UNIVERSIDAD AUTÓNOMA DE GUERRERO
FACULTAD DE CIENCIAS QUÍMICO BIOLÓGICAS
UNIDAD ACADÉMICA DE MEDICINA
UNIDAD DE INVESTIGACIÓN ESPECIALIZADA EN MICROBIOLOGÍA

M A E S T R Í A E N C I E N C I A S B I O M É D I C A S

T E S I S

Expresión de $p16^{INK4a}$, MCM2/TOPIIα y REST y estado físico del VPH 16 en el seguimiento de lesiones tempranas del cérvix uterino en mujeres guerrerenses

PARA OBTENER EL GRADO DE

MAESTRÍA EN CIENCIAS BIOMÉDICAS

P R E S E N T A:

Q.B.P. WENDY AIDE CASTRO MORA

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Chilpancingo, Gro., Enero del 2019.

ACTA DE APROBACIÓN DE TESIS

UNIVERSIDAD AUTÓNOMA DE GUERRERO
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MAESTRÍA EN CIENCIAS BIOMÉDICAS

APROBACIÓN DE TESIS

En la ciudad de Chilpancingo, Guerrero, siendo los 20 días del mes de junio de dos mil dieciocho 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 p16INK^{4a}, MCM2/TOPIIα y REST y estado físico del VPH16 en el seguimiento de lesiones tempranas del cérvix uterino en mujeres guerrerenses"**, presentada por la alumna Wendy Aide Castro Mora, 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 fue realizado en el Laboratorio de Investigación en Citopatología e Histoquímica de la Facultad de Ciencias Químico Biológicas de la Universidad Autónoma de Guerrero.

Bajo la dirección de:

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Durante el periodo en que se cursó la Maestría en ciencias Biomédicas, la C. Wendy Aide Castro Mora, recibió beca CONACYT con no. de registro CVU 778715.

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TÍTULO DEL ARTÍCULO

Viral integration increases the expression of p16INK4a and MCM2/TOPIIα, and decreases the expression of REST in early cervical lesions with persistence of HPV16: follow-up study in women from Southern Mexico

ÍNDICE

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Viral integration increases the expression of p16INK4a and MCM2/TOPIIα, and decreases the expression of REST in early cervical lesions with persistence of HPV16: follow-up study in women from Southern Mexico

Wendy Aide Castro-Mora, MSc; Berenice Illades-Aguiar, PhD; Eugenia Flores-Alfaro, PhD; Carlos Ortuño-Pineda, PhD; Oscar Del Moral-Hernandez, PhD; Marco Antonio Leyva-Vázquez, PhD; Ana Laura Pereira-Suarez, PhD; Ma. Isabel Zubillaga-Guerrero, PhD; Luz del Carmen Alarcón-Romero, PhD

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Viral integration increases the expression of p16INK4a and MCM2/TOPIIα, and decreases the expression of REST in early cervical lesions with persistence of HPV16: follow-up study in women from Southern Mexico

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Abstract

Background: The persistent infection of HPV16 and its DNA integration increase the risk of precancerous lesions progression to invasive cervical carcinoma (ICC) due to viral oncoproteins E6 and E7 overexpression, which are determinants for the malignant phenotype. Nevertheless, not all Low-Squamous Intraepitelial Lesions (LSIL) showing viral DNA integration are able to progress to High-Squamous Intraepitelial Lesions (HSIL) or carcinomes, indicating other factors are necessary for cell transformation. Such molecules could serve as important cellular biomarkers in combination with the physical state of HPV16 DNA, for this reason the goal of this work was to evaluate the relationship between the expression of $p16^{INK4a}$, MCM2/TOPIIα and REST with the physical state of HPV16 DNA in women during one year of follow-up. **Methods:** This study included 50 women with HPV16 determined by INNOLiPA and diagnosed with LSIL by conventional cytology. Physical state of the HPV was determined by in situ hybridization with tyramide amplification, and the expression of $p16^{INK4a}$, MCM2/TOPIIα and REST by immunocytochemistry. The diagnosis, viral genotyping, physical state and protein expression where determined at the begining and after one year of follow-up, and results were analyzed statistically by lineal regression. **Results:** Data showed that after one year of follow-up 98% of women mantained LSIL, showing persistence of HPV16 in 50% of cases. Sixty-eight percent of HPV16 cases had an integrated physical state. On the other hand, the expression of $p16^{NK4a}$ increased 26%, MCM2/TOPIIα increased 17%, whereas REST expression diminished 15%. Importantly, the integrated state was related to the persistence of HPV16 ($p=0.04$), and also to the alteration of $p16^{INK4a}$ ($p<0.001$), MCM2/TOPIIα ($p=0.04$) and REST ($p=0.04$). **Conclusion:** Due to the role of $p16^{INK4a}$ and MCM2/TOPIIα in cell cycle regulation, and the previous association of REST in carcinogenesis, results could evidence an important group of early cervical lesions with potential risk of progression to HSIL; however, a much more robust study must be conducted.

