Arch. Biol. Sci., Belgrade, 65 (1), 81-90, 2013 DOI:10.2298/ABS1301081P

CORRELATION BETWEEN KI-67 AND TELOMERASE EXPRESSION WITH *IN SITU* HYBRIDIZATION FOR HIGH-RISK HUMAN PAPILLOMAVIRUS

ARIANNA VEGA-PEÑA[1], BERENICE ILLADES-AGUIAR[1], EUGENIA FLORES-ALFARO[1], ESTHER LÓPEZ-BAYGHEN[2], ELBA REYES-MALDONADO[3] and LUZ DEL CARMEN ALARCÓN-ROMERO[1*]

[1] *Laboratorio de Investigación en Citopatología y de Biomedicina Molecular, Unidad Académica de Ciencias Químico-Biológicas, Universidad Autónoma de Guerrero, Chilpancingo,* Guerrero, México
[2] *Laboratorio de Genética y Biología Molecular,* CINVESTAV, DF, México
[3] *Laboratorio de Citología, Departamento de Morfología, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional,* DF, México

Abstract - The objective of this research was to evaluate the relationship of Ki-67 and telomerase expression with the progression of cervical intraepithelial neoplasia (CIN) and the physical state of the DNA of high-risk human papillomavirus (HR-HPV) types. A comparative study was done on 80 biopsies of human (female) cervical tissue, distributed in the following manner: 20 CIN-negative biopsies and 60 CIN-positive biopsies of varying grades. The detection of the proteins Ki-67 and telomerase was performed through immunohistochemistry; the detection of HR-HPV, by *in situ* hybridization. The expression of Ki-67 and telomerase increased with the progression of the CIN lesion ($p < 0.001$). The HR-HPV genome was detected in 75% of the cases with CIN, as well as in 20% of the tissues without histological lesions ($p = 0.001$). A significant association was found between the increase in telomerase and Ki-67 expression and the integration of the DNA of HR-HPV. The overexpression of Ki-67, telomerase and the presence the integration of the DNA of HR-HPV are evidenced by more aggressive lesions that may progress to invasive carcinoma.

Key words: Ki-67, telomerase, cervical intraepithelial neoplasia, human papillomavirus, *in situ* hybridization

INTRODUCTION

Invasive cervical carcinoma is preceded by premalignant lesions (cervical intraepithelial neoplasia or CIN), which originate in the squamocolumnar transformation zone. These lesions may progress to cancer when this area presents a high-risk HPV (HR-HPV) viral genome, the most frequent types of which are 16, 18, 31, 33, and 45, among others. At present it is thought that the integration of HR-HPV DNA with the genome of the infected cell promotes the progression of CIN-1 lesions to CIN-2, CIN-3 and/or invasive carcinoma. *In situ* hybridization is a method that allows for the determination of the presence of HR-HPV, as well as for the integration of the DNA of these viruses into the cellular genome, and its association with the progression of cervical lesions, thus supporting clinical diagnosis (Kalof and Cooper, 2006; Evans and Cooper, 2004).

On the other hand, despite histopathological examination being the traditional method utilized to confirm the presence of a cervical lesion, false negative results for CIN have increased due to diagnostic variation among observers (Heatley, 2002), as a result of which the utility of different biomarkers to assist

in the diagnosis of early lesions is being evaluated. The nuclear antigen Ki-67 (MIB-1 being its commonly used monoclonal antibody) is evidenced in all active phases of the cellular cycle, except in the G_0 phase, and is utilized as a good indicator of cellular proliferation (Scholzen and Gerdes, 2000; Cheung et al., 2004; Yang et al., 2006).

The oncoprotein E7 of HR-HPV promotes cellular proliferation upon joining in action with the protein Rb, increasing the kinetics of the cellular cycle and the overexpression of Ki-67 (Doorbar, 2006).

Telomerase is an enzyme that has as a function the replacement of nucleotide sequences that are removed from the telomeric ends of the chromosomes in each replication. Telomerase consists of two subunits: human telomerase RNA (hTR) and telomerase reverse transcriptase (hTERT). hTERT has a catalytic function in the replication of the ends of lineal DNA. In cancer or cellular lines of cancer, an increase in the activity of telomerase in comparison with premalignant lesions and lesionless cells has been found (Smith et al., 2004; Bravaccini et al., 2005).

Studies have been performed to determine the physical state of the HR-HPV DNA of HR-HPVs. In this respect, Cooper et al. (1991) demonstrated that the punctate pattern observed in the nucleus represented the integrated version of the virus in the cellular genome, while the diffuse pattern corresponds to the episomal state of the viral DNA. In cervical lesions, the relationship of the expression of the proteins Ki-67 and telomerase with the physical state of the HR-HPV DNA is still not clear. The aim of this study was to evaluate the relationship of the expression of Ki-67 and telomerase as well as the physical state of HR-HPV DNA with the progression of CIN.

