HPD 95% = 13,511–65,972); D = 33,959 (HPD 95% = 8945–55,849); D1 = 17,711 years (HPD 95% = 2886–24,102) and D2 = 21,421 (HPD 95% = 5065–31,010).

4. Discussion

In Argentina, cervical cancer is the third most frequent cancer among women, with current estimates indicating that 4956 women are diagnosed with cervical cancer and that 2127 die of the disease every year (ICO HPV Information Centre, 2013). Previous studies of HPV16 variation within Argentina have focused on Amerindian populations, such as the Quechua from Jujuy, the Pilagá from Formosa, and the Mbya-Guaraní from Misiones (Picconi et al., 2002, 2003; Tonon et al., 2007; Deluca et al., 2012). These studies neither addressed nor found an association between HPV16 variants and cervical cancer development. However, in light of the limited sample sizes used in those studies, we consider that the issue remains inconclusively resolved. Interestingly, European variants were highly frequent among the Native American populations (from 70% to 80%) and their presence was attributed primarily to contacts with the Spanish conquerors (Picconi et al., 2002, 2003; Tonon et al., 2007; Deluca et al., 2012).

This is the first study conducted in an urban population of northeastern Argentina. Our results show that the composition of HPV16 variants at the LCR region was 93.1% lineage A and 6.9% lineage D. The high prevalence of HPV16 lineage A variants was congruent with previous publications on Amerindian populations from Argentina (~80%), a recently meta-analysis that includes Argentinean study data (90.6%) and other Latin American populations of multiethnic composition such as Paraguay (82%) and Ecuador (85%) (Picconi et al., 2002, 2003; Tonon et al., 2007; Deluca et al., 2012; Cornet et al., 2013; Mendoza et al., 2013; Tornesello et al., 2008). However these findings were different from those reported for Brazil (54%) and Mexico (13%), countries where HPV16 variants of non-European origin are more frequent (Villa et al., 2000; Calleja-Macias et al., 2004).

These differences may be related to the ethnic composition of those populations. For example, in Misiones, genetic admixture as measured with autosomal loci is 13% Native-American, 82% European and 5% African (Corach et al., 2010). Thus, it is possible that the described proportions of infections with HPV16 variants reflect these ancestral contributions to this population. Nevertheless, no lineage B and C (African origin) variants were identified in our sample. Instead, we found a single variant from lineage D1 (formerly NA), one that has been recently reported at highly frequency in North Africa (Cornet et al., 2012). Therefore, D1 may represent an understudied branch of HPV16 African evolution, a hypothesis that will require further testing through the analysis of larger phylogeographic datasets.

Despite their low frequency, HPV16 variants from lineage D were more frequently associated with high-grade lesions and cancer than the HPV16 variants from lineage A, with an OR of 13.8 (1.6–117.0). This is an important finding, as HPV16 variants from lineage D have been associated with invasive cervical cancer in other Latin American countries such as Mexico (Berumen et al., 2001), Costa Rica (Hildesheim et al., 2001; Schiffman et al., 2010), and Brazil (Villa et al., 2000; Sichero et al., 2007), but not in those from Europe (e.g., Portugal, United Kingdom, Italy, among others; Hildesheim and Wang, 2002; Sichero and Villa, 2006; Burk et al., 2013). The existence of conflicting data concerning the linkage between lineage D variants and disease risk has been explained by the ethnic composition of the study population (reviewed in Hildesheim and Wang, 2002; Sichero and Villa, 2006; Burk et al., 2013). For example, in European communities that are ethnically homogeneous, the lack of any association may be due to the overwhelming predominance of lineage A HPV16 variants which, in turn, does not allow a proper evaluation of risk associated with non-European HPV16 variant infections (lineages B, C and D) (Marongiu et al., 2014). On the other hand, studies of more diverse populations such as those of North and Latin America, have indicated that lineage D variants are more aggressive than those of lineage A, with a 2- to 9-fold increased risk of cervical cancer and high-grade cancer precursors (Hildesheim et al., 2001;