Keywords: $p16^{INK4a}$, MCM2/TOPIIα, REST, viral integration, HPV16

Introduction

In Mexico, invasive cervical carcinoma (ICC) remains the second most frequent carcinoma in women [1]. Persistent High-Risk HPV infection (HR-HPV) is the main risk factor for cervical carcinogenesis [2]. The viral infection is characterized cytologically by the presence of coilocytes that includes karyomegaly, binucleation, perinuclear halo and hyperchromasia among other cytological alterations. Bethesda cytological nomenclature uses these characteristics to classify pre-cancerosus lesions as low-grade squamous intraepithelial lesion (LSIL) and high-grade (HSIL) [3]. LSIL is considered the earliest lesion of the uterine cervix that precedes the ICC [2]; however, 80% of the LSIL are transitory and they are eliminated in a period of 6 to 12 months. Certainly, only 10% of HR-HPV infections can persist for years [4], preceding to ICC in cohort studies [5]. A follow-up study by our work group conducted by Vega-Peña et al., including 50 cytologies from women without SIL or with LSIL, both with HPV16, showed the persistence of HPV16 in 42% of the women with LSIL, in which the integrated physical state was found in 52%. Interestingly, the persistence of HPV16 in women without SIL was associated with the risk of progression to LSIL (OR=4.6; $p<0.001$), whereas the integrated physical state was associated to Ki-67 (OR=2.3, $p=0.05$) and E6 expression (OR=3.3, $p=0.01$ [6]. Currently, the determinant factors that can favor the elimination of HPV are unknown, and the viral persistence that can lead to progression of precancerous lesions requires greater understanding of the cellular, viral and host changes that contribute to the progression of the LSIL. Particularly, for viral integration, the HPV episome breaks down in the region of the E2 gene, which is a transcriptional repressor of the E6 and E7 oncogenes, causing overexpression of E6 and E7 [7]. It induces an unscheduled reentry to the S phase of the cell cycle and to the escape of apoptosis [7], [8]. Although the persistence of HPV16 and viral integration contribute to the development of the malignant phenotype and favor the alteration of cellular events for the progression of the LSIL [9], the clinical outcome of the LSIL has not yet been predicted.

Robust evicence shows that $p16^{INK4a}$, which participates in cell cycle control as a negative regulator of the pRb/E2F pathway [10], is associated to LSIL progression and

ICC [11]. High levels of $p16^{INK4a}$ expression have been found in the presence of transcriptionally active HR-HPV [12] and its overexpression has been detected in the presence of cytological alterations caused by the HR-HPV in the LSIL and the cases of ICC [13]. On the other hand, minichromoseome maintenaince protein 2 (MCM2) and topoisomerase IIα (TOPIIα) participate during the replication of DNA in the S phase of the cell cycle [14] and they promote accelerated cell proliferation during SIL progression. Finally, the RE1-Silencing Transcription factor (REST), whose expression is high and ubiquitous in non-neuronal tissues [15], is alterated in several rapidly proliferating cancers such as medulloblastoma, neuroblastoma, colon cancer, breast cancer, small and non-small cell lung carcinoma [15] - [17]. In breast cancer, functional inhibition of REST in epithelial cells increases cell capacity for oncogenic transformation, cell proliferation and survival by promoting Akt phosphorylation [18], [19], whereas in Small Cell Lung Cancer REST promotes the aggressive neuroendocrine phenotype [20]. In cervical carcinogenesis the expression of REST is still unknown, so it is necessary to evaluate it in relation to HPV infection.

In the present study, we evaluated the expression of $p16^{INK4a}$, MCM2/TOPIIα and REST and their relationship with the physical state of HPV16 in women with LSIL in one year of follow-up. In the clinical, these proteins could be useful biomarkers in the early diagnosis of lesions, following to identify women with higher risk of progression and to design proper treatment.

Material and methods

Subjects and sampling

Fifty women from the State of Guerrero, Mexico who attended the Servicio de Diagnóstico Integral en la Detección Oportuna del Cáncer Cérvicouterino y HPV de la de la Facultad de Ciencias Químico Biológicas de la Universidad Autónoma de Guerrero, were followed for a year from June 2016 to April 2018. Two cytological samples were collected, the first specimen at the beginning and the second one after one year. Each participant signed the informed consent in writing, and responded a questionnaire with sociodemographic data and lifestyle behaviors (smoking habit, alcohol consumption and number of sexual partners). Gynecological and obstetric history (parity, use of hormonal contraceptives and condoms) were obtained in the last 2 years also.

Cytological samples were collected with Ayre spatula and disposable cytobrush, ensuring exo/endocervical cellular material from the area of transformation of the uterine cervix. The double take was made for the collection of the cytological samples. With the cellular material obtained in the first shot, a conventional smear was performed for cytomorphological examination by means of Papanicolaou staining for cytological diagnosis, and the cellular material was preserved in liquid based cytology (Liqui-PREPTM) for the determination of physical state of DNA by in situ hybridization (ISH) with tyramide amplification, and the expression of the p16INK4a, MCM2/TOPIIα and REST proteins by immunocytochemistry. The cellular material from the second dose was deposited in an extraction solution for the detection and genotyping of HPV.

At the beginning of the study, the cytological diagnosis, based on the Bethesda System, and the detection and typing of HPV in the 50 women was LSIL with HPV16. The determination of viral persistence was based on the detection and typing of HPV16 in the cytological material collected after one year of follow-up. Women with viral persistence were defined as positive for the specific type of HPV16, with viral reinfection as negative for the specific type of HPV16, but positive for other types of HPV and clearing as negative for any type of HPV.