MATERIALS AND METHODS

Research design and specimens

A cross-sectional comparative study was done on 80 biopsies of cervical tissue in paraffin from women who attended the dysplasia clinic of the Mexican Institute of Social Insurance's (IMSS) General Regional Hospital No 1 in Acapulco, Guerrero, Mexico. Of these samples, 15 had a histological diagnosis of CIN-1, 15 of CIN-2 and 30 of CIN-3. Included in the study as controls were 20 biopsies of cervical tissue without evidence of CIN, which were obtained from women who had undergone hysterectomies because of uterine fibroids. Only women who freely decided to participate and signed a letter of informed consent were included in the study. This project was approved by the Institutional Committee for Bioethics of the University of Guerrero.

Immunohistochemistry for Ki-67 and hTERT

From each sample, 4 to 5 slices of tissue 3 microns in thickness were obtained. To evaluate the expression of Ki-67 and hTERT, the monoclonal antibodies MIB-1 (Dako, Carpinteria, CA, USA) and 2C4 (Novus Biologicals, Littleton, CO, USA) were used respectively with the Cytoscan HRP/DAB immunohistochemical system of detection (Cell Marque Corporation, Hot Springs, AR, USA [now relocated to Rocklin, CA, USA]). The histological slices were deparaffinized and placed in a solution of immunoDNA Retriever (BioSB, Inc., Santa Barbara, CA, USA). Later, the primary antibody, previously diluted in accordance with the manufacturer's instructions, was added; the chromogen diaminobenzidine was added; and finally, the specimens were stained with Mayer's hematoxylin (Merck, USA).

For positive controls, slices of squamous cell carcinoma with overexpression of Ki-67 were utilized as well as slices of mammary carcinoma with overexpression of hTERT. These same tissues, without the addition of the primary antibody, were used as negative controls.

Evaluation of the expression of Ki-67 and hTERT

The expression in the tissues was evaluated in accordance to the distribution and localization of the positive reaction within the cell and within the depth of the epithelium. The expression of Ki-67 was considered positive when a brown ochre color was evi-

dent in the nucleus of the cells: for hTERT, when the coloration was present in the nucleus or both the nucleus and cytoplasm. In respect to the layers of the epithelium, the reaction was classified as negative when the immunostaining was located solely in the deep basal or parabasal cells of the cervix; as a slight positive when the lower third of the cervical epithelium showed coloration; as a moderate positive, when the coloration was localized in the lower two-thirds of the epithelium, and as a high positive when the coloration was located throughout the thickness of the epithelium (Syrjanen, 2005).

In situ hybridization with amplification with tyramide

The detection of the viral genome by *in situ* hybridization was accomplished with the GenPoint™ system of tyramide signal amplification (Deko, Caprinteria, CA, USA). The cervical tissue slices were placed on silanized microscope slides, deparaffinized, and subjected to enzymatic digestion with proteinase K. Later, the probe of biotinylated viral DNA was added for the detection of high-risk viral types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68. The slices were denaturalized and subjected to hybridization for 20 h (hybridizer, Dako, Carpinteria, CA, USA). Later, an astringent solution was added as well as primary streptavidin peroxidase. Biotinyl-tyramide and secondary streptavidin were added. Finally, the chromogen diaminobenzidine (DAB) was added and the samples stained with Mayer's hematoxylin (Merck, USA). The positive reaction was visualized within the nucleus with a dark brown color. The pattern of the obtained signal was classified as diffuse or punctate, as previously described by Kalof et al. (2005).

Cervical squamous cell carcinoma tissue and a culture of SiHa cells, both with HPV-16, served as positive controls.

Statistical analysis

For the comparison of the frequencies, Fisher's exact test was used. Models of multinomial logistic regression were evaluated to determine the relationship of the expression of Ki-67 and telomerase with the progression of the cervical lesions and the physical state of the DNA of the HR-HPV. The statistical analysis was performed with the software STATA version 9.2.

RESULTS

Expression of Ki-67 and hTERT

The expression of Ki-67 in the tissues without CIN was localized solely in some parabasal cells (Fig. 1A) while in the tissues with lesions; this expression was visualized solely in the cells histologically altered by the lesion. In addition, the expression of Ki-67 increased with the progression of the CIN, spreading from a localization in the lower third in CIN-1 to the entire thickness of the epithelium in CIN-3 (Fig. 1A-B) ($p < 0.001$). The expression of hTERT, similar to that of Ki-67, increased with the progression of CIN (Table 1). The highest expression of Ki-67 (56.7%) and hTERT (63.3%) was encountered ($p<0.001$) in the group of women who exhibited CIN-3 lesions. In these cases, the reaction was located throughout the depth of the epithelium and restricted to the histologically altered area of the tissue (Fig. 2A-B). On the other hand, the expression of hTERT was found in the lower third of the epithelium in 20% of the women without lesions (Table 1).

HR-HPV and the physical state of the virus

Of the women analyzed, 61.2% were positive for HR-HPV, with CIN-2 (80%) and CIN-3 (83.3%) cases showing greater frequencies. The punctate pattern of HR-HPV was seen in all CIN groups, being more frequent in CIN-3 (88%) (Fig. 3B). In 20% (4 cases) of the tissues without histological lesions, the presence of HR-HPV was found, 2 with the punctate signal pattern and 2 with a diffuse signal (Fig. 3A, Table 2).

