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Research paper

Analysis of human papillomavirus 16 E6, E7 genes and Long Control Region in cervical samples from Uruguayan women

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ABSTRACT

Objective: This study aims to investigate the HPV16 variant distribution by sequence analyses of E6, E7 oncogenes and the Long Control Region (LCR), from cervical cells collected from Uruguayan women, and to reconstruct the phylogenetic relationships among variants.

Methods: Forty-seven HPV16 variants, obtained from women with HSIL, LSIL, ASCUS and NILM cytological classes were analyzed for LCR and 12 were further studied for E6 and E7. Detailed sequence comparison, genetic heterogeneity analyses and phylogenetic reconstruction were performed.

Results: A high variability was observed among LCR sequences, which were distributed in 18 different variants. E6 and E7 sequences exhibited novel non-synonymous substitutions. Uruguayan sequences mainly belonged to the European lineage, and only 5 sequences clustered in non-European branches; 3 of them in the Asian-American and North-American linage and 2 in an African branch. Additionally, 6 new variants from European and African clusters were identified.

Conclusions: HPV16 isolates mainly belonged to the European lineage, though strains from African and Asian-American lineages were also identified. Herein is reported for the first time the distribution and molecular characterization of HPV16 variants from Uruguay, providing novel insights on the molecular epidemiology of this infectious disease in the South America.

Synopsis: A high variability among HPV 16 isolates mainly belonged to European lineage, provides an extensive sequence dataset from a country with high burden of cervical cancer.

1. Introduction

Genital human papillomavirus (HPV) infection is the most common sexually transmitted disease among women. It is estimated that 300 millions of women are infected worldwide and > 490.000 will develop cervical cancer (Parkin and Bray, 2006). Cervical cancer is the fourth cause of cancer in women, with 528,000 new cases and 266,000 deaths per year (Ferlay et al., 2015). Latin American countries together with other developing areas from Africa and Asia are the regions with the highest incidence and mortality rates of cervical cancer, carrying the 85% of the burden of disease. In these areas, the disease accounts for 12% of female cancers, in contrast to the 3.5% observed in developed countries (Ferlay et al., 2015).

Several studies have compared the nucleotide sequence of the reference virus prototype K02718 with HPV16 isolates from patients coming from different regions of the world and epidemiological settings (Seedorf et al., 1985; Burk et al., 2013). Findings have described the existence of four phylogenetic lineages A, B, C, D. Lineage A comprises four sublineages: A1, A2, A3 (includes European sequences worldwide) and A4 (Asian sequences). Lineage B is further divided into sublineages B1 and B2, which include African sequences, as lineage C. Lineage D, in turn is sub classified into D1, D2 and D3 and comprises Asian-American and North-American sequences isolated worldwide.

This high variability observed among HPV strains has leaded to

Abbreviations: HPV, human papillomaviruses; LCR, Long Control Region; HSIL, High Grade Squamous Intraephitelial lesion; LSIL, Low Grade Squamous Intraephitelial lesion; ASCUS, atypical squamous cells of undetermined significance; NILM, negative for intraephitelial lesion or malignancy; HR-HPV, High Risk - Human Papillomaviruses; LR-HPV, Low Risk - Human Papillomaviruses; A, lineage name; A1, sublineage name, European sequences, E European; A2, sublineage name, European sequences, E; A3, sublineage name, European sequences, E; A4, sublineage name, Asian sequences, As Asian; B, lineage name; B1, sublineage name, African sequences, Af-1a African type 1a; B2, sublineage name, African sequences, Af-1b; C, lineage name, Af-2a African type 2a; D, lineage name; D1, sublineage name, North American sequences, NA1 North American type 1; D2, sublineage name, Asian-American sequences, AA2 Asian American type 2; D3, sublineage name, Asian-American sequences, AA1; SNP, single nucleotide polymorphisms

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several research groups to investigate the potential association between nucleotide substitutions in E6 gene and cancer development (Wheeler et al., 1997). In fact, it is clear that Asian-American variants seem to be more tumorigenic than E variants (Berumen et al., 2001). In this sense, it has been proposed that any mutations in these oncogenes and the LCR may induce changes in the biological properties of the oncoproteins or a deregulation of their expression, thus increasing the risk of progression to cancer (Yamada et al., 1997).

Uruguay, where cervical cancer occupies the fourth position in frequency, has a standardized incidence rate by age of 19 per 100,000 women, involving 402 cases per year and a mortality rate of 4.7 per 100,000, with 175 deaths annually (GLOBOCAN, 2012). Interestingly, this epidemiological picture suggests that Uruguay has incidence rates comparable to that observed in developing countries, but exhibits mortality rates similar to that found in most of the developed countries (Ferlay et al., 2015). Previous studies on the HPV genotype prevalence and distribution in Uruguay have shown that HPV16 genotype is the most frequently found in pre-cancerous lesions and carcinomas (28.6% and 65.9%, respectively) (Berois et al., 2013; Ramas et al., 2013).

This study aimed two goals: first, to determine the distribution and frequency of HPV16 variants among Uruguayan women with different cytological degree; and second, to reconstruct the phylogenetic relationships among HPV16 variants, in order to identify the currently circulating lineages.

