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UNIDAD ACADÉMICA DE CIENCIAS QUÍMICO BIOLÓGICAS

UNIDAD ACADÉMICA DE MEDICINA

MAESTRÍA EN CIENCIAS BIOMÉDICAS

“EXPRESION DE TELOMERASA, KI-67 Y EL VIRUS DEL PAPILOMA HUMANO ONCOGÉNICO EN NEOPLASIAS INTRAEPITELIALES CERVICALES”

T E S I S

QUE PARA OBTENER EL GRADO DE

MAESTRÍA EN CIENCIAS BIOMÉDICAS

P R E S E N T A:

A R I A N N A V E G A P E Ñ A

DIRECTORA DE TESIS:

DRA. LUZ DEL CARMEN ALARCÓN ROMERO

CHILPANCINGO, GRO., OCTUBRE DEL 2009.

UNIVERSIDAD AUTÓNOMA DE GUERRERO
UNIDAD ACADÉMICA DE CIENCIAS QUÍMICO BIOLÓGICAS
UNIDAD ACADÉMICA DE MEDICINA
MAESTRÍA EN CIENCIAS BIOMÉDICAS

APROBACIÓN DE TESIS

En la ciudad de Chilpancingo, Guerrero, siendo los 10 días del mes de julio de dos mil nueve, se reunieron los miembros del Comité Tutoral designado por la Academia de Posgrado de la Maestría en Ciencias Biomédicas, para examinar la tesis titulada **"Expresión de telomerasa, Ki-67 y el virus del papiloma humano oncogénico en neoplasias intraepiteliales cervicales"**, presentada por la alumna Arianna Vega Peña, para obtener el Grado de **Maestría en Ciencias Biomédicas**. Después del análisis correspondiente, los miembros del comité manifiestan su aprobación de la tesis, autorizan la impresión final de la misma y aceptan que, cuando se satisfagan los requisitos señalados en el Reglamento General de Estudios de Posgrado e Investigación Vigente, se proceda a la presentación del examen de grado.

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Este trabajo se realizó en el Laboratorio de Investigación en Citopatología de la Unidad Académica de Ciencias Químico biológicas de la Universidad Autónoma de Guerrero, en la ciudad de Chilpancingo, Gro, México.

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"TELOMERASE EXPRESSION, KI-67 AND HUMAN PAPILLOMAVIRUS ONCOGENIC IN CERVICAL INTRAEPITHELIAL NEOPLASIA"

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Manuscript Received

De: Juan F Madrid <jfmadrid@um.es>
Fecha: 2 de octubre de 2009 12:32
Asunto: HISTOLOGY AND HISTOPATHOLOGY-Reception
Para: Luz Del Carmen Alarcón Romero <luzdelcarmen14@gmail.com>

Our reference: B-3896 (Do not forget to include this number in future correspondence)

Dear Dr. Alarcon Romero et al.,

I am pleased to acknowledge receipt of the paper entitled "Ki-67 and telomerase expression in cervical intraepithelial neoplasia associated with HPV infection" submitted for publication in HISTOLOGY AND HISTOPATHOLOGY (2008 IMPACT FACTOR: 2.194).

As soon as the manuscript has been seen by the referees, you will be informed as to whether it can be accepted.

Yours sincerely,

Prof. Juan F. Madrid, Editor

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Telomerase expression, Ki-67 and human papillomavirus oncogenic in cervical intraepithelial neoplasia

