Full Length Research Paper

Integration event frequency of high-risk human papilloma virus in Mexican women and its association with some risk factors

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Abstract

Persistent infection with the high-risk human papilloma virus (HR-HPV) has been considered as one necessary factor in the development of cervical uterine cancer (CUC). One of the processes that seem to be more involved in the origin of malign cells is the event of the virus integration into the host genome. Consequently, the objectives of this cross-sectional correlational study were: (1) to find out the HR-HPV integration frequency in 216 Mexican women with normal cytology; and (2) to correlate having the oncogenic HR-HPV with six factors (ingestion of hormonal contraceptives, number of pregnancies, age, alcohol use, smoking and number of sexual partners). For this study, 216 samples were analyzed that proved positive to the β-globin gene via endpoint PCR. Three determinations were carried out: PCR-L1 to determine the presence or absence of HPV; PCR-Nested to confirm the HPV positivity and PCR-LCR to determine the HR-HPV. The two physical virus forms were classified afterwards (integrated and/or episomal). According to molecular analysis, the distribution of results obtained was: 29.63% (64/216) of cases amplified for region L1, and 34.37% (22/64) were confirmed with PCR-Nested. Total analysis of samples produced 3.24% (7/216) of oncogenic-positive HR-HPV cases. On revision of the physical condition of the virus in the HR-HPV cases, 0.93% appeared in the integrated form and 2.31% in the episomal form in the host DNA of the 216 women studied. In conclusion, according to the two objectives, we found that the HR-HPV integration frequency in 216 Mexican women with normal cytology was 0.93% and that the number of sexual partners was the only factor that was associated with women with oncogenic HR-HPV.

Keywords: HPV, Cervical cancer, Virus integration, Intraepithelial lesion.

INTRODUCTION

Cervical-uterine (CUC) cancer is the second most common cancer of all types affecting women. During 2002, it is estimated that 493,000 new cervical cancer cases were diagnosed and 274,000 deaths reported in the world (Jemal et al., 2011). It is calculated that about 1.4 million women live with invasive CUC (Baldwin, 2003). In Latin America and the Caribbean, 30,500 women die annually because of CUC, with the majority of women falling in the 35 to 54 years age group (Sherris et al., 2000).

During the 1990s, a number of epidemiological studies were carried out supported by molecular studies that highlighted the causal role of some HPV serotypes in the development of CUC and its precursor lesions (Bosch et al., 2003). All CUC cases contain DNA of high-risk (HR) HPV of some type (Bosch et al., 2002). As a result, in 1995 the International Agency of Research on Cancer

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(IARC) classified the HPV 16 and 18 genotypes as human carcinogens *(IARC, 1995).*

It is thought that the process of integration of the HPV genome into the host cell genome is the key event in cancer progression resulting from overexpression of the E6 and E7 oncoproteins due to the loss of E2, the protein that regulates their expression (Jayshree et al., 2009). There are recent reports of the variation of episomal and integrated forms depending on the type of dysplasia present in cervical samples from a series of cases diagnosed with various degrees of dysplasia (Cortez-López et al., 2012).

Currently, the viral integration process is considered as an important genetic alteration that characterizes malign dysplasia, with potential applications as a progression marker of precursor lesions and diagnostics tool.

As a result of the above, the objectives of this cross-sectional correlational study were: (1) to find out the HR-HPV integration frequency in 216 Mexican women with normal cytology; and (2) to correlate the incidence of oncogenic HR-HPV with six factors (ingestion of hormonal contraceptives, number of pregnancies, age, alcohol use, smoking and number of sexual partners).

MATERIAL AND METHODS

We obtained 216 samples of cervical DNA from women residing in the town of Torreon, Coahuila, Mexico, and registered the women with the human papilloma virus program (PLMCVPH: *Programa Lagunero de Mujeres contra el Virus del Papiloma Humano)*. All women were interviewed and a survey was conducted to assess their risk profile to the acquisition of HPV infection.

Collection of cervical samples

Samples were taken using three sterile swabs: the first swab was introduced in the endocervical channel, the second reached the endo and ectocervix transition zone and the third reached the ectocervix toward the bottom of the uterine cervix sac and turning the swab 360 degrees. Swabs were placed in a 10-ml tube with PBS for homogenization later, all under sterile conditions, and were taken to the Molecular Immunobiology laboratory for processing.

DNA Extraction

DNA was obtained using the salting-out technique (Sheehan et al, 1988).The DNA quantity and quality was determined afterward by spectrophotometry at 260 nm to create standardized aliquots.

HR-HPV detection by PCR: beta-globin

A segment of 250 base pairs was amplified using probes GH20 (5′-GAAGAGCCAAGGACAGGTAC-3′) and PCO4 (5′-CAACTTCATCCACGTTCACC-3′) *(Saiki, 1988).*The positivity of this segment guaranteed the viability of the DNA obtained.