The association of the expression of Ki-67 and hTERT with the physical state of HR-HPV

The expression and localization of Ki-67 was significantly associated with the presence of HR-HPV when

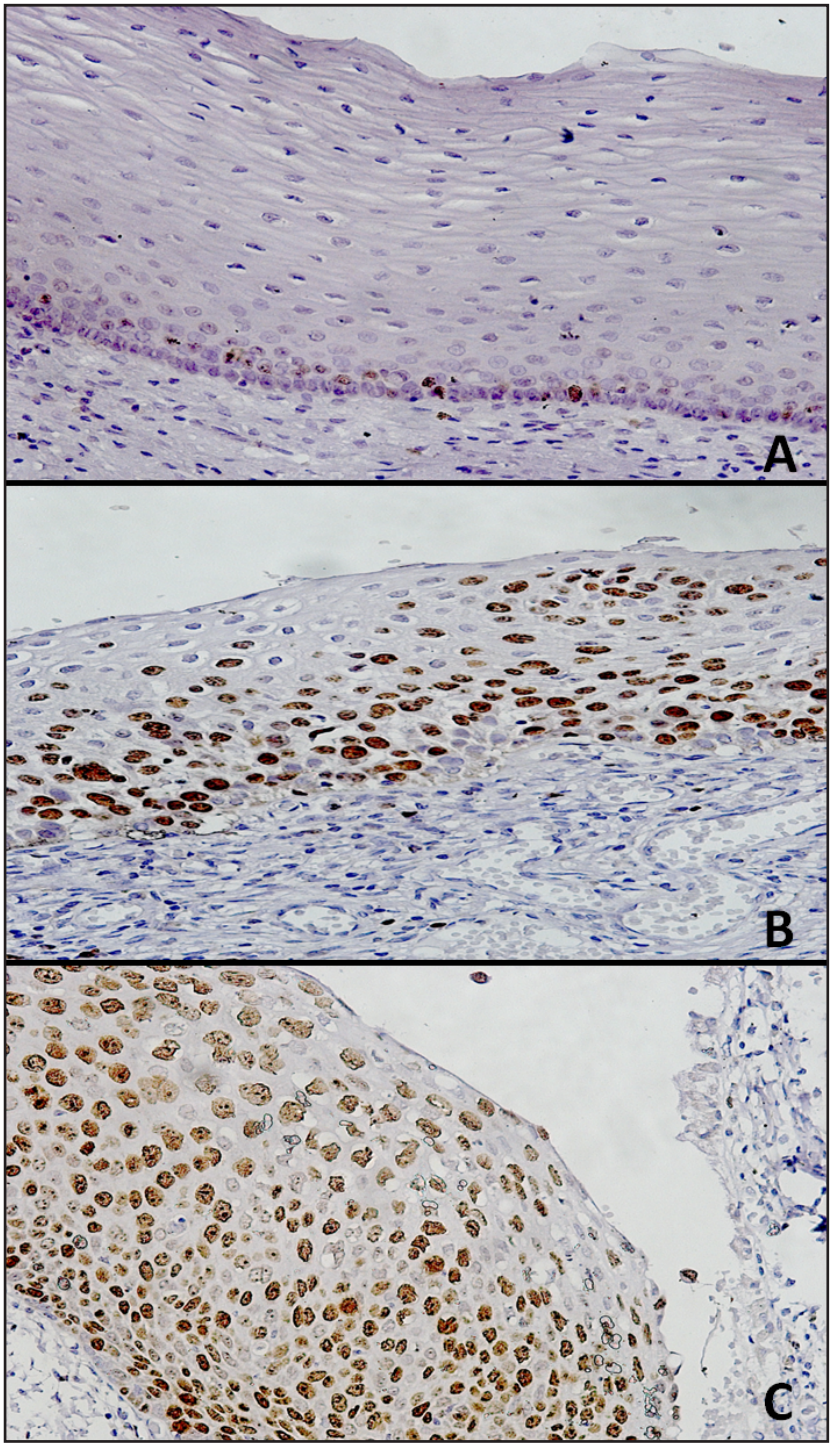

Fig. 1. Expression of Ki-67 (20X). A) Normal cervical tissue showing nuclear immunostaining in some cells, basal and parabasal; B) CIN 1 showing nuclear immunoreactivity in 1/3 lower of epithelium; C) CIN 3 showing strong immunostaining of Ki-67 in the entire epithelial thickness.

