Risk of Progression of Early Cervical Lesions is Associated with Integration and Persistence of HPV-16 and Expression of E6, Ki-67, and Telomerase

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Original Article

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ABSTRACT

Background: Low-grade squamous intraepithelial lesions (LSIL) are the earliest lesions of the uterine cervix, the persistence and integration of high-risk human papillomavirus (HR-HPV) as type 16, which promotes the development of more aggressive lesions.

Aim: To select more aggressive lesions with tendency to progress to invasive cervical cancer.

Materials and Methods: A total of 75 cytological specimens in liquid base (Liqui-PREP) were analyzed: 25 specimens were with no signs of SIL (NSIL) and without HPV; 25 NSIL with HPV-16, and 25 with both LSIL and HPV-16. The expression of Ki-67, telomerase, and viral E6 was evaluated by immunocytochemistry; and the detection of viral DNA was done by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLPs) for genotyping or sequencing of HPV-16. The physical state of HPV-16 was evaluated by *in situ* hybridization with amplification with tyramide.

Results: Of the total group, 58.6% had LSIL associated with persistence and of these 59.3% was associated with integrated state of HPV as intense expression of E6, Ki-67 ($P = 0.013$, $P = 0.055$) has except for the expression of telomerase present a non-significant association ($P<0.341$).

Conclusions: Overexpression of E6 and Ki-67 is associated with the integration of HPV-16, favoring viral persistence, and increasing the risk of progression in women with NSIL and LSIL.

Key words: Cervical lesions; E6; HPV-16; integration; Ki-67; telomerase

Introduction

Invasive cervical carcinoma (ICC) is the second most common cause of death among Mexican women.[1] Mexico's Health Ministry reported a death rate (9.1 per 100,000 women). High mortality rates due to this cancer were reported for Guerrero State, at a rate of 12.5 deaths per 100,000 women.[2] The major cause of cervical intraepithelial neoplasia (CIN) is chronic infection of the cervix with the sexually transmitted human papillomavirus (HPV), especially the high-risk HPV types 16 or 18.[3] In women from Guerrero State, HPV-16 is detected in 68.1% of ICC samples; 27.4% of high-risk squamous intraepithelial lesion (HSIL) samples; 10.6% of low-risk squamous intraepithelial lesion (LSIL) samples; and 2.3% of samples with no signs of squamous intraepithelial lesions (NSIL).[4] Fernández *et al*.[5] reported virus detection in 70.6% of LSIL cases, 54.8% of HSIL cases, and in 70.8% of cervical squamous cell carcinoma cases.

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Arianna Vega-Peña, Berenice Illades-Aguiar[1], Eugenia Flores-Alfaro[2], Esther López-Bayghen[3], Marco Antonio Leyva-Vázquez[1], Eduardo Castañeda-Saucedo[4], Luz Del Carmen Alarcón-Romero

Laboratory for Research in Cytopathology and Histoquemical, [1]Laboratory for Molecular Biomedicine, [2]Laboratory for Chronic-Degenerative Diseases, [4]Laboratory for Cancer Cellular Biology, Academic Unit for Chemical Biological Sciences, Autonomous University of Guerrero, Chilpancingo, Guerrero, [3]Department of Genetics and Molecular Biology, CINVESTAV (Center for Research and Advanced Studies of the National Polytechnic Institute), DF, México

Address for correspondence: Dr. Luz del Carmen Alarcón-Romero, Laboratory for Research in Cytopathology and Histoquemical, Academic Unit for Chemical Biological Sciences, Autonomous University of Guerrero, Avenida Lázaro Cárdenas, Ciudad Universitaria, Chilpancingo, Guerrero, C.P. 39089, México. E-mail: luzcarmen14@gmail.com