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Abstract

Objective. Evaluate the relationship between Ki-67 and telomerase expression with CIN grade and the physical state of high risk human papillomavirus (HR-HPV) DNA. **Methods.** An observational comparative study was done of 80 fragments of paraffin embedded tissues, 20 corresponded to normal cervical tissue and 60 to cervical tissues with different CIN grades. Immunohistochemistry was used to detect the Ki-67 and telomerase proteins, and *in situ* hybridization with tyramide amplification was used to detect the presence of HR-HPV DNA. **Results.** In 100% of the samples the different CIN grades were positive for Ki-67 compared with normal tissue ($p<0.001$), finding the immunostain from the first third (CIN I) to the most superficial epithelial layer (CIN III). Meanwhile, in the normal tissues, positive reaction was only found in some deep parabasal and basal cells. Telomerase expression increases in relation to the CIN grade, in normal tissue, telomerase was found only in 20% of the cases in the first third of the epithelium. HR-HPV DNA presence was detected in 61.3% of the samples including 4 samples of normal tissue ($p=0.001$) and the punctuate signal was more frequent (75.5%). Ki-67 and telomerase expression was found to be significantly associated with HR-HPV presence in the second third and all the thickness of the epithelium. **Conclusions.** The expressions of Ki-67 and telomerase associated with HPV-HR provide evidence of the presence of more aggressive lesions with potential progression to invasive carcinoma.

Keywords: Ki-67 antigen, telomerase, cervical intraepithelial neoplasia, human papilloma virus, *in situ* hybridization

Introduction

Invasive cervical carcinoma is preceded by cervical intraepithelial neoplasias (CIN) that originate from the squamocolumnar transformation zone. Integration of high risk human papillomavirus (HR-HPV) to the host cell genome is a factor that promotes the progression of CIN to invasive carcinoma. It has been proposed that *in situ* hybridization cannot only determine the presence of HR-HPV but also the physical state of the viral DNA, contributing to the knowledge of the molecular biology of HPV associated with CIN and its clinical utility (Kalof and Cooper, 2006) .

On the other hand, even though the histophatological study is the confirmative method of the cervical lesion, the false negative rates of CIN have increased due to the variability in diagnosis between observers, (Heatley, 2002) therefore**,** we are evaluating the clinical utility of the different biomarkers to improve the diagnosis of prognosis of early lesions. The nuclear antigen Ki-67 (MIB-1) is present in all the active phases of the cell cycle, except in the G0 phase and early G1 phase, and represents a good biomarker for the prognosis of tumor growth. (Scholzen and Gerdes, 2000; Cheung *et al*., 2004; Yang *et al*., 2006)

The oncoprotein E7 of HR-HPV promotes cellular proliferation by binding to Rb, thus increasing cell cycle kinetics and overexpression of Ki-67. (Doorbar, 2006)

Another marker in evaluation in various types of cancer is telomerase, an enzyme that replaces small nucleotide sequences that are eliminated in the telomeric ends of the chromosome in each replication. It is composed of two subunits**:** the human RNA telomerase (hTR) and the reverse transcriptase of the human telomerase (hTERT), the latter has a catalytic function in the replication of the ends of linear DNA. In cancer or cancer cell lines, telomerase activity has been found to be increased in comparison to premalignant lesions and normal cells. (Smith *et al*., 2004; Bravaccini *et al*., 2005) The purpose of this study was to evaluate the relation between Ki-67 and telomerase expression with the CIN grade and physical state of HR-HPV DNA.

Materials and methods

Cervical tissue samples

Eighty fragments of paraffin embedded biopsies from women that attended the Vicente Guerrero General Hospital of the IMSS in Acapulco, Guerrero, Mexico were obtained. The histological diagnosis of the samples was: 15 CIN I, 15 CIN II, and 30 CIN III (15 of severe dysplasia and 15 of carcinoma *in situ*), as well as 20 fragments of surgical pieces from hysterectomies due to miomas. The subjects received written and oral information before they gave their consent to participate. The study was approved by the Ethics Committee of the University of Guerrero.