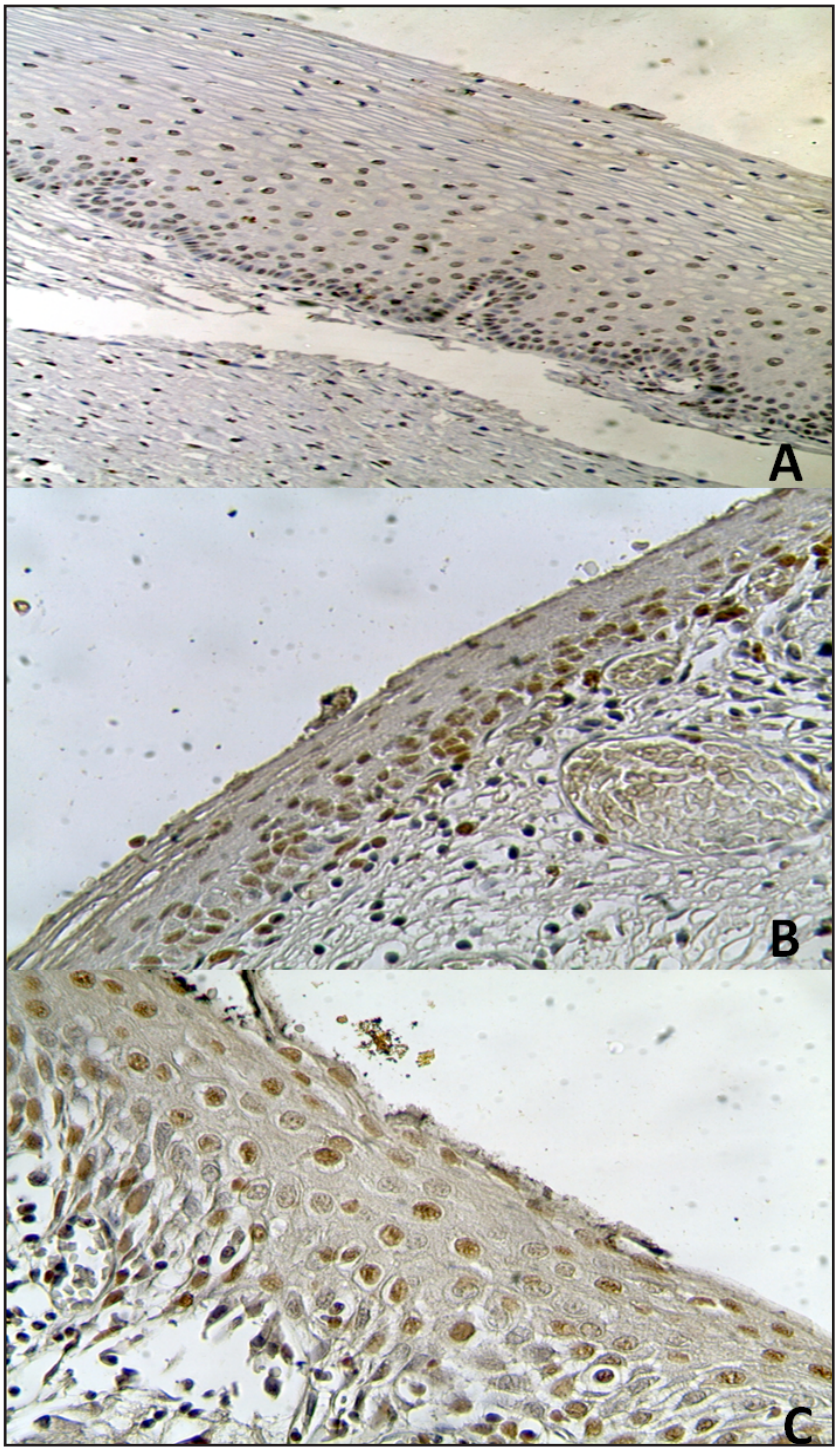

Fig. 2. Expression of telomerase (20X). A) Immunostaining in some cells basal and parabasal of normal cervical tissue; B) CIN 1 showing nuclear immunostaining for telomerase in the 1/3 lower of the epithelium; C) CIN III showing strong nuclear immunostaining of telomerase.