Integration of the viral genome into the host DNA is related to lesion persistence and progression and is considered a late event in cervical carcinogenesis.[6,7] Integration usually occurs within fragile areas of the viral E1 and E2 ORF, with the latter being the most frequent. Elimination of E2 expression results in transcriptional over-regulation of the viral oncogenes E6 and E7, leading to increased expression of both viral oncoproteins that target tumor suppressor proteins, p53 and pRb respectively, among others, resulting in loss of cell cycle control.[8,9] Integration can be evaluated by *in situ* hybridization (ISH) as did Evans *et al*.[10] detected integrated viral DNA in 13.6% cases of cervical intraepithelial neoplasia grade I (CIN I) by ISH amplified with tyramide and using probes for 13 HR-HPV; a similar study also reported that 75% of CIN I cases and 50% of samples without cervical lesions presented with integrated HR-HPV.[11] Zubillaga-Guerrero *et al*.[12] recently reported the HPV-integrated state individually (10%) and in the mixed form (90%) in LSIL using the same methodology.

E6 prevents telomeric shortening by increasing expression of the human telomerase reverse transcriptase catalytic subunit (hTERT), forming a complex with E6AP and binding directly to the hTERT promoter, resulting in transcriptional activation followed by telomerase activation.[13,14] Telomerase has become important because the activity of this enzyme has been found in >90% of different tumors.[15] Among 154 cytological samples, a positive association between telomerase expression and infection with HPV type 16/18 was found (OR 141.1, $P<0.001$), suggesting that telomerase expression detection may serve as an efficient tool for diagnosis and prognosis of cervical lesions, along with cytology and HPV tests.[16]

HPV-16 oncoprotein E7 associates with pRb and promotes the release of the E2F transcription factor, which transactivates cell proteins needed for progression to the S-phase of the cell cycle. Overexpression of $p16^{INK4a}$, MCM, PCNA, Ki-67, p14ARF, and cyclin E, among others, occurs during aberrant proliferation.[17] Yu *et al*.[18] evaluated the diagnostic value of Ki-67, $p16^{INK4a}$, and L1-HPV in cytological samples. They found the expression of Ki-67 in 40% of LSIL, 87% of HSIL, and 100% of ICC cases ($P<0.001$), concluding that Ki-67 evaluation improves detection of premalignant lesions, and that combination with $p16^{INK4a}$ and L1-HPV increases the diagnosis efficacy. Using ISH on CIN2/3 samples has been reported a strong association between intense expression of Ki-67 telomerase and the integrated state of HR-HPV (OR 64.1, $P<0.001$ and OR 50.1, $P = 0.001$, respectively), demonstrating that more aggressive lesions potentially progress to ICC.[11] Follow-up using simultaneous cytological and molecular methods suggests that errors, invasive procedures, and unnecessary expenses could be avoided in diagnosis and prognosis of LSIL. However, this type of testing is not a common practice. The aim of this work was to evaluate whether expression of Ki-67, telomerase, and E6 in LSIL in the presence of integrated HPV-16 during a one-year follow-up that could serve as an early monitoring tool to select women with more aggressive lesions that have a tendency to progression. This would represent an important contribution to understanding cervical cancer pathogenesis and improve diagnosis opportunities among low income Mexican women.

Materials and Methods

Patients and sampling: Cytological specimens were collected from 75 women followed up for one year, who attended the integral diagnostic service for early detection of cervical-uterine cancer and HPV of the Autonomous University of Guerrero, from July 2010 to April 2012. Each participant gave informed consent approved by the Bioethics Committee of the University. Two cytological specimens were collected from each patient to assess the squamous-columnar transformation zone; the first specimen, at the beginning of the study and the second, one year later. Each sample was subjected to Papanicolaou cytology and molecular HPV typing. Each patient completed a questionnaire to gather gynecological data.

Cytological specimens were collected in Liqui-Prep preservation liquid for cytological diagnosis, the detection of viral DNA was done by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLPs) for genotyping or sequencing of HPV-16, and for HR-HPV DNA physical state determination by *in situ* hybridization with amplification with tyramide (ISH). Protein expression was performed using immunocytochemistry. Based on the Bethesda system, they were diagnosed: 25 specimens were with NSIL and without HPV; 25 NSIL with HPV-16 and 25 with both LSIL and HPV-16. Progression risk was assessed only on women with persistent HPV-16 infection after one year of follow up, by evaluating the physical state of viral DNA and protein expression.