Ki-67 and hTERT Immunohistochemistry

Four to five serial sections (3 microns) were obtained. To detect Ki-67 expression the monoclonal antibody MIB1 (Dako, Carpinteria, CA) was used and for hTERT the 2C4 monoclonal antibody (Novus Bio, Littleton, CO). The immunochemical detection system Cytoscan HRP/DAB (Cell Marque Corporation, Hot Springs, AR) was used. The histological sections were deparaffinated and antigenic recovery was done in a ImmunoDNA Retriever solution with citrate (BioSB, Inc, Santa Barbara, CA). After, the primary antibody was added in a 1:50 dilution for Ki-67 and 1:200 for hTERT, diaminobenzidine was added as a chromogen. Finally they were stained with haematoxylin (Merck).

A histological sample of squamous cell carcinoma (SCC) that overexpressed Ki-67 and a case of breast carcinoma that overexpressed hTERT were used as positive controls. The same cases were used as negative control, without adding primary antibody

Evaluation of Ki-67 and hTERT immunostain

Ki-67 and hTERT expression in epithelial tissues was evaluated by the distribution of the positive reaction in thickness of the epithelium and the intensity of the coloration. The reaction was considered positive when the nucleus of the cells showed a brown stain for Ki-67, and nuclear or cytoplasmic for hTERT. In function of the thickness of the epithelium it was evaluated as follows: 0 when the immunostain was found only in deep basal or cervix parabasal cells, 1+ (low),

when 1/3 of the epithelium showed immunoreactivity; 2+ (moderate), when 2/3 of the epithelium showed immunoreactivity and 3+ (high), when the immunostain was observed throughout the entire squamous cervical epithelium. (Syrjanen, 2005)

In situ hybridization with tyramide amplification

The detection of the viral genome by *in situ* hybridization was done with the tyramide signal amplification system GenPoint, (Carpinteria, CA). The cervical tissue sections were placed on silanized slides, then the paraffin was removed. Enzymatic digestion was done with proteinase K, the biotinylated HPV DNA probe that contains base pairs complementary to 13 genotypes of HR-HPV that includes types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 y 68 was then added. I was denatured and hybridized for 20hrs (Dako, Carpinteria, CA). The slides were then covered with astringent solution and then with primary streptavidin peroxidase. Biotinyl-tyramide and secondary streptavidin were added. Finally the slides were covered with the chromagen diaminobenzidine and stained with haematoxylin. The positive reaction was seen as brown intranuclear stains that was either diffused or punctuate signals. Positive controls were cervical squamous carcinoma tissue and SiHa cells, both containing HPV-16.

Statistical analysis

The frequencies were analyzed using the Fisher exact test. The relation between Ki-67 and telomerase expression with HR-HPV was evaluated by logistic regression multinomial models adjusted by age. A $p<0.05$ was considered statistically significant. Statistical analysis was done with the STATA v.9.2 software.

Results

Ki-67 and telomerase immunoreactivity

In table 1, Ki-67 and telomerase expression is related to histological diagnosis (p for trend <0.001). In normal tissues, the immunoreactivity of Ki-67 is limited to some parabasal cells (Figure 1A). The intensity of Ki-67 immunostaining augmented with the CIN grade, being present from the deep first third, middle and throughout the thickness of the epithelium (Figure 1 B-F) in comparison to normal tissue (p< 0.001). Telomerase expression was predominantly nuclear and in some cases cytoplasmic and increased with CIN grade, showing intense expression throughout the thickness of the epithelium in the carcinoma *in situ* (Figure 3 A-D) in comparison to the normal tissues (p<0.001). It is important to mention that in 20% of normal tissues, telomerase expression was observed in the first third of the epithelium.

Table 1. Ki-67 and telomerase inmunoreactivity by histological diagnosis

	Histological diagnosis					
	Normal tissue n(%)	CIN I n (%)	CIN II n (%)	CIN III† n (%)	CIN III‡ n (%)	p value*
Ki-67 expression						
Negative	20 (100)	0	0	0	0	
1st third	0	12 (80.0)	1 (6.7)	0	0	
2 thirds	0	3 (20.0)	14(93.3)	8 (53.3)	5 (33.3)	0.001
All epithelium	0	0	0	7 (46.7)	10 (66.7)	
Telomerase expression						
Negative	16 (80.0)	0	0	0	0	
1st third	4 (20.0)	11 (73.3)	1 (6.7)	0	0	
2 thirds	0	4 (26.7)	12(80.0)	9 (60.0)	2 (13.3)	0.001
All epithelium	0	0	2 (13.3)	6 (40.0)	13 (86.7)	

p for trend <0.001 of both biomarkers. CIN: Cervical intraepithelial neoplasia; †severe dysplasia; ‡ carcinoma *in situ*. *Fisher exact test