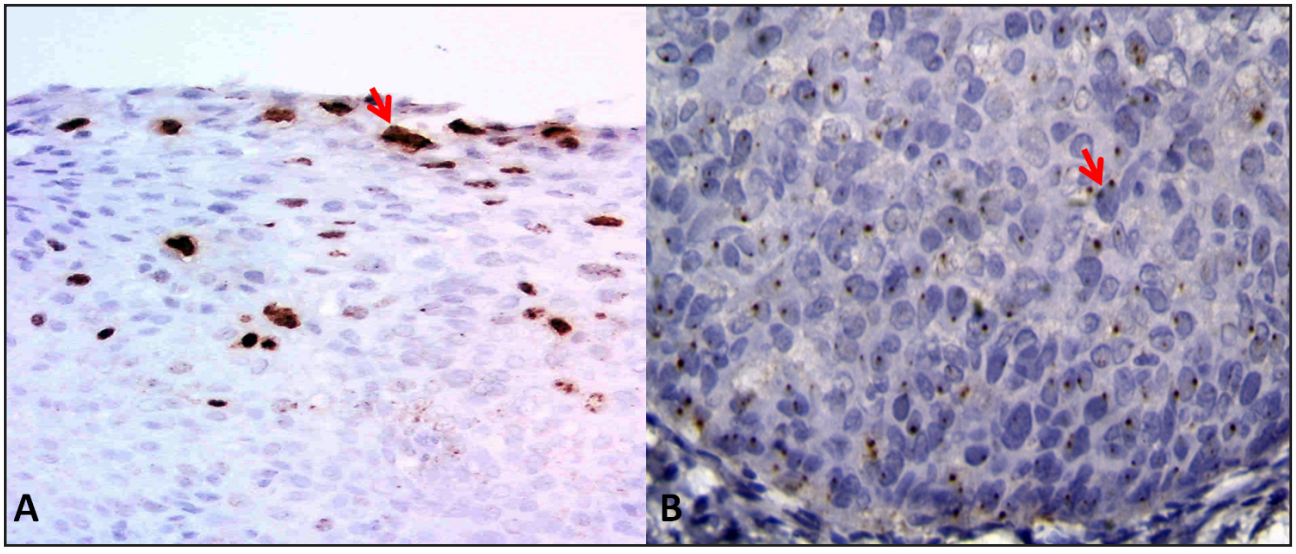

Fig. 3. DNA signal high risk HPV (40 X). A) CIN 1 showing signal diffuse in superficial epithelial cells (arrow); B) Cervical intraepithelial neoplasia 3 with punctate signal in the nuclei of cells (arrow).

Table 1. Expression of Ki-67 and hTERT by histological diagnosis

Location in the epithelium	Histological diagnosis				p value*
	No CIN (%) n	CIN 1 (%) n	CIN 2 (%) n	CIN 3 (%) n	
Ki-67 expression					
Negative	(100) 20	0	0	0	<0.001
lower 1/3	0	(80.0) 12	(6.7) 1	0	
lower 2/3	0	(20.0) 3	(93.3) 14	(43.3) 13	
Full thickness	0	0	0	(56.7) 17	
hTERT expression					
Negative	(80) 16	0	0	0	<0.001
lower 1/3	(20) 4	(73.3) 11	(6.7) 1	0	
lower 2/3	0	(26.7) 4	(80.0) 12	(36.7) 11	
Full thickness	0	0	(13.3) 2	(63.3) 19	

* *Fisher exact test. P*<0.001, test for *trend for both markers.* CIN: Cervical intraepithelial neoplasia.

Ki-67 was expressed and localized within the lower two-thirds of the epithelium (OR=17.5; p<0.01) as well as up to the total depth of the epithelium (OR=37; p<0.001) (Table 3). This association was still greater when the HR-HPV DNA showed a virus-integrated pattern (punctate signal) and was located within the lower 2/3 of the epithelium (OR=21.9; p=0.001) as well as throughout the total strata of the epithelium (OR=64.1; p<0.001) (Table 4). A similar trend was manifested by the overexpression of hTERT which was significantly associated with the integrated state of HR-HPV when it was located in the lower third of the epithelium (OR=10.7; p<0.049) and in the lower two-thirds of the epithelium (OR=50.2; 0=0.001), as well as throughout the total depth of the epithelium (OR=50.1; p=0.001) (Table 4).

DISCUSSION

The treatment of patients with precursory lesions of the uterine cervix depends upon the histological diagnosis. This diagnosis, although reliable in the ma-

Table 2. HR-HPV and physical state of virus by histological diagnosis.

	TOTAL (%) n	Histological diagnosis				p value*
		No CIN (%) n	CIN 1 (%) n	CIN 2 (%) n	CIN 3 (%) n	
HR-HPV						
Negative	(38.8) 31	(80.0) 16	(46.7) 7	(20.0) 3	(16.7) 5	<0.001
Positive	(61.2) 49	(20.0) 4	(53.3) 8	(80.0)12	(83.3) 25	
Signal pattern HR-HPV						
Diffuse	(24.5) 12	(50.0) 2	(25.0) 2	(41.7) 5	(12.0) 3	0.194
Punctuate	(75.5) 37	(50.0) 2	(75.0) 6	(58.3) 7	(88.0) 22	

* *Fisher exact test.* CIN: Cervical intraepithelial neoplasia.

Table 3. Association between Ki-67 and hTERT expression with HR-VPH

Location in the epithelium	HR-HPV		OR (95%CI)	p value
	Negative (%) n	Positive (%) n		
Ki-67				
Negative	(51.5) 16	(8.2) 4	1.0*	
Lower 1/3	(22.6) 7	(12.2) 6	4.2 (0.8-21.3)	0.083
Lower 2/3	(19.4) 6	(49.0) 24	17.5 (4.0-77.0)	<0.001
Full thickness	(6.5) 2	(30.6) 15	37.0 (5.5-250.0)	<0.001
hTERT				
Negative	(45.1) 14	(4.0) 2	1.0*	
Lower 1/3	(22.6) 7	(18.4) 9	12.4 (1.8-85.1)	0.011
Lower 2/3	(19.4) 6	(42.9) 21	31.5 (4.8-204.6)	<0.001
Full thickness	(12.9) 4	(34.7) 17	35.9 (5.0-254.9)	<0.001