Molecular detection and HPV typing: DNA extraction was performed using the SDS-K proteinase-phenol-chloroform standard method. PCR was carried out with 1.5 μg total DNA with degenerate oligonucleotides MY09 and MY11.[19] PCR products were analyzed by electrophoresis in 1.5% agarose gels and visualized with ethidium bromide. As positive controls, 1 pg and 1 ng of recombinant pHPV16 plasmid DNA was used. As negative control, a reaction with sterile water instead of DNA was used. PCR products of HPV-positive samples were digested

with restriction enzymes *BamHI, DdeI, HaeIII, HinfI, PstI, RsaI*, and *Sau3AI* (Invitrogen, Carlsbad, CA). Viral type was determined by restriction fragment length polymorphism (RFLPs)[20] When samples were negative or the HPV type could not be identified by RFPLs, the PCR system GP5+/6+ was used,[21] and the products were sequenced. PCR products were purified using 75% isopropanol Centri-Sep Spin Columns (Applied Biosystems, CA), and sequenced in an automatic sequencer (310 ABI PRISM Genetic Analyzer, Applied Biosystems, CA). All sequences were compared to known sequences for HPV types, available at the NCBI site (http://www.ncbi.nlm.nih.gov).

In situ hybridization: Viral genome detection by ISH was performed using the signal amplification system with tyramide (Dako, Carpintería CA, USA) adjusted to the indicated times. Cytology specimens were permeabilized and enzyme-digested. A drop of biotinylated viral DNA probe (Dako) suited for detecting 13 genotypes of HR-HPV (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) was added. SiHa and CaSki cell cultures were used as positive controls and samples with no probe were used as negative controls. A positive signal pattern was visualized as a brown deposit completely covering the nucleus (diffuse form) that indicates the episomal viral DNA state or a dotted signal corresponding to the integrated viral DNA state or mixed (diffuse and punctate).[10]

Immunocytochemistry for Ki-67, telomerase, and viral E6: Ki-67, telomerase, and viral E6 expression was determined using a streptavidin-biotin peroxidase technique (Cell Marque Corporation, Hot Springs, AR). The following monoclonal antibodies were used: MIB1 for Ki-67 (Dako, CA, USA), 2C4 for telomerase (Novus Bio, Littleton, CO), and C1P5 for E6-HPV-16 (Santa Cruz, USA). Antigen recovery was performed, and the primary antibody was added at a 1:50 dilution for Ki-67 and viral E6, and at 1:200 for telomerase. An anti-antibody coupled to biotin was added, and then streptavidin peroxidase followed by diaminobenzidine. CaSki cells were used as positive control in each run and the same type of cells without primary antibody were used as negative controls. Evaluation of Ki-67, telomerase, and viral E6 expression was based on the visualization of cells acquiring brown nuclear staining based on Cheung's criteria: 0-10% cells with basal staining was considered negative, 11-50% positive cells was considered moderate immunostaining, and >50% positive cells was considered strong immunostaining.[22]

Statistical analysis

Correlation of cytological diagnosis, HPV typing, and physical state of viral DNA during one year of follow-up was evaluated. Relative frequency for the different variables was determined and Fisher's exact test was used to evaluate correlation. A logistic regression model for repeated measures adjusted for age was used to evaluate the association between cytological diagnosis and physical state of HPV-16 and between the expression of E6, Ki-67, and telomerase, obtaining OR with confidence intervals at 95%. A $P \leq 0.05$ was considered significant. SPSS v13.0 and Stata v11.0 software were used for the statistical analysis.

Results

Cytological and molecular findings during follow-up

Cytological and molecular results of the one-year follow-up are shown in Table 1. Cytological diagnosis of LSIL was based on visualizing cervical cells with the following HPV infection characteristics: karyomegaly, double nuclei, hyperchromicity, and koilocytosis, whereas samples of NSIL had inflammatory changes [Figure 1]. After one year of follow-up, results show that 28% (7) women NSIL and without HPV-developed LSIL, and four of them were found to have HPV-16. 52% (13) women initially diagnosed with NSIL and with HPV-16 after one year were diagnosed with LSIL, and only 7 showed persistent infection ($P < 0.001$). For women initially diagnosed with LSIL, 96% had no changes during the follow-up. Only one woman with LSIL returned to normal as assessed by cytology after one year.