L1 Region consensus of the HPV genome

To detect virus presence or absence in the samples under study, a segment of 450 base pairs was amplified utilizing probes MY09 (5′-CGTCCMAARGGAWACTGATC-3′) and MY11(5′-GCMCAGGGWCATAAYAATGG-3′) (Qu, 1997)*.*

PCR-Nested

To corroborate the HPV positivity in the sample using probes GP5+ (5′-AAGGGAGTAACCGAAAACGGT-3′) and GP6+ (5′-TCATCCTCCTCCTCTGAG-3′) (de Roda Husman, 1995)*.*

Determination of the HPV oncogenic type by PCR specific to the LCR region (E6, E7, ORF)

Utilizing probes PU1M (5′-Cy5-TGTCAAAAACCGTTGTGTCC-3′) and PU2R (5′-GAGCTGTCGCTTAATTGCTC-3′) (Fujinaga et al., *1991)* that amplify a fragment of 231–268 base pairs in the LCR region.

Visualization of the physical condition of the HPV inside the host cell us in agarose gel electrophoresis (Figure 1). DNA from HR-HPV-positive samples underwent 1.5% agarose gel electrophoresis to recover DNA bands in each one for purification afterward through an affinity column. This procedure was carried out following the protocol established by the commercial Wizard ®SV Gel and PCR Clean-Up System kit (Promega, Madison, WI. 53711 USA). DNA obtained is subjected to a second PCR-LCR utilizing PU1M and PU2R primers for oncogenic HPV to verify integrated HPV cases (Cortez-Lopez et al., *2012).*

Association with risk factors

We assessed six risk factors such as ingestion of hormonal contraceptives, number of pregnancies and age, use of alcohol and tobacco, as well as sexual behavior, including age at first sexual relationship and number of sexual partners (Nazzal et al., 2006; Franco et al., 1999), with the aim to find a correlation with the presence of the HR-HPV.
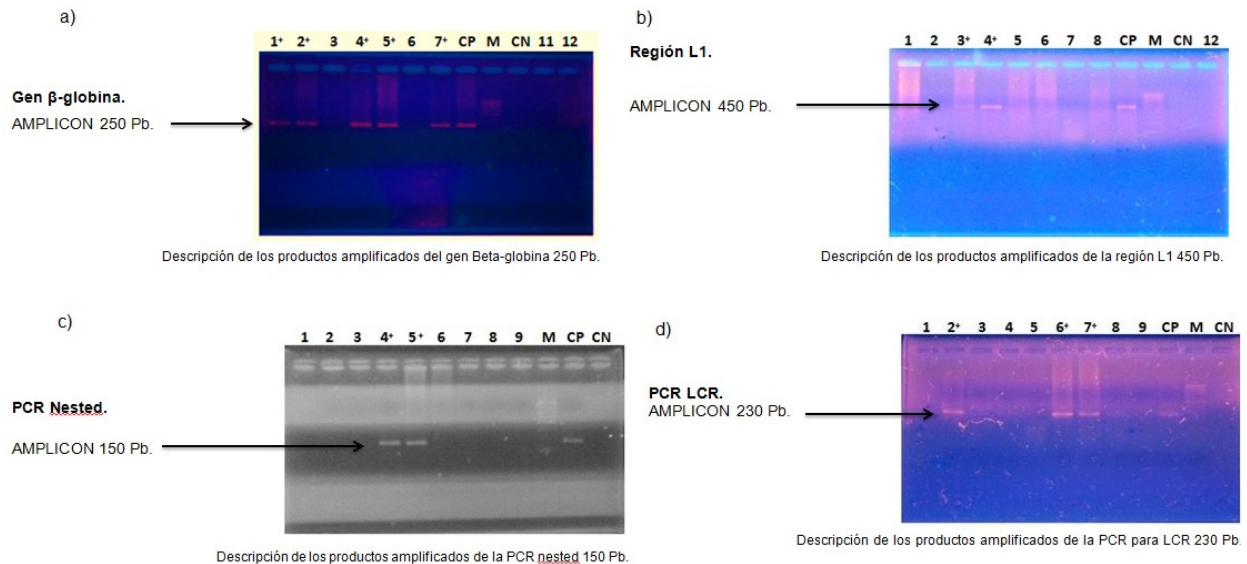
Figure 1. Molecular results from amplifications of each one of the HPV regions by PCR, in 1.5% agarose gel and dyed with Ethidium Bromide.