This project was approved by the Bioethics Committee of the Universidad Autónoma de Guerrero, México, and all procedures were carried out in accordance with the ethical guidelines for medical research through the Declaration of Helsinki updated in 2013.

HPV genotyping

The determination of the HPV genotype was made with the HPV genotyping test INNOLiPA (Innogenetics NV, Ghent, Belgium), following the manufacturers instructions. This trial can identify 28 different genotypes of HPV, such as HR-HPV (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59), probable high risk (26, 53, 66, 68, 73 and 82), low risk HPV (LR-HPV) (6, 11, 40, 43, 44, 54 and 70) and several additional types (69, 71 and 74). This assay is based on the amplification of a fragment of the L1 region of the HPV genome by nested PCR [21].

In situ hybridization with tyramide amplification

For ISH with tyramide signal amplification (GenPoint Dako Cytomation, Carpinteria, CA, USA), the cytological smears were subjected to permeabilization and enzymatic digestion with proteinase K. A biotinylated DNA probe was used and detects 13 types of HR-HPV (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) (Dako Carpintería, CA, USA). These smears were subjected to desnaturation and hybridization (Hybridizer Dako, Carpinteria, CA. USA). The samples were counterstained with Mayers hematoxylin. The positive reaction was visualized as an ocher brown deposit inside the nucleus. This brown deposit was classified as diffuse (episomal state), punctate (integrated state) or mixed (episomal and Integrated) [22]. The SiHa cell line (squamous cell carcinoma with HPV16) containing the integrated HPV16 genome was used as a positive control.

Immunocytochemistry

The expression $p16^{INK4a}$, MCM2/TOPIIα and REST was determined by the immunocytochemical method using the ImmunoDetector HRP/DAB kit (BioSB, Inc.,

Santa Barbara, CA, USA). The antibodies used were anti-p16^{INK4a} (clone G175-405; 1:50; BD Pharmingen), a recombinant monoclonal antibody ProEx C (clones MCM 26H6.19, MCM2 27C5.6, TOPIIα SWT3D1; Tripathlmagning, Burlington, NC) and anti-REST/NRSF (ab21635, 1:500, Abcam). Liquid-based cytology smears were subjected to antigen retrieval (Declere, 1:20; CELL MARQUE; USA, for p16^{INK4a} and REST; and EDTA decloaker; 1: 5; BIOCARE Medical; USA; MCM2/TOPIIα) at 120°C. Samples were counterstained with Mayers hematoxylin. For p16^{INK4a} and REST, nuclear or nuclear/cytoplasmic immunostaining was considered positive, and for MCM2/TOPIIα only nuclear was considered. The cell line SiHa (squamous cell carcinoma with HPV16) was used as positive control. As negative control was used cervical cytology without SIL and HPV. The quantification of the immunostaining of the expression of p16^{INK4a}, MCM2/TOPIIα and REST was performed by means of a digital image analysis with the program Image-Pro Plus 6.0 (Mediacybernetics, USA), using the parameter of average optical density. Digital imaging of each immunocytochemistry was performed with a Leica microscope model DM750 P, coupled with a Leica EC3 camera. The digital images were captured from 5 different areas of each cytological spread processed by immunocytochemistry, for the determination of intensity of the immunostaining of the expression of p16^{INK4a}, MCM2/TOPIIα and REST.

Statistic analysis

The statistical analysis was performed using the statistical program STATA v.13.0 (Stat Corporation, College Station, TX, USA). We used the X2 test, student t test for paired data, and ANOVA analysis of variance. To evaluate the relationship between the expression of p16INK4a, MCM2/TOPIIα and REST with the presence of viral HPV16 persistence and the physical state of viral DNA, we used a linear regression model for longitudinal data (panel data) to obtain coefficients and standard error. A value of $p<0.05$ was considered significant.

Results

At the beginning of the study, the average age of women diagnosed with LSIL and HPV16 was 39 years old. After one year of follow-up, the cytological diagnosis of LSIL was maintained in 98% of women, which showed the presence of cellular alterations such as cariomegaly, perinuclear halos, binucleation and hyperchromasia that are cytological features of the coilocytes (Additional file 1: Table S1). Notably, only 2% of women did not present SIL, but incipient non-specific inflammatory changes, which were not associated to any viral type, indicating the clearence of the viral infection (Additional file 2: Fig S1, Table 1). In cases with LSIL after follow-up, the persistence of HPV16 was found in 50% of the women and did not coexist with other types of HPV. Forty-eight percent of the LSIL cases eliminated the HPV16 but showed viral reinfection by other HR-HPV types (17/24 cases, 70%), low risk (3/24, 12%) and non-identified HPV (4/24, 18%) (Table 1).

Table 1. Frequency of persistence of HPV16 after follow-up.