Of the 50 women initially diagnosed with HPV-16, 46% showed persistent infection (21 with LSIL and 6 with NSIL) and the remaining 54% acquired new infections with HR-HPV types such as 18, 31, 45, and 55; with LR-HPV types 6, 11, 72, and 81, or multiple infections. Four cases (16%), initially NSIL and without HPV, acquired HPV-16 after a year of follow-up

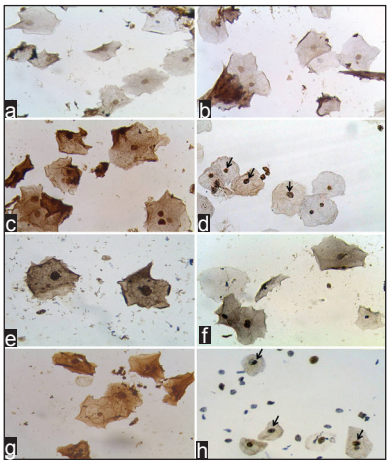

Figure 1: Expression of E6, Ki-67, and telomerase in cell from NSIL and LSIL with the HPV-16 integrated state Immunostaining for: A: viral E6, B: Ki-67 both in the nucleus of cervical cells, C: hTERT in nucleus/cytoplasm of the cells, D: integrated viral DNA (fine dots, black arrows) in cell from NSIL. E: E6 in cells binucleated, F: Ki-67 in binucleated cells, G: telomerase in cells with binucleation and karyomegaly, H: cells with integrated HPV-16 (punctate signal, black arrows), (Technique: *In situ* hybridization with tyramide amplification and Streptavidin biotin peroxidase, ×400).

($P < 0.001$). 27 persistent samples were used to evaluate the progression risk associated with HPV-16.

Physical state of HPV-16 DNA

Viral integration was found in 83% of women with NSIL and HPV-16 and 52% of women with LSIL. The integrated state was most frequent in the 27 samples evaluated by ISH (59.3%), compared to 40.7% of samples showing the episomal state ($P < 0.001$, Table 1).

Association of E6, Ki-67, and telomerase expression with early lesions

Table 2 summarizes the expression of cell proteins associated with early lesions. Intense expression was considered when >50% of the cells present showed staining for E6, Ki-67, or telomerase. Intense expression was found in 67% of samples with NSIL and HPV-16, whereas telomerase expression was found in 86% cases, suggesting a strong association (OR = 4.6; IC: 95%: 2.1 to 9.8) with progression to LSIL in comparison with women who persisted with NSIL, $P < 0.001$. Intense expression of E6 and Ki-67 was found in 63% LSIL cases. Expression levels of both proteins are associated with the progression to LSIL (E6: OR = 2.6; IC 95%: 1.4 to 4.9, $P = 0.003$; Ki-67: OR = 2.2; IC 95%: 1.2 to 3.8, $P = 0.008$). Positive staining for E6, Ki-67, and telomerase was related to cytological alterations caused by HPV-16 infection in samples with LSIL [Figures 1a-c and e-g].

Association of expression of E6, Ki-67, and telomerase with the physical state of HPV-16

Increased expression of E6 and Ki-67 was associated with the integrated state of HPV-16, especially in women with LSIL (81%) for both proteins, E6 (OR = 3.3; IC 95%: 1.2 to 8.5, $P = 0.013$) and Ki-67 (OR = 2.3; IC 95%: 0.9 to 5.8, $P = 0.055$). Intense expression of telomerase was found 1.7 times more frequently with the integrated state, but this association was not significant ($P = 0.341$, Table 3) [Figures 1 d and h].