Table 1. Number and percentage distribution of the integrated and episomal forms in 216 women

Beta Globin	HPV			Forms	
	1	2	3	Integrated	Episomal
Positive	64 (29.63)	22 (10.19)	7 (3.24)	2 (0.93)	5 (2.31)
Negative	152 (70.37)	42 (19.44)	15 (6.94)	5 (2.31)	2(0.93)
TOTAL	216	64 (29.63)	22 (10.19)	7 (3.24)	7 (3.24)

1. HPV determination via the PCR-L1 technique
2. HPV determination via the PCR-Nested technique
3. High Risk HPV determination via the PCR-LCR technique

Statistical analysis

The information collected was analyzed with the SPSS Statistics v.20. Statistical software. Frequency analyses were carried out as well as correlation analyses of (positive and negative) PCR-LCR values with each of the six risk factors (dichotomous scale) utilizing Fisher's exact test. A p value of < 0.05 was considered statistically significant.

RESULT

Our study included 216 women that participated in the PLMCVPH. Average age of the whole group was 46 ± 8.15 and the average age of commencement of their active sexual life was 19± 4.43. Only 24.07% (52) used contraceptives. On the other hand, only 19.90% (43) had had a colposcopy, while 80.09% had never had one. With regard to tobacco consumption, 12.68% (27) said they smoked and 87.32% did not. Regarding alcohol consumption, only 21.83% (47) said they drank versus 78.17% who did not.

Results from the molecular analysis

We found a total of 216 samples that tested positive to the β-globin gene via endpoint PCR. According to molecular analysis, the distribution of results obtained was as follows: 29.63% (64/216) of cases amplified for region L1, 34.37% (22/64) of which were confirmed by PCR-Nested. The total analysis of the samples produced 3.24% (7/216) of oncogenic-positive HR-HPV cases. On revision of the physical condition of the virus, 28.57% (2/7) appeared in the integrated form and 71.42% (5/7) in the episomal form in the host DNA of the 216 women under study (Table 1).

Association with risk factors

Results obtained did not show any significant association between the presence of HR-HPV and the use of contraceptives, or with ingestion of contraceptives,

Table 2. Contingency table to correlate risks factors to positive high-risk HPV cases

RISK FACTORS		PCR-LCR		TOTAL	Probability**
		+	−		
NSP*	1	1	122	123	
	2 or more	6	87	93	0.044
OBESITY	yes	2	79	81	
	no	5	130	135	0.714
CONTRACEPTIVES	yes	2	41	43	
	no	5	168	173	0.628
COLPOSCOPY	yes	0	52	52	
	no	7	157	164	0.200
SMOKING	yes	1	30	31	
	no	6	179	185	1.000
ALCOHOL	yes	3	48	51	
	no	4	161	165	0.360
	TOTAL	7	209	216	

*NSP: Number of sexual partners
**Fisher's exact test

obesity or use of alcohol or tobacco. However, it can be inferred that the number of sexual partners is the higher inherent risk factor, as it is this factor that presents the highest trend to acquire the HPV infection (Table 2).

Of the total population of women in the study, 56.94% (123) manifested monogamous behavior and 43.06% (93) confirmed between two to six sexual partners throughout their active sexual life. Of the positive HR-HPV cases (7), 85.7% experienced polygamous behavior. Data from Fisher's exact test (Table 2) show a significant correlation (p= 0.044) between having oncogenic HR-HPV and polygamous behavior.

DISCUSSION

In Mexico, CUC represents a serious public health issue (Mohar et al., 2000).Papers published about the prevalence of the HPV in the country have reported different positivity frequencies (Hernandez-Ávila et al., 1997; Torroella-Kouri et al., 1998). Results from this study provide a vision of HPV types and frequency in a population that includes patients with a normal cytology registered in the PLMCVPH. Normal cytology participants showed 10.18% HPV infection frequency, of which 31.81% belong to the oncogenic HR-HPV group. This matches other studies (Kiviat, 1989; Lazcano-Ponce, 2001), and confirms the high frequency of asymptomatic carriers.

The integration rate in this study was 0.92% in patients with presumably normal epithelia, which suggests that the frequency of HPV forms integrated into the host cell increases as the cervix disease progresses, according to results that show much higher frequencies in high-degree intraepithelial lesions *(Peitsaro et al., 2002)*.Experimental results show that integration is common in invasive cancer cases and rarely frequent in

normal epithelia and in cervical intraepithelial neoplasia (Chen et al., 2005).

One of the main risk factors for acquiring infection is sexual behavior, the greater number of sexual partners (NPS) increased risk of acquiring the infection during sexual activity (Castellsague, 2008).

CONCLUSIONS

Cancer is a complex disease that appears as a result of genetic alterations that interfere with cell functions responsible for regulation of cell proliferation, apoptosis and ageing. The validation of the integration event rate as a molecular marker of progression toward CUC will contribute directly and effectively to the decision-making process regarding the management and follow-up of HPV carriers, thus reducing the risk of CUC.

A significant correlation was found in women with oncogenic HR-HPV and polygamous behavior. This highlights the importance of considering the HPV integration as a diagnostics tool by utilizing it as a predictive risk biomarker, especially in women with high-grade squamous intraepithelial lesion and low-grade squamous intraepithelial lesion, and even in those various dysplasia grades that may progress to CUC.

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