

Human papillomavirus type 16 molecular variants in Guarani Indian women from Misiones, Argentina

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KEYWORDS

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Summary

Objective: To identify human papillomavirus type 16 (HPV16) E6 and L1 molecular variants infecting Guarani Indian women settled in Misiones, Argentina, a region with a high prevalence of cervical cancer. Some intratypic molecular variants of HPV16 have been associated with greater oncogenic risk, but their implication in the etiology of cervical cancer is still uncertain.

Methods: Seventy HPV16 positive cervical samples from Guarani Indian women settled in two different areas of Misiones, Argentina, (34 from the northern area and 36 from the central area), were analyzed. Thirty-seven had normal cytology, 18 had a low-grade squamous intraepithelial lesion (LGSIL), and 15 a high-grade squamous intraepithelial lesion (HGSIL). HPV16 E6 and L1 molecular variants were identified by PCR, followed by dot blot hybridization with 23 and 12 biotinylated oligonucleotide probes, respectively.

Results: The frequency of HPV16 variants over the Guarani population was 51% EP (European prototype), 32% E-350G, 9% Af1-a (African 1), 4% E-6862C, 3% Af2-a, and 1% AA-a (Asian-American). The distribution of variants was not homogeneous in the two areas under analysis, with the northern area being more diverse showing 74% of European variants, while the central area presented exclusively E variants. No statistically significant association was found between any particular variant and grade of cervical lesion.

Conclusion: This study reports for the first time HPV16 E6 and L1 molecular variants infecting women from an aboriginal community inhabiting a rainforest region of South America. The presence of E class variants could be attributed primarily to contacts with the Spanish

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conquerors, and Af variants from African slaves introduced later in the South American continent.

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Introduction

More than 490 000 new cases of cervical cancer occur among women worldwide each year. Incidence rates of this disease vary from about 5 cases per 100 000 women per year in many industrialized countries to more than 50 per 100 000 in some developing nations.¹ Approximately 80% of all cases occur in less-developed countries, because prevention programs are either non-existent or poorly conducted.

High-risk types of mucosotropic human papillomaviruses (HPVs) are associated with more than 96% of cervical cancers worldwide, with HPV16 detected in about 50% of them.² However, HPV16 is also the most common type of HPV infecting the normal population, and only a minority of women with HPV16 infection develop invasive cervical cancer.³ It has now been well established that high-risk HPV cervical infection plays a significant role in the initiation and progression through various stages of cervical intraepithelial neoplasias (CINs) to invasive cancer.^{4,5} Nevertheless, the evolution of primary viral infection to CINs and cancer is dependent on several factors, many of which are still unknown.^{6,7}

HPV genotypes have been commonly used as the exposure measure for HPV-associated risk for invasive cervical carcinoma, considering that different types of HPVs vary in tissue affinity and pathogenicity.² It becomes important then to consider biological modifiers of HPV-associated disease risk, such as the presence and role of sequence variation within HPVs as it may relate to risk for invasive cancer.⁸

For all HPV types studied to date, intratypic variants have been identified.^{9–12} These variants differ from the reference viral type sequence in the L1 coding region by up to 2.0% and show different geographic distribution.¹³ In particular, HPV16 variants are distributed differently among the five continents: Asian (A) variants are mainly in Southeast Asia, African (Af) variants are mainly in Africa, European (E) variants are in all regions other than Africa, and Asian-American (AA) variants are located mainly in Central, South America and Spain.^{11,14}

The possibility that differences in biologic characteristics might also be present among intratypic HPV16 variants deserves consideration. Some of them have already been associated with CINs or more aggressive cervical cancers.^{15–20} Therefore determining the natural HPV16 variants present in populations showing high incidence of cervical lesions and cancer is of particular interest in view of their potential association, and for the design of HPV16 vaccine strategies.