Group n=50	**At the beginning of the study** Presence of HPV16	**After one year of follow-up** Presence of HPV16	**After one year of follow-up** Other types of HPV	**n (%)**
Viral persistence	**+**	**+**	**-**	**25 (50)**
Viral reinfection	**+**	**-**	**+**	**24 (48)**
HR HPV[1]	+	-	+	17 (70)
LR HPV[2]	+	-	+	3 (12)
HPV X[3]	+	-	+	4 (18)
Clearance	**+**	**-**	**-**	**1 (2)**

Viral persistence was defined as positive to the specific type of HPV16 both at the beginning and after the follow-up, while reinfection was negative as to the specific type of HPV16 but other types of HPV positive after the follow-up; and clearance as negative to any type of HPV after follow-up. The viral type was determined through the INNOLiPA test.
(+) Positive
(-) Negative
[1] HR-HPV 18, 31, 33, 35, 39, 45, 51, 52, 56 y 59
[2] LR-HPV 6, 54, 61, 62
[3] HPV X: Viral type not identified by INNOLiPA

Frequency of the physical state of viral DNA after follow-up

After one year of follow-up, frequency of the integrated state increased from 34% to 50%, 17 cases corresponding to HPV16 and 8 cases to reinfection by other HR-HPV (Fig 1A-B). In cases with viral reinfection by low risk and HPV X (non-determined HPV by INNOLiPA), the episomal state was found in 17% and mixed state were in 16%,

respectively (Fig 1B). Notably, the frequency of the integrated state in cases with viral persistence with HPV16 was significantly higher than in cases with viral reinfection (p=0.04). In cases with persistent HPV16, as well as in cases with viral reinfection by other HR-HPV, the integrated state was found in a small number of cells (2 to 4 nuclei) (Fig. 2A-B).

Expression of $p16^{INK4a}$, MCM2/TOPIIα and REST after follow-up

In the LSIL with persistence of HPV16, we found increased expression of p16INK4a and MCM2/TOPIIα, and a diminished expression of REST compared to the expression observed at the beginning of the study. Differences in the expression p16INK4a, MCM2/TOPIIα and REST were 26%, 17%, 15%, respectively, which were statistically significant (p16INK4a and MCM2/TOPIIα, p<0.001 and REST, p=0.001) (Fig 3A). In the presence of the integrated and mixed state we found a significantly high expression of p16INK4a and MCM2/TOPIIα and decreased REST compared to the episomal state (Fig 3B).

Differences in the expression of p16INK4a, MCM2/TOPIIα and REST in cases with LSIL were observed in cells with cariomegaly, both in the persistence of HPV16 and viral reinfection (Fig 4).

The integrated physical state of HPV16 associated with the expression of $p16^{INK4a}$, MCM2/TOPIIα and REST

When evaluating the association of the expression $p16^{INK4a}$, MCM2/TOPIIα and REST in LSIL with persistence of HPV16 compared with cases with viral reinfection, we found association of the high expression of $p16^{INK4a}$ in cases with viral persistence (p=0.02) (Table 2). In addition, we determined the association of the expression $p16^{INK4a}$, MCM2/TOPIIα and REST with physical state in the cases of LSIL with persistence of HPV16, we found that the integrated state of HPV16 was associated with the increase in the expression of $p16^{INK4}$ and MCM2/TOPIIα, and with the decrease in REST (p<0.001, p=0.04 and p=0.04, respectively). The integrated state of HPV16 increases the expression of $p16^{INK4a}$ in 42 au and of MCM2/TOPIIα in 15 au, and decreases the

expression of REST in 6 au, suggesting the early activity of the viral oncoproteins of HPV 16 in the LSIL with viral persistence (Table 2).

Table 2. Association of the integrated state of HPV16 with the expression of $p16^{INK4a}$, MCM2/TOPIIα and REST.

	Expression of $p16^{INK4a}$ (a.u.)				**Expression of MCM2/TOPIIα (a.u.)**				**Expression of REST (a.u.)**			
	***β**	**SE**	**IC 95%**	***P***	***β**	**SE**	**IC 95%**	***p***	***β**	**SE**	**IC 95%**	***p***
Type of infection												
Viral reinfection	Ref.	-	-	-	Ref.	-	-	-	Ref.	-	-	-
Vital persistence	14	8	-2; 29	**0.02**	-4	5	-15; 7	0.7	-10	6	-21; 2	0.1
Physical state of viral DNA												
Episomal	Ref.	-	-	-	Ref.	-	-	-	Ref.	-	-	-
Integrated	42	11	20; 64	**<0.001**	15	8	-2; 31	**0.04**	-6	7	-21; 7	**0.04**
Mixed	33	11	10; 56	**0.004**	11	8	-5; 28	0.1	-0.6	7	-16; 14	0.8

*β coefficients obtained by a linear regression model for longitudinal data (panel data) adjusted for age.
Value of p <0.05 was considered statistically significant.
A.U: Arbitrary units of staining intensity.

Discussion

Despite some cytological characteristics and viral DNA integration in cell genome are related to an increased risk for cancer development, the management of the earliest precursor lesion identified by cervical exfoliative cytology is difficult, in part due to the absence of other predictive biomarkers. Although viral DNA integration is a key event during carcinogenesis, not all cases of LSIL progress to HSIL or ICC [23]. Here we searched association between physical state of HPV16 and the expression of $p16^{INK4a}$, MCM2/TOPIIα and REST in women with viral persistence after one year of follow-up. The persistence of HPV16 (50%) in LSIL observed in this study (Table 1), was lower than that reported by Zhang co-workers. They found the persistence of HPV16 in 72% of the 376 women with CIN 1 after 4 years of follow-up, which had an average age of 48 ± 8 years [24]. Since age is an important factor for the elimination of infection by HR-HPV [25], the average age of women in our study (age 39) could be a reason for the low frequency of viral persistence, due to the fact that elimination of HPV infection can occur in reproductive women [2].