Discussion

As a screening method, uterine cervix exfoliative cytology has effectively reduced the incidence of ICC in different countries.[23] However, this technique has a low sensitivity

Table 1: Cytological and molecular results during follow up

	Final evaluation			
Initial cytological diagnosis	**NSIL without HPV**	**NSIL with HPV**	**LSIL with HPV**	**Total (%)**
NSIL without HPV	17 (22.7)	14 (18.7)	44 (58.6)	75
NSIL with HPV	17 (68)	1 (4)	7 (28)	25 (100)
LSIL with HPV	0	12 (48)	13 (52)	25 (100)
	0	1 (4)	24 (96)	25 (100)
Type of initial HPV	Negative	HPV-16*	Other HPV†	Total
Negative	17 (22.7)	27 (36)	31 (41.3)	75
HPV-16	17 (68)	4 (16)	4 (16)	25 (100)
	0	23 (46)	27 (54)	50 (100)
Physical state of HPV-16*		NSIL with HPV	LSIL with HPV	Total
Episomal		6 (22.2)	21 (77.8)	27 (100)
Integrated		1 (17)	10 (48)	11 (40.7)
		5 (83)	11 (52)	16 (59.3)

NSIL: No sign of intraepithelial lesion, LSIL: Low-risk squamous intraepithelial lesion, HPV: Human papilloma virus. $P<0.001$, Fisher's exact test performed to compare the initial and final diagnosis. *physical state evaluated only for women positive to HPV-16 or persistent. †includes high-risk HPV (18, 31, 45, and 55), low-risk HPV (6, 11, 72, and 81), and multiple infections excluded for later evaluation

Table 2: Association between E6, Ki-67, and telomerase expression and cytological diagnosis

	Cytological Diagnosis				
E6, Ki-67 and telomerase expression	**NSIL without HPV**	**NSIL with HPV**	**LSIL with HPV**	**OR (IC 95%)†**	***P* value**
$n = 44$‡	17 (38.6)	6 (13.6)	21 (47.8)		
0-10% positive cells	17 (100)	0	0	1*	
>50% cells E6+	0	4 (67)	13 (62)	2.6 (1.4-4.9)	0.003
>50% cells Ki-67+	0	4 (67)	13 (62)	2.2 (1.2-3.8)	0.008
>50% cells telomerase+	0	4 (67)	18 (86)	4.6 (2.1-9.8)	<0.001

NSIL: No sign of intraepithelial lesion, LSIL: low-risk squamous intraepithelial lesion, >50% cells: refers to an intense expression of the proteins evaluated. †P value determined with a logistic regression model for repeated measures adjusted for age. *Reference category. ‡Frequencies are given row.

Table 3: Association between E6, Ki-67, and telomerase expression and HPV-16 physical state

	HPV-16 physical state				
E6, Ki-67 and telomerase expression	**Negative**	**Episomal**	**Integrated**	**OR (IC 95%)†**	***P* value**
$n=44$‡	17 (38.6)	11 (25)	16 (36.4)		
0-10% positive cells	17 (100)	0	0	1*	
>50% cells E6+	0	4 (36)	13 (81)	3.3 (1.2-8.5)	0.013
>50% cells Ki-67+	0	4 (36)	13 (81)	2.3 (0.9-5.8)	0.055
>50% cells telomerase+	0	7 (64)	15 (94)	1.7 (0.5-4.7)	0.341

>50% cells: refers to an intense expression of the proteins evaluated. †P value determined with a logistic regression model for repeated measures adjusted for age. *Reference category. ‡Frequencies are given row.

(50 to 75%) to detect high-risk cervical lesions, has ample variability among observers, and produces a high percentage of false-negative results.[24,25] Histopathological studies have also shown variability among observers.[26] It is not only desirable to improve opportune diagnosis but also to provide control and follow-up of cases before instituting possible unnecessary patient treatments. The use of biomarkers in cytological and histological samples has improved the results of cervical screening.[27]

