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HUMAN PAPILLOMAVIRUS GENOTYPES IN WOMEN WITH CERVICAL CYTOLOGICAL ABNORMALITIES FROM AN AREA WITH HIGH INCIDENCE OF CERVICAL CANCER

Gerardo Daniel DELUCA(1), Raúl Horacio LUCERO(1), María T. MARTIN de CIVETTA(2), Lilian VICENTE(3), Ofelia L.Z. de GORODNER(4), Eduardo SCHELOVER(4) & José Mario ALONSO(1)

SUMMARY

It has been well demonstrated the relationship between the infection with high-risk human papillomavirus (HPVs) genotypes and cervical cancer. In Northeastern Argentina a high incidence of this pathology has been described and therefore a high prevalence of HPV infection is expected. In order to identify HPV genotypes associated with malignant and pre-malignant cervical lesions present in the area, 53 ecto-endo cervical cell specimens obtained from women with cytohistological alterations were studied by a PCR-RFLP technique. Out of 53 patients, 34 (64.2%) were positive for HPV infection, being HPV-16 (32.3%) the most frequently found genotype, followed by HPV-58 (14.7%), -6, -18 and –45 (5.9%), -33, -52, -53, -54, -56, -66, -MM4 and –LVX100 (2.9%). Also 5 cases of infection caused by multiple genotypes were found, which corresponded to 14.7% of the positive cases. Results indicate that besides HPV-16 and –18, the most prevalent high-risk HPV genotypes worldwide, others like –45 and –58 as well as co-infection cases are frequent between women of Northeastern Argentina, and a particular attention should be paid to this circumstance because it could be an epidemiological feature of regional importance and a useful information for a future vaccination program.

KEYWORDS: HPV; Cervical cancer; Papillomavirus; PCR-RFLP.

INTRODUCTION

Human papillomavirus (HPVs) are natural occurring DNA tumor viruses which induce epithelial proliferation during the course of a productive infection and are known to be consistently associated with cervical cancer[18,31].

Human papillomavirus invade epithelium germinal cells through microlesions and the resulting infection may be transient or persistent[4,10,27].

Even though infection with oncogenic HPV genotypes is frequent among sexually active women, most of the cases are autolimited[28]; the development of malignant cervical lesions only occurs in a small proportion of infected women that harbor persistent infections with oncogenic genotypes[9,17,26]. It has been demonstrated the integration of viral genome within malignant cells in all cervical cancer cases, which is thought to be a necessary condition for the development of neoplasia[11,12,21].

More than 80 HPV genotypes have been identified up to the present days, and about 40 of them infect the anogenital region. Within the anogenital genotypes, HPV-16, -18,- 31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -68, -73, and -82 were classified as high-risk group, because they were clearly identified in patients with malignant cervical lesions[16,24].

Uterine cervical cancer is the second most common type of cancer among women worldwide, but in Latin America the prevalence rates are about 4 fold higher than in USA and in other developed countries[6].

Although available epidemiological information about this disease is somehow scarce in Argentina, data show a significant variation in the mortality rates for the different regions of the country; from 7 cases out of 100,000 women in the metropolitan area of Buenos Aires city to 17-22 cases out of 100,000 women in Northeast Argentina[15]. In spite of this feature, few epidemiological studies were performed in this last region related to HPV infection and they exclusively focused on a limited number of genotypes, mainly –6, -11, -16 and –18[30]. For these reasons we considered relevant to investigate the presence of other genotypes also associated with cervical malignancy. The aim of this work was to evaluate the presence of HPV genotypes infecting sexually active adult women with cervical abnormalities, and correlate genotypes with the cytohistological type of lesions.

MATERIALS AND METHODS

Subjects: All women self-referring for gynecological examination at the Gynecology Service of "J.R.Vidal Hospital" in Corrientes city and at a private gynecological clinic in Resistencia city, were enrolled in a HPV study from August 2001 to August 2002. A routine gynecological examination including colposcopy was completed, and exfoliated cervical

(1) Area de Biología Molecular. Instituto de Medicina Regional. Universidad Nacional del Nordeste. Resistencia, Argentina
(2) Servicio de Ginecología. Hospital "J. R. Vidal". Corrientes, Argentina.
(3) Instituto Médico 9 de Julio. Resistencia, Argentina.
(4) Servicio de Anatomía Patológica. Hospital "J. R. Vidal". Corrientes, Argentina.
Correspondence to: Gerardo D. Deluca, Instituto de Medicina Regional, Av. Las Heras 727, (3500), Resistencia, Argentina. Fax: 54-3722-422793. E-mail: gerdeluca@hotmail.com

cells were taken for PAP smear. Within the next 30 days, patients who presented any cytohistological lesion, excluding inflammatory PAP smears, were called again and asked for authorization for a new cervical cell sampling to evaluate HPV infection. Upon this procedure, 53 women were studied belonging to the following groups: 3 women with atypical squamous cells of undetermined significance (ASCUS), 36 with low-grade cervical intraepithelial lesions (L-SIL), 5 with high-grade cervical intraepithelial lesions (H-SIL) and 9 with uterine cervical cancer in different stages.