Unlike other studies evaluating only the persistence of HPV, we determined viral DNA integration at the beginning and after one year of follow-up. We found that the integrated state was related to the persistence of HPV16 (68%, p=0.04; Fig 1B). In this respect, Huang and co-workers reported the viral integration of HPV16 in 80% of CIN 1, 90% of CIN II / III and in 94% of cases of cervical carcinoma in stage II- IV, indicating that the integration of the viral DNA of HPV 16 is carried out from the early stage of cervical carcinogenesis and that these early events can play an important role in the malignant transformation [26]. A much more similar study conducted by Gallo and co-

workers showed 53% integration of HPV16 in LSIL, some of these presented dysplasia after two years of follow-up suggesting that integration of viral DNA precede the morphological characteristics leading to malignancy [27]. Based on these facts we hypothetized that the relationship between viral DNA integration and persistence of HPV16 are early key events in cervical carcinogenesis.

Interestingly, we also found that the integrated state of HPV16 in the LSIL with persistence was associated with increased expression of $p16^{INK4a}$ ($p<0.001$) and MCM2/TOPIIα ($p=0.04$), and with decreased REST expression ($p=0.04$). It is demonstrated that viral integration is essential in the deregulation of the cell cycle and apoptosis inhibition, through the overexpression of oncoproteins E6 and E7, which can bind to their cellular targets p53 and pRB, respectively [3], [28], alterating $p16^{INK4a}$ and of MCM2/TOPIIα expression by the activation of cell proliferation [29]. In cases with reinfection by other types of HR-HPV or LR-HPV, no significant change in the expression of these proteins was found after one year of follow-up, possibly due to biochemical properties of E7 protein encoded by HPV16 [8],[30].

Some longitudinal studies have reported that women with CIN 1 who express $p16^{INK4a}$ have a higher risk of progression to precancerous [31]. Cortechia, et. al, reported that 29% of the LSIL with expression of $p16^{INK4a}$, had the highest rate of progression (13%) compared to the 523 LSIL that did not express this protein where only 2% progress after 3 years of follow-up [32]. Similarly, the expression of MCM2 and TOPIIα correlated with the increase in dysplasia and the severity of the lesion [33], [34]. In normal cervical epithelium, MCM2 and TOPIIα are expressed in proliferating cells [35]. In HR-HPV infection, the interaction of oncoproteins E6 and E7 with their cellular targets p53 and

pRB respectively induce an aberrant S phase [3]. Both two proteins have correspondence with the cellular morphological alterations, helping to identify lesions with risk for malignant transformation [36]. Given these relationships, we consider that overexpression of $p16^{INK4a}$ and MCM2/TOPIIα detected by immunohistochemistry in combination with persistence and HPV16 DNA integration can be sensitive markers for the identification for LSIL progression.

Besides, although the mechanism by which HR-HPV could deregulate the expression of REST in LSIL is unknown, we found a significant decrease in nuclear expression after of the year of follow-up in the cases with viral persistence. The decreased expression of REST was only associated with the integrated state of HPV16, thus suggesting that the action of the oncoproteins E6 and E7 may be involved in the control of the REST degradation. Supporting this hypothesis, studies reported that the regulation of REST protein levels is determined by the ubiquitination and deubiquitination system. Deubiquitination controls the levels of REST to direct the determination of cell fate during neurogenesis [18], [37]. The E6 oncoprotein may be playing an important role in the decreased expression of REST, since this oncoprotein manipulates the ubiquitin-proteasome pathway to promote the degradation of proteins [38]. It is important to investigate the role of REST in the earliest lesions of cervical cancer because its role in other epithelial cancers induces a neuroendocrine phenotype and contribute to tumor development and progression.

Conclusion

In conclusion, we showed that the early integration of HPV16 in LSIL during one year of follow-up increases the expression of $p16^{INK4a}$ and MCM2/TOPIIα and diminishes the expression of REST, which could be associated with the risk of progression. However, further analyses increasing the number of samples and the time of follow-up are necessary. It is also necessary to investigate whether REST targets contribute to phenotype transformation.

Additional material

Additional file 1: Table S1. General characteristics of the study subjects.
Additional file 2: Fig S1 Cervical cytology without SIL and LSIL after one year of follow-up.

Abbreviations

ICC: Invasive cervical carcinoma. LSIL: Low-grade squamous intraepithelial lesion. HSIL: High-grade squamous intraepithelial lesion. HR-HPV: High-risk human papillomavirus. LR-HPV: Low-risk human papillomavirus. MCM2: Minichromoseome maintenaince protein 2. REST: RE1-Silencing Transcription factor. ISH: In situ hybridization.

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Availability of data and materials

All data generated or analysed during this study are included in the published article and its supplementary information files.

Author contributions

WACM, LCAR, MALV and BIA: conceptualization and design of experiments for research. WACM and LCAR: conducting experiments. WACM, EFA, LCAR, MIZG and OMH: analysis of data. LCAR, BIA, MALV, ALPS and COP: contribution of reagents, materials and analysis tools. WACM, LCAR, EFA, MIZG and COP: wrote the paper. WACM, COP, ALPS, OMH and LCAR: critical revision of the article.