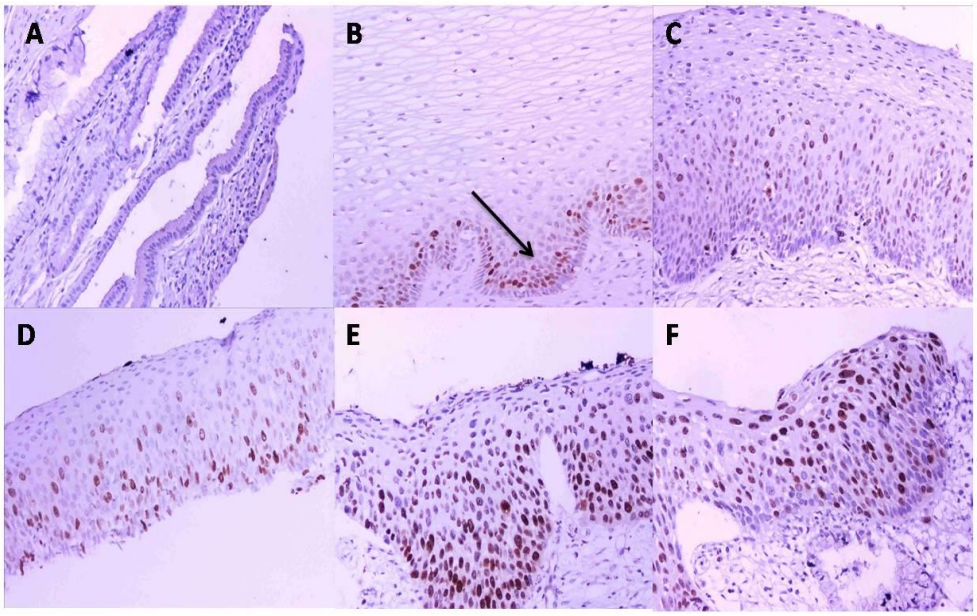

Figure 1. Ki-67 expression. A) Absence of Ki-67 expression in columnar cells, B) Normal squamous epithelium with nuclear expression in some basal and deep parabasal cells (arrow), C) Cervical intraepithelial neoplasia I (CIN I) showing nuclear immunoreactivity, D) CIN II showing nuclear immunoreactivity (with greater intensity than CIN I), E) CIN III showing nuclear immunoreactivity in two thirds of the epithelium, F) Carcinoma *in situ* (CIN III) showing strong immunoreactivity of Ki-67 in the entire epithelial thickness. 10X.