PCR for HPV detection: For viral analysis, cell samples were collected by scraping the uterine ecto-endo cervix with a cytobrush. Cells were washed in sterile buffered saline and the final cellular pellet was kept at -70 ºC until DNA extraction.

DNA was obtained by treating the pellet with 400-700 µl of homogenization solution (2% cetyltrimethylammonium bromide-CTAB, 1.4 M NaCl, 0.2% β-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl pH 5.0), extracted with phenol:chlorophorm:isoamyl alcohol method, then precipitated with ethanol and resuspended in 50-100 µl bidestilled sterile water. All samples were tested for quality and integrity of DNA by PCR with the use of exon III from the human ß-actin gene that produces a 289 bp amplimer. Samples negative for this primer were considered unadequate and discarded.

PCR was performed in a reaction mixture of 100 µl containing 10 mM Tris- HCl pH 8.3, 50 mM KCl, 2.5 mM Mg Cl_2, 0.2 mM of each dNTP *(Promega Inc. – USA),* 0.5 Units of *Taq* DNA polymerase *(Promega Inc. – USA)*, 0.5 mM of generic primers MY09 and MY11 *(Cyber-Sin, USA),* and 5 µl of sample purified DNA. Each PCR was carried out in a DNA thermal cycler *(MJ Research Inc.,* PTC-150*)*, with first denaturation step at 94 ºC for 4 min and final extension step at 72 ºC for 5 min. DNA amplification was performed during 35 cycles which included the denaturation at 94 ºC for 60 s, the annealing at 52 ºC for 60 s, and the primer extension at 72 ºC for 60 s. To avoid false negatives and false positives a reagent control (no template DNA) and known HPV DNA from infected HPV-16 CasKi cells were included in each amplification. PCR product was electrophoresed on 3% agarose gel, stained with ethidium bromide, and photographed under UV light.

Restriction digestion, RFLP analysis: Positive samples products were digested as described by BERNARD *et al.*[3], with 8-10 Units of each one of the following enzymes: Bam HI, Dde I, Hae III, Hinf I, Pst I, Rsa I and Sau3AI (*Promega, USA)*. The digested products were electrophoresed on 3% agarose gel, stained with ethidium bromide and photographed under UV light. Restriction fragment length polymorphism (RFLP) patterns were analyzed and compared with published data[3].

RESULTS

Out of the 53 samples analyzed, 34 (64%) were positive for HPV-DNA. Its distribution according to the type of cytohistological lesions is referred in Table 1.

In regard to the genotypification, HPV infecting types could be identified in 26/34 positive samples. From the 8 remaining samples, the identification could not be resolved in 5 cases due to the fact that they showed a very weak restriction pattern with a blurred image unable for a distinct viral identification; 2 showed restriction maps which did not correspond to none of the patterns described by BERNARD *et al.*[3], and the last one was a case of mixed infection with a very complicated restriction pattern that could not be elucidated (Table 2).

Table 1
PCR for HPV-DNA in women with uterine cervical lesions

	ASCUS	L-SIL	H-SIL	Cervical cancer	Total
Positive	2	19	4	9	34
Negative	1	17	1	-	19
Total	3	36	5	9	53

Table 2
Distribution of HPV genotypes according to histopathological cervical lesions, in 34 HPV-DNA positive samples

Genotype	ASCUS n = 3	L-SIL n = 36	H-SIL n = 5	Cervical cancer n = 9	Total (%)
6		2			2 (5.9)
16		3	3	5	11 (32.3)
18		2			2 (5.9)
33		1			1 (2.9)
45		1		1	2 (5.9)
52		1			1 (2.9)
53		1			1 (2.9)
54		1			1 (2.9)
56		1			1 (2.9)
58		2	1	2	5 (14.7)
66		1			1 (2.9)
MM4		1			1 (2.9)
LVX100	1				1 (2.9)
Not identified*	1	3		1	5 (14.7)
Undetermined		2	1		3 (8.8)

* RFLP not done

Out of the 34 positive samples, 5 (14.7%) were multiple infection cases produced by more than one viral genotype. In 4 of them final genotyping could be successfully resolved by RFLP: HPV-58/45, HPV-6/33, HPV-16/58, HPV-16/66, remaining one case undetermined.