Competing interests

The authors declare that they have no competing interests.

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Figures legends

Fig 1. Physical state of HPV after follow-up. A) Cases with episomal state at the beginning of the study showed changes in viral DNA state, while cases with integrated and mixed states were maintained more frequently after follow-up. B) After the year of follow-up, in cases with viral persistence the integrated state is observed more frequently and in cases with viral reinfection the mixed state. * The category with clearance was excluded, since after the follow-up it was negative to HPV and the physical state was not determined.

Fig 2. Representative cases of the physical state integrated of HPV16 and HPV31 in LSIL after follow-up. The integrated state of HPV16 in a case with viral persistence (A) and of HPV31 in case with viral reinfection (B), shows the punctate pattern (red arrow) and negative nuclei (black arrow). In situ hybridization with tyramide amplification. 40X objective.

Fig 3. Expression of $p16^{INK4a}$, MCM2/TOPIIα and REST during follow-up evaluated by immunocytochemistry. A) After one year of follow-up, the expression of $p16^{INK4a}$ and MCM2/TOPIIα was significantly higher and that of REST significantly lower in cases with viral persistence compared to the expression of these proteins at the beginning of the study. B) The expression of $p16^{INK4a}$ and MCM2/TOPIIα was significantly higher and that of REST significantly lower in the cases with integrated and mixed state than in the cases with episomal status after one year of follow-up. AU: Arbitrary units of staining intensity. Levels of statistical significance in A) *p<0.001, **p<0.001, ***p=0.001, ****p=0.002, *****p=0.04 and ******p=0.001; and B) *p<0.001, **p<0.001, ***p=0.01, ****p=0.03, *****p=0.05 and ******p=0.03. NS, not significant. *P* value obtained through the student's t test for paired data and ANOVA analysis of variance.

Fig 4. Expression of $p16^{INK4a}$, MCM2/TOPIIα and REST in LSIL with persistence and viral reinfection after follow-up. In cases with persistent viral infection, a greater

intensity of nuclear/cytoplasmic p16INK4a expression is observed in cells with karyomegaly (A), whereas increased nuclear expression of MCM2/TOPIIα is observed in cells with karyomegaly and normal cells (C). Lower intensity of nuclear/cytoplamic REST in cells with karyomegaly (E). Compared expression of cases with viral reinfection by HPV31 (B, D and F). 40X objective.