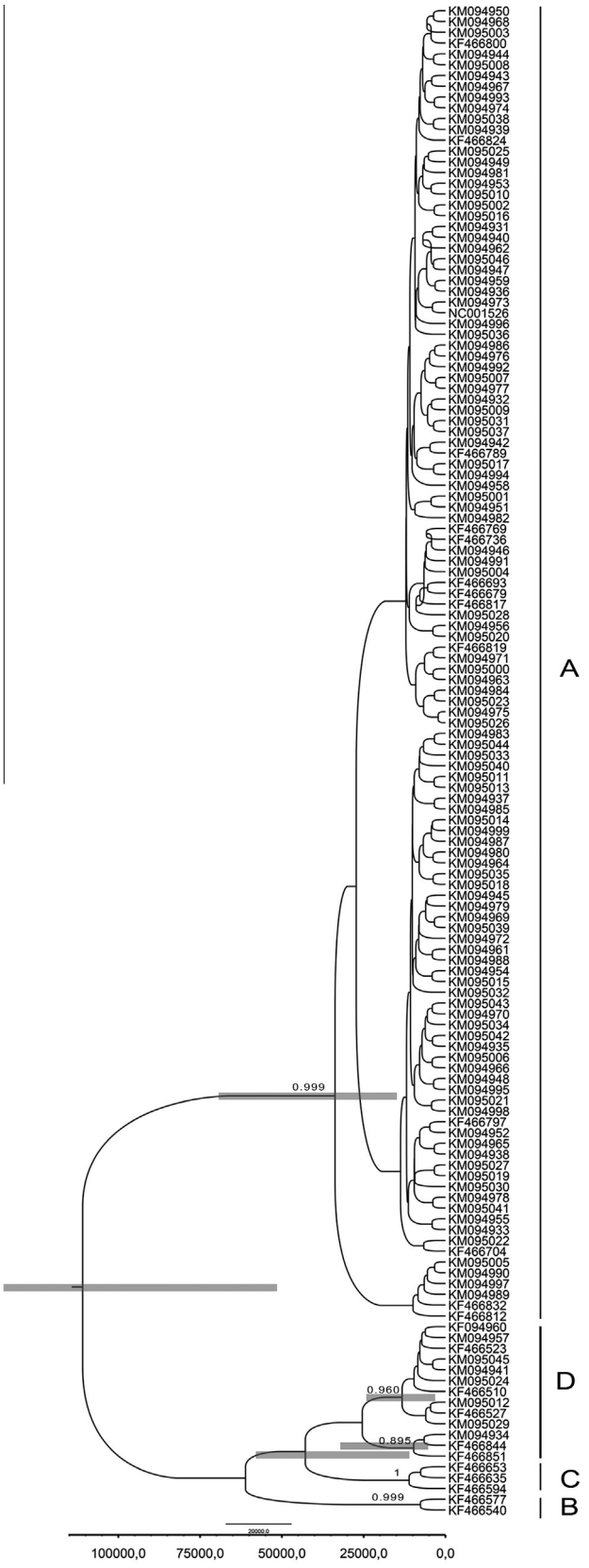

Fig. 1. Phylogenetic analysis and molecular dating of HPV-16 variants from this study. The evolutionary history was inferred using the Bayesian method. The maximum clade credibility tree is shown. The analysis involved 140 nucleotide sequences. The final dataset included a total of 322 positions. Timeline: the *X* axis indicates years ago. The posterior probability values ($\geqslant 0.89$) are shown next to the branches. The HPD 95% values for the t_{MCRA} are shown in the shaded areas.

Table 2
Distribution of HPV16 variants according to the lesion grade.

HPV16 Variant	NLIM	L-SIL	HSIL[a]	Odds ratio (CI 95%)[b]
Lineage A	16	61	31	1 (Ref)
Lineage D	0	1	7	13.8 (1.6–117.0)

[a] H-SIL+ includes H-SIL, CIS and ISCC.
[b] The odds ratio was calculated for L-SIL versus H-SIL. The lowest grade of cervical lesions (L-SIL) is taken as a reference. NLIM cytology could not be computed because one of the cells is zero.

Berumen et al., 2001; Sichero et al., 2007; Smith et al., 2011). Therefore, the distribution of HPV16 variants worldwide and their relative risks for cervical cancer appear to be population dependent.

These differences may be attributed to several factors, including viral genetics, human genetics and viral co-evolution within human ethnic groups (Zehbe et al., 2001; Hildesheim et al., 2001; Beskow et al., 2001; Burk et al., 2003; Xi et al., 2006; de Araujo Souza et al., 2008; Zuna et al., 2009; Cornet et al., 2013). Given that viral co-evolution within human populations is a long held hypothesis, we explored the temporal framework for the origin of HPV16 variants with a Bayesian analysis. In our population, all variants coalesced to a t_{MCRA} of 119,103 years, a date that is consistent with the emergence of anatomically modern humans (*Homo sapiens*) prior to their expansion out of Africa (Behar et al., 2008). Two additional time points are worth highlighting. Lineage D2 coalesced to a t_{MCRA} of 21,421 years ago and lineage A to a t_{MCRA} of 41,793 years ago. The median values of these t_{MCRA}s were similar to those estimated with the mtDNA lineages of Amerindian and European populations (Achilli et al., 2008; Torroni et al., 2006). Therefore, HPV16 variants from lineage D2 may have evolved in association with Amerindian populations, beginning thousands of years ago. On the other hand, HPV16 variants from lineage A were probably brought to Misiones during the Spanish colonization (15th century) of the region and later European immigration in the 19th century.

In this regard, an ongoing analysis of larger fragments of the genome (E6, E7 and L1 genes) is showing that several different European variants are circulating within the urban population, although most of them not detected with the 350 bp LCR sequences. Therefore, the hypothesized introduction of European variants into this population is plausible and needs to be further studied.