Here, we show that 28% of women without cytological or molecular evidence of HPV developed LSIL related mainly to HPV-16 after a year of follow-up. 52% of cases with NSIL but with molecular diagnosis of HPV-16 progressed to LSIL, but 96% of women with LSIL showed no further cytological changes during the follow-up year. Several authors report that after two years, 90% of premalignant lesions reappear.[27,28] In the present study of women with LSIL, only a single case showed regression (4%). In the end, prevalence of LSIL was 58.7%, of which 47.7% presented with HPV-16 ($P < 0.001$). Of the cases initially diagnosed with HPV-16, 46% persisted during the one-year follow-up. Schmeink *et al*.[29] in 2011 found that only 11% of LSIL/CIN1 cases persisted with HR-HPV, among which HPV-16 had a prevalence of 30.2% in a follow-up period of 12 months, and 54.4 % persisted after two years.

Castle *et al*.[30] (2005) reported that 21.1% of women with LSIL have infection with HPV-16 (6). In 2008, Rodríguez *et al*.[31] reported the persistence of HPV-16 in 26.3% of 209 women, and 12% of these had multiple HR-HPV infections that persisted more than a year. Similar results were found in the present study, with multiple infections present in 18.7% of cases, with high-risk viral types (14.7%) and low-risk HPVs (8%) ($P < 0.001$). These results differ from those reported by Fernández-Tilapa *et al*.[5] in 2007 with a prevalence of 0.98% for low-risk HPV and multiple infections in LSIL cases.

In the present study, the integrated state of HPV-16 viral DNA was found most frequently (59.3%) in cytological samples evaluated by ISH. It was also detected in 83% of NSIL samples, as well as in 52% of LSIL cases. These results are consistent with those reported in other studies.[11] Similar to that reported by Kulmala *et al*.,[32] this supports the hypothesis that integration is an early event in cervical carcinogenesis. However, the integrated state of HPV-16 in early lesions has been associated with progression to HSIL and ICC.[33,34]

ISH technique amplified with tyramide has high sensitivity, capable of detecting as few as two copies of integrated HPV-16 DNA in SiHa cells,[35] which makes it useful for cytological samples in liquid base, conserving cell morphology. Besides, HPV physical state diagnosis (differentiating between the episomal and integrated states) is a useful technique.[36-38]

In this study, we found that in the presence of the integrated state, there is a strong association between the expression of E6 (OR 3.3; IC 95%: 1.2 to 8.5, $P = 0.013$) and Ki-67 (OR 2.3; IC 95%: 0.9 to 5.8, $P = 0.055$). This demonstrates the importance of integration in the cell proliferation process mediated by E6, resulting in an increase of Ki-67 expression in cervical cells infected by HPV-16. Nam *et al*.[39] (2008) and Yu *et al*.[18] (2010) proved that Ki-67 is the most important indicator of cell proliferation in cervical lesions, and may be used as an additional marker of cytological diagnosis. However, it was found that association between telomerase expression and the integrated state was not significant (OR 1.7; IC 95%: 0.5 to 4.7, $P = 0.341$), suggesting that telomerase expression is independent of viral genome integration but not from the HPV-16 infection and progression to LSIL. In 2006, Kailash *et al*.[16] reported telomerase activity in samples with slight dysplasia (63.6%) and in 100% of moderate and severe dysplasia and ICC ($P < 0.001$) associated with HPV-16/18, suggesting that telomerase activity starts in the early stages of HPV-16/18 infection and progresses with the lesion. It may also serve as a diagnostic and prognostic tool for cervical lesions. As for the association of the expression of E6, Ki-67, and telomerase with the persistence and progression of LSIL, we found that expression of these proteins is related to cytological alterations and the presence of integrated HPV-16 results in a longer persistence of LSIL, and increases the risk of progression to more aggressive lesions.

In summary, we find that telomerase overexpression is associated with development of LSIL. Infection with HPV-16 and overexpression of the viral products E6 and Ki-67 is significantly related with the integration of HPV-16, which could promote viral persistence and increase the risk of progression over the following year in women with NSIL and LSIL. This study is based on data from 75 patients, but it suggests the need to confirm these relationships in a larger population with a longer follow-up period to identify early uterine cervical lesions with a high risk of progression associated with HR-HPV, which are becoming more frequent in LSIL.

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