Figures

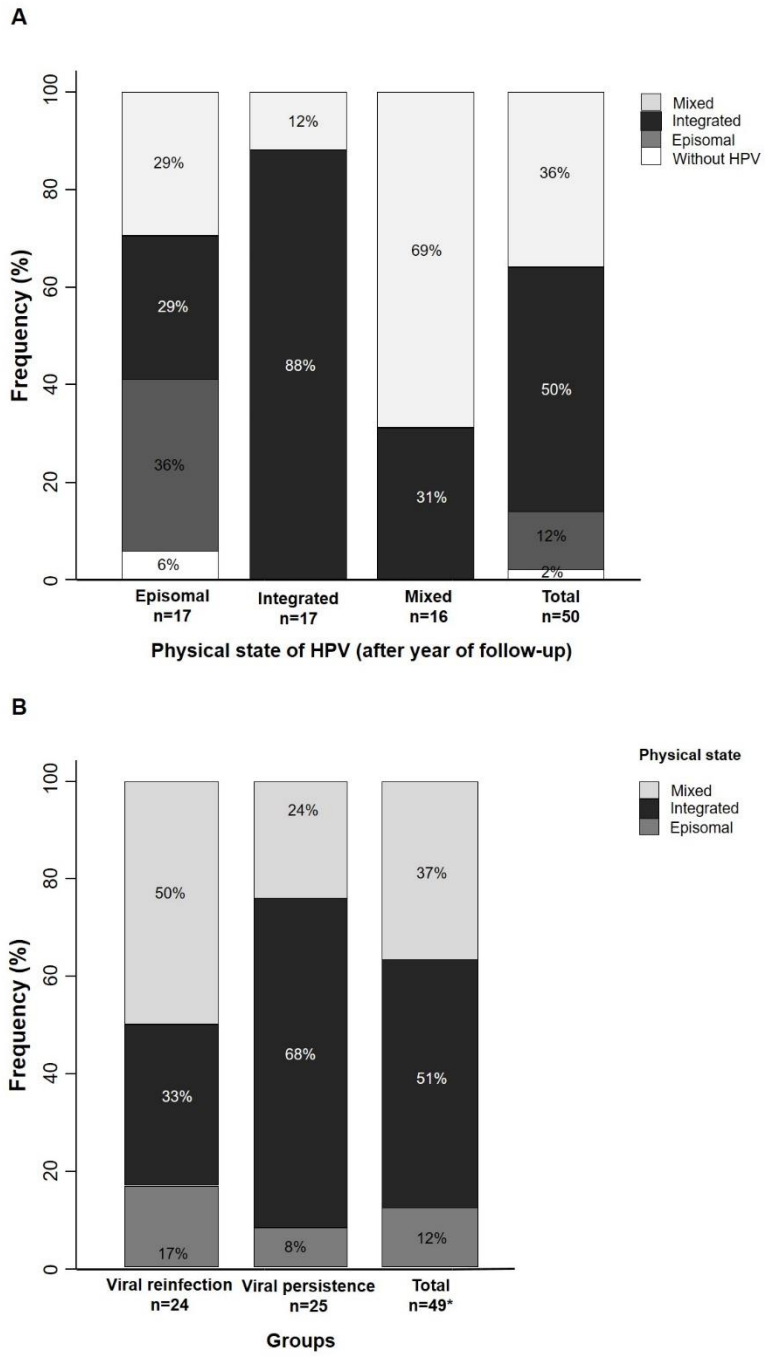

Fig 1

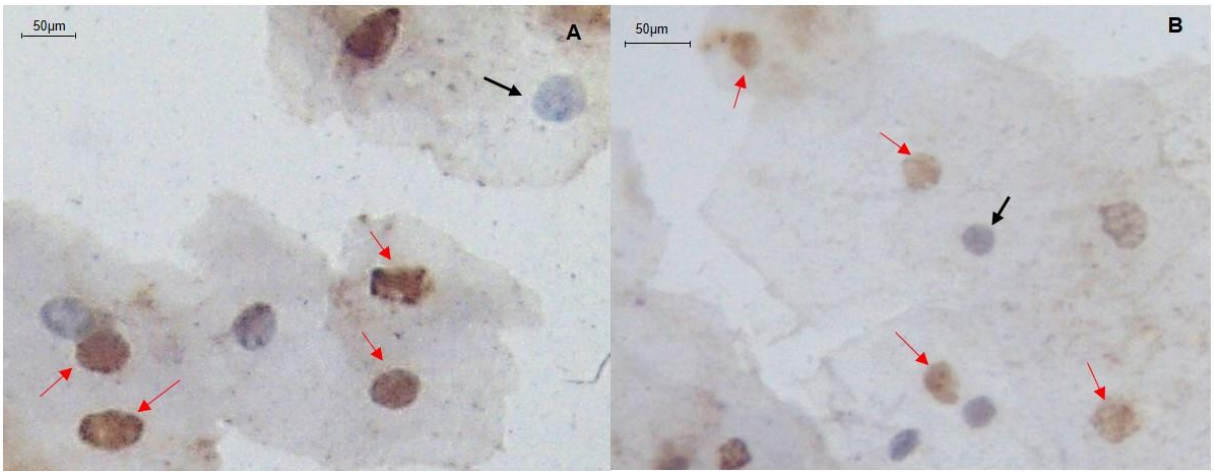

Fig 2

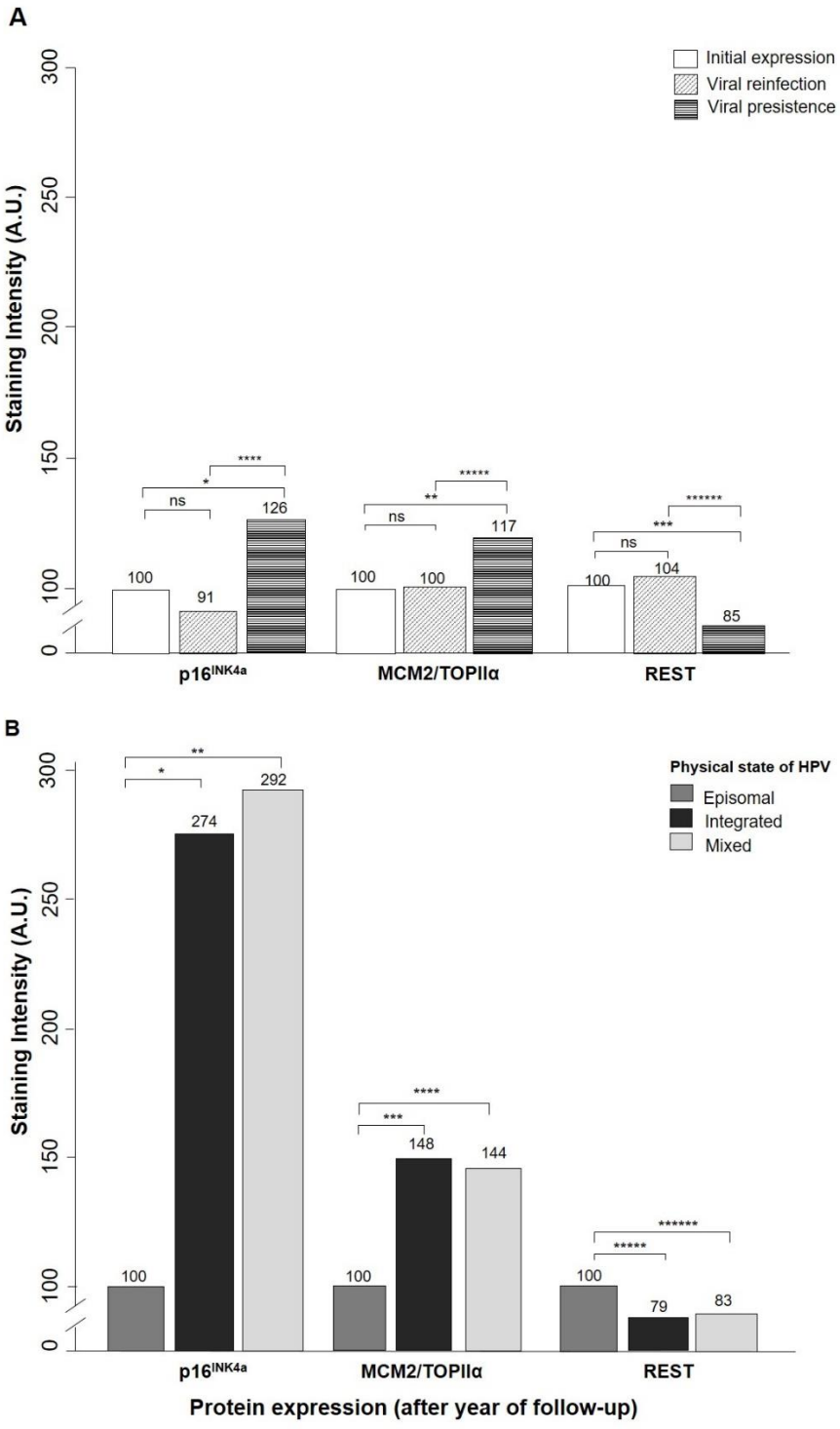
A
Initial expression
Viral reinfection
Viral presistence
Staining Intensity (A.U.)
0
100
150
200
250
300
****
*
ns
126
100
91
*****
**
ns
117
100
100
******
***
ns
104
100
85
p16INK4a
MCM2/TOPIIα
REST
B
Physical state of HPV
Episomal
Integrated
Mixed
Staining Intensity (A.U.)
**
*
292
274
100
****
***
148
144
100
******
*****
100
79
83
p16INK4a
MCM2/TOPIIα
REST
Protein expression (after year of follow-up)