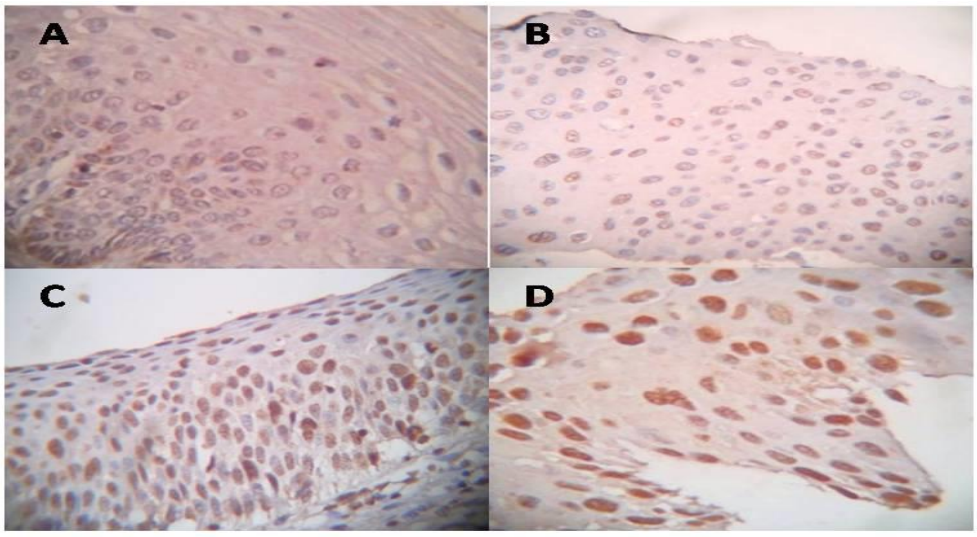

Figure 2. Telomerase expression: A) Expression in some parabasal cells in normal tissue, B) Cervical intraepithelial neoplasia I (CIN I) showing nuclear immunoreactivity in the first third of the epithelium, C) CIN II showing nuclear immunoreactivity in more two-thirds of the epithelium, D) CIN III showing immunoreactivity the entire thickness of the epithelium.40X

In situ hybridization

Table 2 summarizes the results obtained about the physical state of the HR-HPV DNA. Thirty eight point seven percent (31/80) of the tissues included in this study were negative for HR-HPV and 61.3% were positive. In 20% (4/20) of the normal tissues HR-HVP was found, two of these showed a punctate signal of the virus. In 77.8% (35/45) of the positive CIN punctate signal of HR-HPV was seen.

Table 2. Frequency and signal pattern of HR-HPV by histological diagnosis

	Histological diagnosis						
	Normal tissue n(%)	CIN I n(%)	CIN II n (%)	CIN III† n (%)	CIN III‡ n (%)	Total	p value*
HR-HPV							
Negative	16(80.0)	7 (46.7)	3 (20.0)	2 (13.3)	3 (20.0)	31 (38.7)	0.001
Positive	4 (20.0)	8 (53.3)	12(80.0)	13 (86.7)	12 (80.0)	49 (61.3)	
Signal patters HR-HPV							
Diffuse	2 (50.0)	2 (25)	5 (41.7)	2 (15.4)	1 (8.3)	12(24.5)	0.20
Punctate	2 (50.0)	6 (75.0)	7 (58.3)	11(84.6)	11 (91.7)	37 75.5)	

CIN: Cervical intraepithelial neoplasia; †severe dysplasia; ‡ carcinoma *in situ*. *Fisher exact test.