The province of Misiones is located at the northeastern tip of Argentina sharing international borders with Paraguay and Brazil, and retains the last remnant of rainforest of the area. Misiones is considered a region with both a high incidence of cervical carcinoma (33/100 000) and HPV genital infection in the urban population.²¹

The Guaraní Indians inhabit the rainforest of Misiones, being around 1700 in total, concentrated in more than 20 small communities. Until the last decade, the Indian social

units had been self-sustaining, avoiding direct exchanges with the white population of urban areas. However, recent economic and ecological factors have generated an important change in the Indian–white relationship, compelling male Indians to integrate with the rural work-force in white-run agricultural establishments.

Guaraní women begin sexual activity early, after menarche; they have multiple sexual partners and are multiparous. Some of their partners have already had intercourse with white women of neighboring towns. This abrupt social change has had a direct impact on the Indian community health status, bringing a significant increase in sexually transmitted and respiratory diseases.

This situation led us to conduct the first reported cervical HPV infection screening study among sexually active Guaraní women resident in the region, representative of a particular community beginning to interact with the urban population.²² In this research, a cross-sectional cytological and HPV screening study of the nine main Indian settlements was conducted. Demographic data, information about sexual behavior, and gynecological history were recorded. Nearly all Guaraní women screened had some grade of cervical disease, including *in situ* carcinomas. Generic HPV infection prevalence was elevated (64%), with predominance of high-risk types 16/18 (26%). A large variety of other viral types were also detected, including high- to intermediate-risk types not found previously in the urban population of the region. These epidemiological results led us to propose the existence of an HPV high-risk type viral reservoir in the Indian community.

The purpose of the present study was to characterize class and subclass variants of HPV16 from this group of Guaraní Indian women based on the analysis of the E6 and L1 viral genes, and to analyze putative associations with observed cervical lesions.

Materials and methods

Study population

Seventy cervical samples from Guaraní Indian women infected with HPV16 were obtained from a community-based cervical HPV infection screening conducted by the authors during the years 2002–2003.²² Informed consent was obtained from their guardians.

The general population characteristics are presented in Table 1. The median age was 20 years, 60% being younger than 30. This particular age distribution is common among Guaraní Indians, mainly due to high mortality rates of those above 40 years of age caused by different infectious diseases. The mean age of first intercourse was 13 years and first pregnancy was 15 years. Polygamy reached 80% and the illiteracy rate 87%, as expected in this particular semi-closed society. Ninety-four percent had had no previous gynecological examination.

The 70 HPV16 infected women were distributed over two different geographical regions; 34 in the northern area of the

Table 1 General characteristics of the group of Guarani women under study

Characteristic	
Age range (years)	15–45
Median (years)	20
Educational level	
No instruction	87%
Elementary instruction	13%
Complete general instruction	0%
Previous gynecological examination	
Yes	6%
No	94%
Age of first intercourse	
>19 years old	1.6%
17–19 years old	7.8%
14–16 years old	44.3%
<14 years old	46.3%
Partners	
1	20.8%
2–5	65.7%
>5	13.5%
Pregnancies	
1	13.9%
2–5	44.4%
>5	41.7%

Misiones province, at the borders with Brazil and Paraguay, and 36 in the central area, deep into the rainforest, far away from the borders (Figure 1).

Samples

HPV16 positive samples selected for variant analysis were previously identified and typed by PCR and PCR–RFLP (restriction fragment length polymorphism).^{22,23} Histopatho-

logic diagnoses for these samples included 37 within normal limits, 18 low-grade squamous intraepithelial lesions (LGSIL), and 15 high-grade squamous intraepithelial lesions (HGSIL).

Identification of HPV16 E6 and L1 variants

The identification of HPV16 E6 and L1 variants was done by the method proposed by Wheeler et al.²⁴ In brief, a one-tube nested PCR was performed to amplify a 585 bp region of the E6 gene and a two-tube nested PCR for a 423 bp region of the L1 gene from HPV16. The amplification products were identified by dot blot hybridization. Twenty-three and 12 biotinylated oligonucleotide probes were used for E6 and L1 fragment hybridization, respectively. The nucleotide variations targeted by these probes included HPV16 E6 nucleotide positions 109, 131, 132, 143, 145, 178, 183, 286, 289, 335, 350, 403, and 532 and L1 nucleotide positions 6695, 6721, 6803, 6854, 6862, and 6694.