Multinomial regression model adjusted for age. CI: Confidence interval. * Reference category,

Table 4. Association between Ki-67 and hTERT expression with the physical state of HR-HPV,

Location in the epithelium	HR-HPV						
	Negative (%) n	Diffuse (%) n	OR (95%CI)	p value	Punctuate (%) n	OR (95%CI)	p value
Ki-67							
Negative	(51.5)16	(16.7) 2	1.0*		(5.4) 2	1.0*	
Lower 1/3	(22.6) 7	(16.7)2	3.3 (0.3-33.0)	0.310	(10.8) 4	5.0 (0.7-34.5)	0.106
Lower 2/3	(19.4) 6	(66.6) 8	12.0 (1.7-85.9)	0.013	(43.2) 16	21.9 (3.8-127.1)	0.001
Full thickness	(6.5) 2	0	10.0 (0.4 - ∞)	0.160	(40.5) 15	64.1 (7.8-524.9)	<0.001
hTERT							
Negative	(45.2)14	(7.7) 1	1.0*		(2.7) 1	1.0*	
Lower 1/3	(22.6) 7	(30.7) 4	13.3 (0.9-188.5)	0.056	(13.5) 5	10.7 (1.0-113.3)	0.049
Lower 2/3	(16.1) 5	(38.5) 5	19.4 (1.4-268.8)	0.027	(46.0) 17	50.2 (5.2-489.3)	0.001
Full thickness	(16.1) 5	(23.1) 2	11.9 (0.8-189.7)	0.079	(37.8) 14	50.1 (4.9-512.0)	0.001

Multinomial regression model adjusted for age. CI: Confidence interval. * Reference category

jority of the cases, depends on the quality and accuracy of the CIN diagnosis, as well as on an adequate interpretation of the benign reactive changes that may be confused with a CIN. The joint evaluation of the histology and some biomarkers, as those used in this work, may be complementary helpers in the diagnosis and prognosis of a lesion. In four specimens of tissue without CIN we encountered the presence of HR-HPV. Two of these specimens showed a punctate signal pattern and the expression of telomerase localized in the lower third of the epithelium, indicating an evident alteration of the cellular cycle, possibly due to the integration of the HR-HPV DNA into the cellular genome, with a high probability of these women showing a CIN within a short time (Mittal et al., 1993; Kruse et al., 2001; Pirog et al., 2002). This observation will be checked by following these women closely.

We found a significant association between the increase in the expression of Ki-67 and the progression of CIN. This overexpression correlated with the integration of the HR-HPV DNA into the cellular genome. This correlation confirms that the determination of Ki-67, upon being a marker of cellular proliferation, is useful in the detection of early lesions that emerge through the interaction of the HR-HPV oncoproteins E6 and E7 with the genome of infected cells, corroborating the discoveries already described in other studies (Agoff et al., 2003; Bahnassy et al., 2006).

In a manner similar to Ki-67, the increased expression of hTERT is associated significantly with CIN progression; similar results have been obtained by Frost et al. (2000) and Keating et al. (2001). These researchers found high levels of hTERT expression in cases of CIN-3 and squamous cell carcinoma (SCC). Nowak (2000) observed the expression of telomerase in 66% of high-grade lesions and in 100% of cervical carcinomas, suggesting that this marker may be important in the diagnosis and prognosis of invasive carcinoma. He thus proposes that the overexpression of the telomerase may be a useful indicator in the early detection of cervical carcinoma. In our study, we encountered telomerase expression in CIN-1 lesions, including from the lower first third to the lower two-thirds of the epithelium. It was also expressed in the 4 cases negative for CIN but positive for HR-HPV. Although there were, of course, only four cases with such characteristics, these results indicated to us that the telomerase may be a good marker for incipient infections by HR-HPV.

We also encountered the overexpression of telomerase in CIN of high grade, in the presence of HR-HPV integrated DNA. This indicates that the oncoproteins E6 and E7 of these viruses cooperate to promote a proliferative state and cellular immortalization through telomerase activation (Shay and Bacchetti, 1997; Munger et al., 2004; Liu et al., 2008; Bellon and Nicot, 2008).

The result that 25% of the cases with CIN were HR-HPV negative may be because the probe used does not include all the HPV considered as likely high risk, such as types 53 and 66. HPV-56 and 66 have been detected by our workgroup in women in the south of Mexico (State of Guerrero) in specimens with CIN-negative cytologies: 0.7% of these specimens had HPV-53 and 0.06% HPV-66 while in the cases with low-grade squamous intraepithelial lesions (LSIL), 1.7% exhibited HPV-53 and 1.1%, HPV-66 (Illades-Aguiar et al., 2010). These results are why it is suggested that the probe should contain these VPH types.