DISCUSSION

Recent advances in molecular techniques allowed the use of highly sensitive methodologies for the detection of HPV-DNA, such as filter *in situ* hybridization (FISH), southern hybridization (SH), Hybrid Capture™ (HC) and the polymerase chain reaction (PCR), being the latter one of the most frequently employed[2,7,13]. However, these methodologies for virological diagnosis do not replace classical gynecological exploration techniques for cytohistological disturbances (PAP, colposcopy and biopsies), but rather, they complement them[22,25].

The high incidence of HPV infection previously reported in the Northern region of Argentina[20,30] is consistent with a high incidence of uterine cervix cancer present in the area. In this work, only patients with cytohistological

alterations and colposcopy compatible with HPV infection were studied. As expected, we certainly found a high frequency of HPV infection, particularly among H-SIL and cancer cases, while in the L-SIL group virus DNA was found only in 52% of the cases. This percentage may be regarded as low if compared with the findings of authors who referred infection rates as high as 70%[8]. Nevertheless, in other reports the presence of viral DNA in this type of patients is considered uncertain[23]. Our results could be directly related to the number of cases analyzed and a better estimation could be probably reached studying a greater number of L-SILs .

The PCR-RFLP technique employing MY generic primers allows the recognition of more than 30 genital genotypes in a single amplification reaction[3]. This is its major advantage in comparison to other methodologies that use specific primers, but its disadvantage is just not to be able to define certain cases of infection caused by multiple genotypes, which frequently produce blurred restriction patterns[1]. In such cases final identification may be achieved by PCR with subsequent Dot-Blot hybridization, by PCR with specific primers, or by nucleotide sequence analysis[1,5,29].

In this work, RFLP analysis could not be performed in five positive samples because genomic material did not amplify enough to obtain a definite pattern with clear-cut bands. We suppose these cases may belong to those whose initial viral genomic load stands on the limit of the technique's threshold[14]. Also, two samples showed patterns that could not be associated with any of the genotypes described by the methodology employed; therefore, it would be necessary to perform DNA sequence analysis to assess if they correspond to intratypic variations, subtypes or to novel genotypes[19].

In agreement with the reports of other epidemiological surveys[20,30], HPV-16 was the most frequent genotype in our series, but HPV-58 was in the second place instead of HPV-18, -31, -33 and –45 as in the most of the series. However, geographical variations in the occurrence of the HPV genotypes are well known and depends on the epidemiological features of each region[24].

The knowledge of the most prevalent HPV genotypes present in a region with a high incidence of cervical cancer is relevant in order to design an effective HPV nationwide vaccination program. In this way, our research may be a contribution for a better knowledge of the problem in an area without many data on HPV infection.

We may conclude that in Northeastern Argentina, an important number of high-risk HPV genotypes are circulating, and the results of this study show that besides HPV-16 and HPV-18, other types like HPV-58 are also frequent, as well as infections produced by more than one genotype. Attention should be focussed on these circumstances since they could be of local epidemiological significance.

RESUMEN

Genotipos de virus papiloma humano en mujeres con alteraciones citológicas cervicales de un área con alta incidencia de cáncer cervical

La relación entre la infección por los virus papiloma humanos (HPVs) de alto riesgo y el cáncer de cuello de útero ha sido bien demostrada. En el Nordeste de Argentina se observa una alta incidencia de esta patología y en consecuencia se estima una alta prevalencia de infección por HPV. A fin de identificar los genotipos de HPV presentes en el área, asociados a casos de lesiones malignas y premalignas de cuello de útero, se estudiaron 53 muestras ecto-endo cervicales de mujeres con alteraciones citohistológicas residentes permanentes de las ciudades de Resistencia y Corrientes. De las 53 pacientes estudiadas, 34 resultaron positivas para HPV (64.2%), correspondiendo la mayor frecuencia a HPV-16 (32.3%), seguido por HPV-58 (14.7%), HPV-6, -18 y –45 (5.9%), -33, -52, -53, -54, -56, -66, -MM4 y –LVX100 (2.9%). Además, se encontraron 5 casos de infecciones mixtas causadas por mas de un genotipo, lo que resulta de importancia ya que representan el 14.7% del total de los casos positivos. Los resultados demuestran que, además de HPV-16 y –18 que son los genotipos de alto riesgo de mayor prevalencia a nivel mundial, otros como el HPV-45 y –58 y los casos de infecciones múltiples son frecuentes en mujeres del nordeste argentino, lo que podría constituir un rasgo epidemiológico de importancia regional y ser de utilidad en el futuro en los programas de vacunación.

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