Immediately prior to hybridization with HPV16 lineage-specific oligonucleotide probes, membranes were pretreated at 65 °C in 0.1X SSPE containing 0.5% sodium dodecyl sulfate (SDS) under vigorous shaking conditions. Hybridizations with biotinylated probes (1.0 pmol of probe per 50 mL of hybridization solution) were conducted overnight in 5X SSPE containing 0.1% SDS at 42.5 °C. Membranes were then subjected to two consecutive 10-min washes with 2 liters of 2X SSPE that had been prewarmed at a previously standardized temperature, and further incubated with 2X SSPE–0.1% SDS containing 30 ng/mL of streptavidin peroxidase for 20 min at room temperature and constant shaking. Later, the membranes were washed twice with 2X SSPE–0.1% SDS with constant shaking for 10 min each and incubated for 1 min in 10 mL of a mixture for enhanced chemiluminescent substrate reagents (ECL; Amersham, Arlington Heights, IL, USA) following the manufacturer's instructions. Exposure to Kodak X-OMAT AR-5 film was for 2 and 10 min or for 2 h. Positive controls used included HPV16 clones of each variant under study.

E6 and L1 hybridization assay results were interpreted from the chemiluminescent signals recorded on X-ray film. Linked results were used to establish an overall hybridization

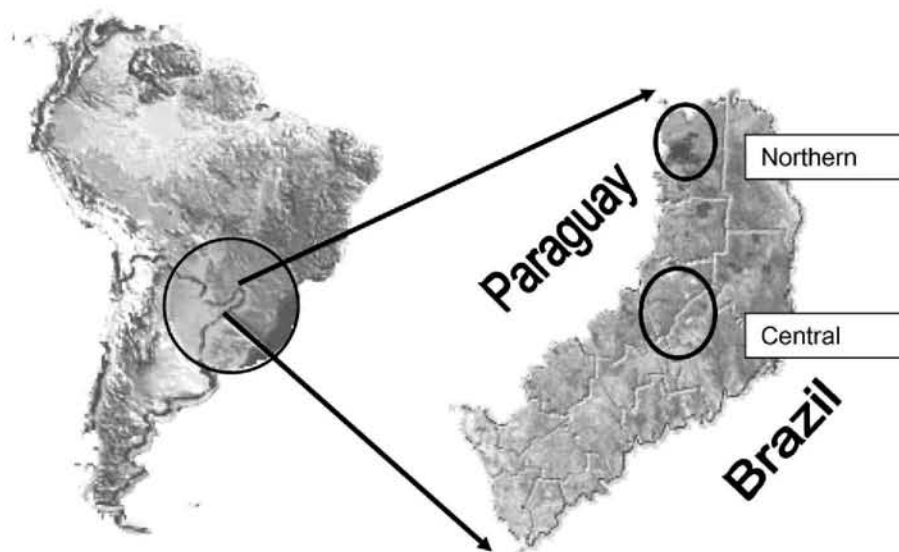


Figure 1 Geographical locations of Guarani settlements included in this study.

pattern for the targeted HPV16 E6 and L1 regions in each specimen examined. Combined hybridization patterns were assigned on the basis of the predicted HPV16 class, subclass, or minor class variants reported previously.¹¹

Statistical analysis

Association analysis between histopathological status and HPV16 variants was carried out by χ^2 , calculated using the Epi-Info 6.0 program (Epidemiology Program Office, Division of Public Health Surveillance and Informatics, Centers for Disease Control and Prevention, USA).

Results

Study population characteristics

Seventy samples from Guaraní Indian women, catalogued as mono-infected with HPV16 from a previous epidemiological HPV screening analysis carried out during the years 2002–2003 were selected for this study.²²

There was no statistically significant association between any of the population characteristics and HPV16 infection or cervical disease as analyzed by means of χ^2 . Furthermore, the distribution of lesions over the population regarding settlement area showed no statistically significant differences (Table 2).