2. Materials and methods

2.1. Study population

Forty-seven HPV16 positive samples were selected from 568 cervical smears screened by standard procedures as previously reported (Ramas et al., 2013). Patients (mean age 33 years, 18–53) were enrolled from the cervical screening medical centres which are part of the National Cervical Cancer Prevention Sub-Program, Ministerio de Salud Pública, Uruguay. The study design had been approved by Faculty of Medicine's Ethics Committee (Res. 071140-000927-07) and all participants gave their written consent.

Smears were cytologically diagnosed according to the Bethesda classification system (Solomon et al., 2002). Samples included 7 high grade intraepithelial lesions (HSIL), 16 low grade intraepithelial lesions (LSIL), 6 atypical squamous cells of undetermined significance (ASCUS), 7 with no lesion or malignancy (NILM) and 11 without cytological data or undetermined result.

2.2. HPV DNA detection

A 364 bp region within the LCR was amplified for the 47 specimens studied, as previously described (Chan et al., 1992). From these, 12 samples with sufficient amount of DNA were further analyzed for E6 and E7 oncogenes. HPV16 E6 and E7 PCR assays were performed with the specific primers reported by Pande et al. (2008) with modifications: for HPV16-E6, 5′-GAAACCGGTTAGTATAAAAGCAGAC-3′ and 5′-AGC TGGGTTTCTCTACGTGTTCT-3′, with an amplicon size of 476 bp (nucleotide positions 83-559); and for E7 5′-CCATAATATAAGGGGTCGG TGGA-3′ and 5′-TTTTTCCACTAACAGCCTCTACAT-3′, with an amplicon size of 296 bp (nucleotide positions 562-858). Nucleotide positions refer to the prototype HPV strain K02718.

Specific PCR products were purified by NucleoSpin® Gel and PCR kit (Macherey-Nagel) according to manufacturer's protocol, and sequenced in both directions on an automated DNA sequencer (3130 Genetic Analyzer; Applied Biosystems) in the Sequencing Service from Institut Pasteur de Montevideo. In order to avoid misinterpretations of the substitutions due to polymerase errors, PCR and sequencing reactions were performed twice with newly extracted material.

2.3. HPV16 genetic variability

Viral variants were identified by comparative sequence analysis with the prototype sequence of HPV16 (GenBank accession number K02718), which belongs to the A1 sublineage within A lineage (European strain) (Seedorf et al., 1985; Burk et al., 2013). Sequence analyses and alignment of LCR, E6 and E7 of HPV16 isolates were performed with MUSCLE, included in MEGA v6.0 software.

The E6, E7 and LCR sequences obtained from the viral strains detected in this work were submitted to the GenBank database (Supplementary material, Table S1).

2.4. Phylogenetic analysis

Phylogenetic reconstructions were carried out by the Neighbor-Joining method with MEGA v6.0. JModel Test was used to estimate the optimal evolutionary model that best fitted to the dataset (data not shown). Trees reliability was assessed by bootstrapping and consensus trees were generated. Bootstrap values > 50% provided significant evidence for phylogenetic grouping (Fig. 1).

For comparison, LCR, E6 and E7 sequences from the four phylogenetic branches as proposed by Burk et al. (2013) were retrieved from GenBank. In addition, regional HPV16 variants sequences from Argentina, Brazil and Paraguay were specifically selected and included (Picconi et al., 2003; Burk et al., 2013; Mendoza et al., 2013; Gurgel et al., 2015).

3. Results

3.1. LCR nucleotide sequences analysis

Forty-seven HPV16 samples were compared with the prototype clone (K02718), by analysis of the genome region comprising nucleotides 7478 to 7841. Study of HPV16 LCR showed 30 single nucleotide polymorphisms (SNP) within this region; 15 transitions and 15 transversions. Eighteen variants were detected and 37 sequences differed from the prototype clone in at least one position (Table 1). Of these 18 isolates, 6 sequences (URU4, 18, 19, 39, 55 and 62) that had not been ever reported were newly identified in this work.

3.2. E6 and E7 nucleotide sequences analysis

Twelve HPV16 E6 and E7 gene sequences were amplified with good quality and compared with the prototype clone from nucleotides 83 to 559 and 562 to 858, respectively. Analysis of E6 region evidenced 7 variable sites; 3 transitions (A131G, A289G and C335T) and 4 transversions (G145T, T286A, T350G and T387A). Eight sequences, grouped in 4 variants, displayed at least one single nucleotide change compared to the K02718 reference strain. Five of the seven SNPs were missense mutations, R10G (A131G), Q14H (G145T), H78Y (C335T), L83V (T350G) and D98E (T387A).

In turn, 4 of the 5 SNPs detected in E7 (transitions T732C, C785T and T789C and transversion T795G) were synonymous mutations. Additionally, a non-synonymous change, histidine for asparagine, was identified in the position 51 (C712A). Alignment of E6 and E7 sequences is shown in Table 1.

3.3. Phylogenetic reconstruction

Phylogenetic analysis of the 47 Uruguayan strains and reference sequences performed with the 364-pb region within the LCR, showed that most (89.4%) of the HPV16 strains belonged to A lineage (A1 sublineage), and clustered with the prototype clone K02718. Non-European variants accounted for 5 (10.6%) infections; 3 were included in D lineage, 2 (within D1 sublineage and 1 in D3 sublineage) and 2 clustered within C lineage. Novel Uruguayan variants (URU4, 18, 19,

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