As a final point, it is important to recall that the results of the coalescence analyses with the 322 bp sequences do not provide the definitive evolutionary history of HPV16. However, they do provide a glimpse in this matter. In fact, given the large confidence intervals with our estimates, those results should be interpreted cautiously (also see Section 6).

5. Conclusions

The ongoing implementation of vaccination programs in Argentina has reinforced the need for more knowledge about the regional prevalence of HPV16 types and variants in this population. To date, only fifty sequences of HPV16 LCR from our country were available in Genbank. This work will significant enlarge that dataset by adding 116 sequences, some of which are presumed to be novel variants. Our results also show that HPV16 variants from lineage D are more pathogenic than isolates from the A lineage in a multiethnic population of northeastern Argentina. The increased odds ratio data obtained in this study provides us with important insights into the oncogenic potential of the variants detected in this population, but due to our limited sample size future pooling efforts are needed to confirm the patterns of genetic diversity and disease association observed in this project. Whether

the observed association is due to a direct effect by the LCR variants detected here or to an indirect effect resulting from "lineage fixation" (co-variations with other regions of the viral genome) (Chen et al., 2005; Smith et al., 2011; Burk et al., 2013), further analysis of other genes of clinical importance such as E6, E7 and L1 will help to clarify these evolutionary and clinical questions about HPV16 infection.

6. Study limitations and future research

Thus far, our analysis suggests that HPV16 lineages have co-evolved with human populations and provides a potential congruence between HPV16 and demographic histories in Misiones. However, there is some concern regarding the mutation rate used in this study. In fact, the rate of 1×10^{-7} s/s/y is a theoretical estimate based on the assumption that the convergence hypothesis is correct (Halpern, 2000). Therefore, we tested our data with two different rates that were independent of the co-evolution hypothesis: (1) 3.94×10^{-3} s/s/y, a higher nucleotide substitution rate estimated by using time-structured data (Firth et al., 2010); and (2) 1.95×10^{-8} s/s/y, a value estimated using fossil calibration points for *Felidae Papillomavirus* tree (Rector et al., 2007). The two rates imply different scenarios for HPV evolution. On the one hand, with a substitution rate of 3.94×10^{-3} s/s/y, our data coalesce to a root of nearly 3 years, with the divergence of the D2 lineage occurring during the last few months, which is a very unlikely epidemiological scenario for human papillomavirus.

On the other hand, when using a fixed rate of 1.95×10^{-8} s/s/y, all HPV16 lineages diverged from their common ancestors within the last million years (mean = 560,240 ybp; HPD 95% 254,550–1,010,200), which corresponds to a period of time when several species from the genus *Homo*, including *Homo erectus* and *Homo heidelbergensis*, lived on earth (Tattersall, 1995). Interestingly, Chen et al. (2009) obtained a similar t_{MCRA} for HPV18 variants (<0.7 Myr). Moreover, the divergence of the HPV16 D2 lineage can be estimated at nearly 85,951 ybp, close to the date at which modern humans expanded out of Africa. Under this scenario, the current patterns of viral infection are the result of a combination of dispersal events (migration and founder effects) and viral co-evolution with humans. By extension, in scenarios involving the 1×10^{-7} s/s/y and 1.95×10^{-8} s/s/y mutation rates, Asian-American variants evolved in association with modern human populations, beginning thousands of years ago (data available upon request).

An additional point that needs to be addressed regarding viral/host evolution is the tree topology. While specific lineages do predominantly exist within certain populations and the t_{MCRA}s appears to match their population histories—at a fixed rate of 1×10^{-7} s/s/y—the overall shape of the tree is not consistent with pattern of human evolution, with one of the main discrepancies being that the HPV16 B and C lineages (which are associated with African populations) are not basal to the HPV phylogeny. This lack of congruence between virus and host phylogenies favors the hypothesis of dispersal over strict co-evolution, giving additional support to the lower rate of 1×10^{-8} s/s/y. An alternative explanation to the phylogenetic incongruence is that either a basal African strain of HPV has gone extinct or has not yet been isolated, the being a possibility given that the African continent is a more poorly studied geographical region compared to Europe, North America and Latin America. However, this discrepancy cannot be resolved with our current data set. It will be difficult to statistically test the degree of congruence between phylogenetic trees of virus and host genomes with our short DNA sequences and relatively small sample of viral and human ethnic diversity.

Nevertheless, the present study provides new ways of thinking about HPV16 evolution. In addition, our investigation of the rates and processes of HPV16 evolution independently of the hypothesis of co-divergence constitutes an important avenue for future research.

Author contributions

Conceived and designed the experiments: IB, ACAC, RHC. Performed the experiments in molecular biology: IB, MET, DJS, ICB, AR, JB. Performed the experiments in phylogeny/coalescence: ACAC. Analyzed the data: IB, ACAC. Contributed reagents/materials/analysis tools: IB, TGS, MAP, RHC, DJL. Wrote the paper: IB, ACAC, TGS, RHC, DJL. All authors have read and approved the final manuscript. The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2014.11.013.

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