HPV16 variants

The data allowed the identification and classification of the major class and subclass variants of HPV16 infecting this aboriginal population. The distribution of HPV16 variants was 36 (51%) EP (European prototype), 22 (32%) E-350G, six (9%) Af1-a (African 1), three (4%) E-6862C, two (3%) Af2-a, and one (1%) AA-a (Asian-American).

The distribution of variants was not homogeneous in the two geographical regions under analysis, the northern area

Table 4 Distribution of HPV16 variants among samples regarding histopathological status

HPV16 variant	Normal	Total in lesions	LGSIL	HGSIL
EP	20 (56%)	16 (44%)	8 (22%)	8 (22%)
E-350G	11 (50%)	11 (50%)	7 (32%)	4 (18%)
E-6862C	2 (nc)	1 (nc)	1 (nc)	—
Af1-a	3 (50%)	3 (50%)	3 (50%)	—
Af2-a	1 (nc)	1 (nc)	1 (nc)	—
AA-a	—	1 (nc)	—	1 (nc)

No statistically significant difference detected in the frequency distribution of variants regarding histopathological sample status. LGSIL: low-grade squamous intraepithelial lesion; HGSIL: high-grade squamous intraepithelial lesion; nc: not compared due to low data registry.

being more diverse, showing 74% of European variants and 26% of other classes, while the central area presented exclusively European variants (Table 3). In both areas the most frequent variant was EP, followed by the sub-class E-350G.

The distribution of variants among samples regarding their histopathological status showed no statistically significant association when analyzed by means of χ^2 (Table 4).

Discussion

In this study the distribution of HPV16 E6 and L1 variants in a particular indigenous population – Guaraní women inhabiting the last remnant of rainforest in Argentina – has been analyzed. This ethnic group presents particular characteristics from the social, geographical, nutritional, and health related standpoint. Their limited access to public health programs and relatively scarce contact with the urban population lasting for decades is changing rapidly, bringing important modifications in their way of living and affecting their health status. The northern area, at the borders with Brazil and Paraguay, has been experiencing an explosive migratory flux during the last decade. This situation has pushed male Guaraní Indians to leave their ancient settlements for extended periods in order to work in rural duties or to sell their products to tourists or white neighbors.

The recently reported epidemiological survey of HPV carried out in this population²² detected a high prevalence of generic HPV cervical infection (64%) and the presence of oncogenic HPV types (26%). Also noteworthy was the important viral diversity detected (24 different viral types), including high- to intermediate-risk types not previously found in the region. This situation contrasted sharply with the only six HPV types found in the white urban population residing in nearby areas.²¹

All Guaraní women detected as mono-infected with HPV16 in the screening program were selected for variant analysis and included in this work. This particular group showed a high prevalence of cervical lesions, with 47% of them being affected with some squamous intraepithelial lesion, 19% of these high-grade.

It has already been well established that a persistent cervical infection with HPV16 is associated with the development of premalignant lesions and cervical cancer.^{15,25} Several findings suggest that different natural variants of

Table 2 Histopathological profile of the population of Guaraní women under study

Region	n	Normal	LGSIL	HGSIL
Northern area	34	17 (24%)	11 (16%)	6 (9%)
Central area	36	20 (28%)	7 (10%)	9 (13%)
Total	70	37 (53%)	18 (26%)	15 (21%)

LGSIL: low-grade squamous intraepithelial lesion; HGSIL: high-grade squamous intraepithelial lesion.