On the other hand, upon correlating the expression of Ki-67 and/or telomerase with HR-HPV infection, a significant association of the expression of Ki-67 and telomerase with HR-HPV was found when this expression is localized in the lower two-thirds or the entirety of the epithelium's thickness. These results suggest that the expression of telomerase and/or cellular proliferation is associated with cervical carcinogenesis, being markers important and useful for appraising the progression of a cervical lesion. Cheung et al. (2004) described how the determination of Ki-67, telomerase and chromosomal *in situ* hybridization are methods that permit the detection of cancerous and precancerous cells, and thus they may be used as markers of these lesions.

The integration of the viral genome into the cellular genome is a key event for the development and progression of CIN. Few studies have been done where the physical state of HR-HPV DNA has been analyzed in cervical samples in paraffin. In our study, we found 75.5% of the CIN cases had a punctate signal pattern. Kalof et al. (2005) studied 25 CIN-1 cases and 17 CIN-2 and CIN-3 cases, with the results of the punctate signal being detected only in 3 CIN-1 cases (13.6%) infected with HR-HPV and in 100% of the CIN-2 and CIN-3 cases similarly infected. Presently it is known that the integration of the HR-HPV DNA with the DNA of the host cell is visualized with a punctate pattern within the cellular nucleus, while the expression of a diffuse signal in the nucleus of the infected cells is related to the episomal state of the virus, a productive phenotype of HPV, and koilocytic changes observed with greater frequency in low-grade lesions (Cooper et al., 2003). While the integration of the viral genome into the host cell is a common event in high-grade lesions, it is practically absent in low-grade lesions (CIN-1). In this study, the integrated state of the viral genome was present in 75% of the CIN-1 lesions positive for HR-HPV, indicating that these women show an early lesion that will surely evolve into high-grade one, where the significant association of the overexpression of telomerase with the progression of the CIN and the integration of viral DNA suggests that the activation of telomerase is an event very early in cervical carcinogenesis which correlates with the presence of HR-HPV.

The cellular proliferation, evaluated by Ki-67 expression, in association with the overexpression of telomerase and HR-HPV DNA integration in different grades of CIN indicate a high risk of progression into invasive carcinoma. The simultaneous assessment of these biomarkers in biopsies allows for detection of early and incipient lesions in underdeveloped countries such as ours, where cervical invasive carcinoma continues being one of the principal problems of public health.

Acknowledgments - Arianna Vega-Peña, Master in Science of the Academic Unit of of Chemistry and Biology Sciences of the Autonomus University of Guerrero was grateful for the Conacyt scholarship. We thank all of the Surgical Pathology Service of Hospital General Regional Vicente Guerrero of the Instituto Mexicano del Seguro Social, in Acapulco, Guerrero who helped with this study at the clinic sites. We thank Tomás Hernández-Quijano for patient management and specimen collection and Dr Gloria Fernández-Tilapa for critically reviewing this manuscript. We would also like to acknowledge the finalcial support provided by Promep /SEP Nov-2007-2009 (Programa Exbecarios Promep UA-GRO-EXB-106).

REFERENCES

Agoff, S.N., Lin, P., Morihara, J., Mao, C., Kiviat, N.B. and *L.A. Koutsky* (2003) p16(INK4a) expression correlates with degree of cervical neoplasia: a comparison with Ki-67 expression and detection of high-risk HPV types. *Mod Pathol*. **16**, 665-73.

Bahnassy, A.A., Zekri, A.R., Alam El-Din, H.M., Aboubakr, A.A., Kamel, K., El-Sabah, M.T. and *N.M. Mokhtar* (2006) The role of cyclins and cyclin inhibitors in the multistep process of HPV-associated cervical carcinoma. *J Egypt Natl Canc Inst*. **18**, 292-302.

Bellon, M. and *C. Nicot* (2008) Regulation of telomerase and telomeres: human tumor viruses take control. *J Natl Cancer Inst*. **100**, 98-108.

Bravaccini, S., Sanchini, M.A., Amadori, A., Medri, L., Saragoni, L., Calistri, D., Monti, F., Volpi, A. and *D. Amadori* (2005) Potential of telomerase expression and activity in cervical specimens as a diagnostic tool. *J Clin Pathol*. **58**, 911-4.

Cheung, A.N., Chiu, P.M., Tsun, K.L., Khoo, U.S., Leung, B.S. and *H.Y. Ngan* (2004) Chromosome *in situ* hybridisation, Ki-67, and telomerase immunocytochemistry in liquid based cervical cytology. *J Clin Pathol*. **57**, 721-7.

Cooper, K., Evans, M. and *S. Mount* (2003) Biology and evolution of cervical squamous intraepithelial lesions: a hypothesis with diagnostic prognostic implications. *Adv Anat Pathol*. **10**, 200-3.

Cooper, K., Herrington, C.S., Stickland, J.E., Evans, M.F. and *J.O. McGee* (1991) Episomal and integrated human papillomavirus in cervical neoplasia shown by non-isotopic *in situ* hybridisation. *J Clin Pathol*. **44**, 990-6.