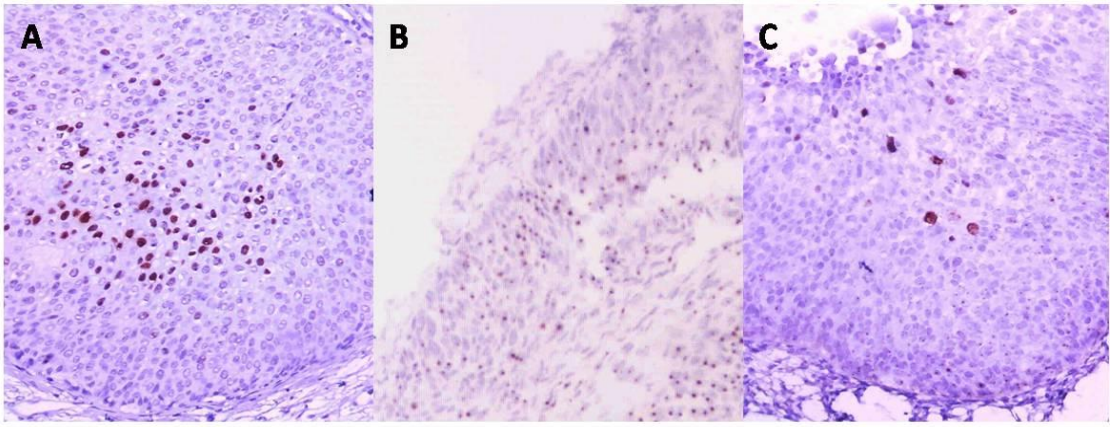

Figure 3. Signal pattern of the HR-HPV DNA. A) Carcinoma *in situ*, showing diffuse signal the entire nucleus. B) Severe dysplasia is presented punctate signal throughout the thickness of the epithelium, C) Severe dysplasia, showing the expression of diffuse and punctate signal. 10X.

Association between Ki-67 and telomerase expression with physical state of HR-HP

Ki-67 expression was associated significantly with physical state and the presence of HR-HPV (Table 3, 4), with an increase in expression from the deep second third to the entire thickness of the epithelium (p for trend <0.001). Similarly, telomerase overexpression was associated significantly with physical state and the presence of HR-HPV (p for trend <0.001).

Table 3. Association between Ki-67 and telomerase expression with physical state of HR-HPV

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

	HR-HPV						
	Negative n (%)	Diffuse n (%)	OR (95%CI)	p value	Punctate n (%)	OR (95%CI)	p value
Ki-67							
Negative	16(51.5)	2(16.7)	1.0*		2 (5.4)	1.0*	
1st third	7 (22.6)	2(16.7)	3.3 (0.3-33.0)	0.310	4 (10.8)	5.0 (0.7-34.5)	0.106
2 thirds	6 (19.4)	8(66.6)	12.0 (1.7-85.9)	0.013	16(43.2)	21.9 (3.8-127.1)	0.001
All epithelium	2 (6.5)	0	10.0 (0.4-∞)	0.160	15(40.5)	64.1 (7.8-524.9)	<0.001
Telomerase							
Negative	14(46.7)	1 (7.7)	1.0*		1 (2.7)	1.0*	
1st third	7 (23.3)	4(30.8)	13.3(0.9-188.5)	0.056	5 (13.5)	10.7 (1.0-113.3)	0.049
2 thirds	5 (16.7)	5(38.5)	19.4 (1.4-268.8)	0.027	17(46.0)	50.2 (5.2-489.3)	0.001
All epithelium	4 (13.3)	3(23.1)	11.9(0.8-189.7)	0.079	14(37.8)	50.1 (4.9-512.0)	0.001

Table 4. Association between Ki-67 and telomerase expression with HR-HPV

	HR-HPV				
	Negative n (%)	Positive n (%)	Total	OR (95%CI)	p value
Ki-67 expression					
Negative	16 (51.5)	4 (8.2)	20	1.0*	
1st third	7 (22.6)	6 (12.2)	13	4.2 (0.8-21.3)	0.083
2 thirds	6 (19.4)	24 (49.0)	30	17.5 (4.0-77.0)	<0.001
All epithelium	2 (6.5)	15 (30.6)	17	37.0 (5.5-250.0)	<0.001
Telomerase expression					
Negative	14 (45.1)	2 (4.0)	16	1.0*	
1st third	7 (22.6)	9 (18.4)	16	12.4 (1.8-85.1)	0.011
2 thirds	6 (19.4)	21 (42.9)	27	31.5 (4.8-204.6)	<0.001
All epithelium	4 (12.9)	17 (34.7)	21	35.9 (5.0-254.9)	<0.001

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

Discussion

Treatment of precursor lesions of the uterine cervix depends on histological confirmation. This strategy, although effective, is linked to the precision of the histological diagnosis of precancerous lesion and the adequate interpretation of the benign reactive chances that imitate a lesion. Thus, the overall evaluation of diverse biomarkers can be a complementary alternative for the diagnosis and prognosis of the progression of the lesion. When we evaluated Ki-67 and telomerase expression as well as the signal patterns of the HR-HPV in CIN and normal tissues, we found 4 samples of normal tissue with HR-HPV presence, 2 with punctate signal and telomerase expression in the deep third of the epithelium, which can indicate an alteration in the cellular cycle and a risk of progression to CIN (Mittal, 1993; Kruse *et al*., 2001; Pirog *et al*., 2002), therefore these women need to be carefully monitored. Cooper *et al.,* 1991 showed that the punctate pattern in the nucleus represent the integrated version of the virus to the cellular genome, while the diffuse pattern indicated an episomal state.