Table 3 Distribution of HPV16 E6 and L1 variants in the regions under study

HPV16 variant	Northern area	Central area
EP	17 (50%)	19 (53%)
E-350G	5 (15%)	17 (47%)
E-6862C	3 (9%)	—
Af1-a	6 (18%)	—
Af2-a	2 (6%)	—
AA-a	1 (2%)	—

HPV16 in a population may not have the same biologic behavior. Recent research has found that certain HPV16 variants tend to be more strongly associated to high-grade intraepithelial lesions and cancer^{26,16} and that their distribution, defined on the basis of geographical relatedness, is associated to ethnicity.¹⁸

The present study detected a high prevalence of European class variants, representing 87% of the total over the entire HPV16 infected Guaraní Indian population, with the prototype being the most common strain found (51%), followed by the variant E-350G (32%). These results are in agreement with the only worldwide study of HPV16 variants in cervical cancers done to date. In that work, Yamada et al.¹¹ found 76% of European class variants with 52% of subclass E-350G in 228 samples from Central and South America. Recently, del Refugio González-Losa et al.²⁷ reported 69% of European class, with 27.5% being of the E-350G subclass in 40 samples of LGSIL and cervical cancer from Mexican women.

Few non-European class variants were detected in the Guaraní population, accounting for 12% of the total. The African class 1 was the most detected, representing 9% among them. Surprisingly only one Asian-American variant was detected. These results contrast with those reported by Yamada et al.,¹¹ where Asian-American variants accounted for 20% of the total present in carcinomas from Central and South America, and African variants only 3%.

Of particular interest is the distribution of these non-European class variants, for all of them were found in one geographical area, the northern region. The Indian settlements of this region are situated over the borderlines with Paraguay and Brazil, being separated only by rivers, and exposed to strong migratory flux over the last two decades. This situation contrasts sharply with those Indian settlements situated in the central region, these being more isolated and avoiding periodical contact with urban populations. Noticeably, only European class HPV16 variants were detected in Guaraní women inhabiting this area.

Taking into account that recombination events between HPV16 variants are nonexistent or very rare,¹¹ these findings may represent different waves of HPV viral spread associated to historical human migration patterns – E class variants being introduced primarily by the Spanish conquerors that colonized these areas, and Af variants from African slaves introduced later in the South American continent.²⁸

The only study of HPV16 variants performed on Indians inhabiting Argentina reported to date is that of Picconi et al.²⁹ They analyzed 106 HPV16 positive cervical samples, including 33 LGSIL, 28 HGSIL, nine invasive cervical cancer, and 36 samples from women with normal colposcopy and cytology from the Quechua Indian population inhabiting the northwest part of Argentina, at the borders with Bolivia and Chile in the Andean mountains. Their results showed that only about 10% of the specimens had non-European variants, but these included eight Asian-American, two Asian, one North-American-1 and no African variants, and found a statistically significant association between the E-350G variant presence and severity of lesion.

This report provides preliminary support to the hypothesis that human migration at the western side of Argentina brought in the original ancient variant from Asia, while that at the eastern side has incorporated variants as a result of more recent movements such as the introduction of African

slaves in Brazil. However the influence of several other factors like founder effects, co-evolution of HPVs with human races, and viral transmissibility cannot be ruled out. In particular, specific human leukocyte antigen (HLA) alleles may influence the immune response to HPV infection, making possible a preferential HPV type selection by hosts associated to defined genetic backgrounds.^{26,30} The question of whether particular ethnic groups are more susceptible to specific HPV types/variants is still controversial and should be further studied.³¹

Although several reports have suggested that the risk of developing a cervical lesion is not the same with all variants of HPV16, the non-prototype ones being more carcinogenic,^{16,18,20,27,29} no statistically significant difference among these variables was detected in the Guaraní population analyzed. Despite the fact that the number of samples analyzed is small, they represent more than 90% of the local sexually active Indian female population, establishing their epidemiological profile. It will be of interest to compare the dynamics of the particular HPV16 variants actually present in these aboriginals with those circulating in the white urban population of the region.

The World Health Organization has recommended extending HPV epidemiological studies particularly to those populations showing high prevalence of cervical cancer, in order to contribute to vaccine formulations. This work provides primary data regarding HPV16 variants circulating in a semi-closed aboriginal society, the Guaraní Indians from north-eastern Argentina. The identification of the variants present in this particular community adds valuable information to worldwide epidemiological surveys and will allow, in the near future, the generation of a map of HPV circulation in northern Argentina and nearby nations.

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