Doorbar, J. (2006) Molecular biology of human papillomavirus infection and cervical cancer. *Clin Sci (Lond)*. **110**, 525-41.

Evans, M.F. and *K. Cooper* (2004) Human papillomavirus integration: detection by in situ hybridization and potential clinical application. *J Pathol*. **202**, 1-4.

Frost, M., Bobak, J.B., Gianani, R., Kim, N., Weinrich, S., Spalding, D.C., Cass, L.G., Thompson, L.C., Enomoto, T., Uribe-Lopez, D. and *K.R. Shroyer* (2000) Localization of telomerase hTERT protein and hTR in benign mucosa, dysplasia, and squamous cell carcinoma of the cervix. *Am J Clin Pathol.* **114**, 726-34.

Heatley, M.K. (2002) How should we grade CIN? *Histopathology*. **40**, 377-90.

Illades-Aguiar, B., Alarcon-Romero, L.C., Antonio-Vejar, V., Zamudio-Lopez, N., Sales-Linares, N., Flores-Alfaro, E., Fernandez-Tilapa, G., Vences-Velazquez, A., Muñoz-Valle, J.F. and *M.A. Leyva-Vazquez* (2010) Prevalence and distribution of human papillomavirus types in cervical cancer, squamous intraepithelial lesions, and with no intraepithelial lesions in women from Southern Mexico. *Gynecol Oncol*. **117**;(2):291-6.

Kalof, A.N. and *K. Cooper* (2006) p16INK4a immunoexpression: surrogate marker of high-risk HPV and high-grade cervical intraepithelial neoplasia. *Adv Anat Pathol*. **13**, 190-4.

Kalof, A.N., Evans, M.F., Simmons-Arnold, L., Beatty, B.G. and *K. Cooper* (2005) p16INK4A immunoexpression and HPV in situ hybridization signal patterns: potential markers of high-grade cervical intraepithelial neoplasia. *Am J Surg Pathol*. **29**, 674-9.

Keating, J.T., Ince, T. and *C.P. Crum* (2001) Surrogate biomarkers of HPV infection in cervical neoplasia screening and diagnosis. *Adv Anat Pathol*. **8**, 83-92.

Kruse, A.J., Baak, J.P., de Bruin, P.C., Jiwa, M., Snijders, W.P., Boodt, P.J., Fons, G., Houben, P.W. and *H.S. The* (2001) Ki-67 immunoquantitation in cervical intraepithelial neoplasia (CIN): a sensitive marker for grading. *J Pathol*. **193**, 48-54.

Liu, X., Dakic, A., Chen, R., Disbrow, G.L., Zhang, Y., Dai, Y. and *R. Schlegel* (2008) Cell-restricted immortalization by human papillomavirus correlates with telomerase activation and engagement of the hTERT promoter by Myc. *J Virol*. **82**, 11568-76.

Mittal, K.R., Demopoulos, R.I. and *S. Goswami* (1993) Proliferating cell nuclear antigen (cyclin) expression in normal and abnormal cervical squamous epithelia. *Am J Surg Pathol.* **17**, 117-22.

Munger, K., Baldwin, A., Edwards, K.M., Hayakawa, H., Nguyen, C.L., Owens, M., Grace, M. and *K. Huh* (2004) Mechanisms of human papillomavirus-induced oncogenesis. *J Virol.* **78**, 11451-60.

Nowak, J.A. (2000) Telomerase, cervical cancer, and human papillomavirus. *Clin Lab Med*. **20**, 369-82.

Pirog, E.C., Isacson, C., Szabolcs, M.J., Kleter, B., Quint, W. and *R.M. Richart* (2002) Proliferative activity of benign and neoplastic endocervical epithelium and correlation with HPV DNA detection. *Int J Gynecol Pathol.* **21**, 22-6.

Scholzen, T. and *J. Gerdes* (2000) The Ki-67 protein: from the known and the unknown. *J Cell Physiol*. **182**, 311-22.

Shay, J.W. and *S. Bacchetti* (1997) A survey of telomerase activity in human cancer. *Eur J Cancer*. **33**, 787-91.

Smith, D.L., Soria, J.C., Morat, L., Yang, Q., Sabatier, L., Liu, D.D., Nemr, R.A., Rashid, A. and *J.N. Vauthey* (2004) Human telomerase reverse transcriptase (hTERT) and Ki-67 are better predictors of survival than established clinical indicators in patients undergoing curative hepatic resection for colorectal metastases. *Ann Surg Oncol*. **11**, 45-51.

Syrjanen, K.J. (2005) Immunohistochemistry in assessment of molecular pathogenesis of cervical carcinogenesis. *Eur J Gynaecol Oncol*. **26**, 5-19.

Yang, J., Ramnath, N., Moysich, K.B., Asch, H.L., Swede, H., Alrawi, S.J., Huberman, J., Geradts, J., Brooks, J.S. and *D. Tan* (2006) Prognostic significance of MCM2, Ki-67 and gelsolin in non-small cell lung cancer. *BMC Cancer*. **6**, 203.