We found and increase in Ki-67 positive cells as the histological grade of the CIN increases, this overexpression correlated with the presence of HR-HPV, showing the high sensitivity with which Ki-67 identifies abnormal cellular proliferation and indicates that hyperproliferation is the results of cellular interaction with the oncoprotein E6 and E7 of HR-HPV. Similar results were found in other studies. (Agoff *et al*., 2003; Bahnassy *et al*., 2006)

Like Ki-67, telomerase expression increased with the advance of the lesion's CIN histological grade, similar to that found by Frost *et al*., 2000 and Keating *et al*., 2001. They reported high levels of hTERT expression in high grade lesion (HSIL) and squamous cell carcinoma (SCC), similar to that reported by Nowak (2000) in cytological samples, who found telomerase in 66% of the HSIL and 100% of cervical carcinoma, suggesting that this marker can be important in diagnosis and prognosis of invasive carcinoma. Telomerase overexpression can be useful in the early detection of cervical carcinoma, therefore its applications need to be further explored.

Telomerase overexpression in CIN II and III associated with the presence of HR-HPV DNA indicate that the oncoproteins E6 and E7 of these viruses cooperate simultaneoustly to promote cellular immortalization, throuth the activation of the telomerase and contribute to a proliferative state. (Shay and Bacchetti, 1997; Munger *et al*., 2004; Liu *et al*., 2008; Bellon and Nicot, 2008)

Viral genome integration to the host cells is a key event in the development of cervical neoplasia. Few studies have been done to analyze the physical state of HPV DNA in paraffin included cervical samples. In the evaluation of the physical state of HR-HPV we found the punctuate form in 77.8% of CIN, these results are similar to those reported by Kalof *et al.,* 2005 in which 100% of CIN II and III showed punctate signal acroos the epithelium. HPV integration is related to the punctate form in high grade lesions and cervical cancer. (Cooper *et al*., 1991) The CIN cases with diffuse signal pattern (22.2%) in that study have been related with the episomal state of the virus and it has been reported that this pattern correlates with a productive HPV phenotype and coilocytic changes, observed with more frequency in low grade lesions. (Cooper and Evans, 2005)

Twenty five percent of the CIN were negative for the presence of HR-HPV with the probe used that does not include low risk HPV and others of probable high risk such as 53 and 66 that have been found in normal cytologies and cervical cancer in women from the state of Guerrero by our research group (Fernández-Tilapa *et al*., 2007; Illades-Aguiar *et al*., 2009). Including these viral types in the probe used for *in situ* hybridization is recommended because it would benefit Mexican women in which prevalence of these viruses is seen.

In this study, we found an association between Ki-67 and telomerase expression in the second third and across all epithelium and HR-HPV. Our findings suggest that telomerase expression and cellular proliferation are associated with cervical carcinogenesis, thus being useful markers to evaluate the cervical lesion grade.

Cheung *et al.*, 2004 reported that determination of Ki-67, telomerase and chromosomal *in situ* hybridization can detect cancerous and precancerous cells, and could be good markers for precancerous lesions.

In summary, overexpression of telomerase and the increase in cellular proliferation indicated by Ki-67 overexpression and the presence of HR-HPV DNA in the different CIN grades, indicate a greater risk of invasive carcinoma. On the other hand, simultaneous evaluation of these biomarkers allows the identification of cellular alterations. It is important that the clinical utility of these and other markers for early detection of cervical invasive carcinoma are further studied.

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Competing interests

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