Fig 3

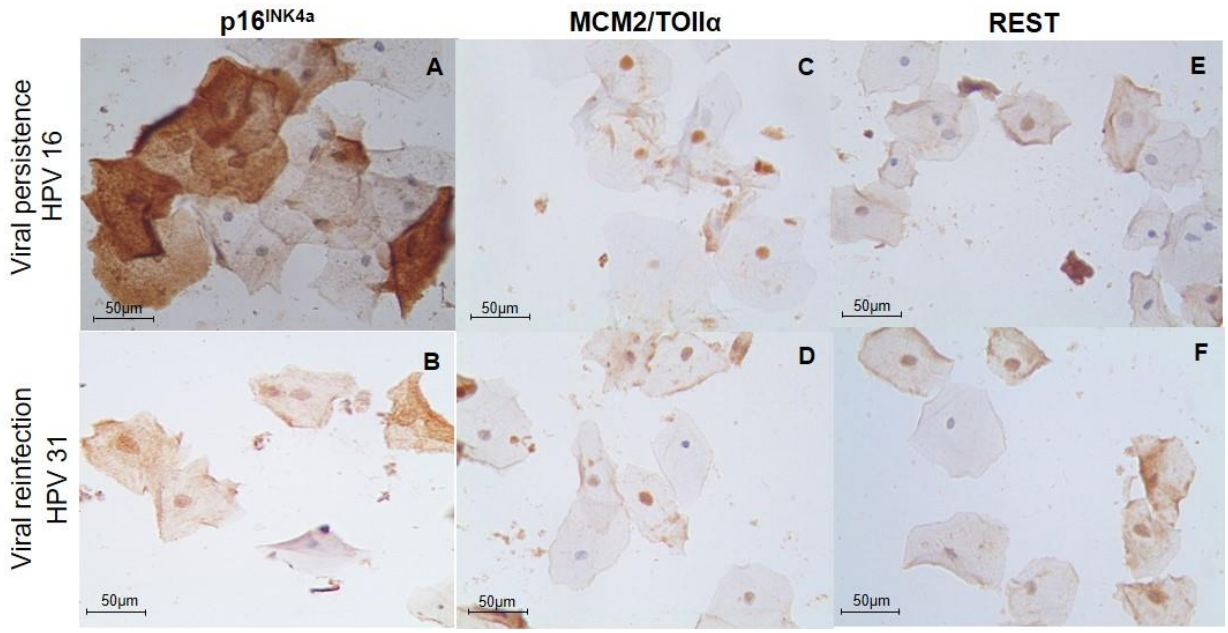
p16INK4a
MCM2/TOIIα
REST
Viral persistence HPV 16
Viral reinfection HPV 31
A
B
C
D
E
F
50µm

Fig 4

Additional file

Table S1. General characteristics of the study subjects.

Characteristic	1 year follow-up n=50*
Age (years)	39 ±10
Parity (%)	
None or 1	10 (20)
≥ 2	40 (80)
No. of sexual partners (%)	
1	21 (42)
2-3	19 (38)
≥4	10 (20)
Condom use (%)	
No	39 (78)
Yes	11 (22)
Use of hormonal contraceptives (%)	
No	24 (48)
Yes	26 (52)
Smoke (%)	
No	36 (72)
Yes	14 (28)
Alcohol consumption (%)	
No	17 (34)
Yes	33 (66)
Cytological diagnosis (1 year follow-up) (%)	
LSIL	49 (98)
Without SIL	1 (2)

Without SIL, no squamous intraepithelial lesion. LSIL, low-grade squamous intraepithelial lesion.* The case in which an clearance was found after a year of follow-up is not included.

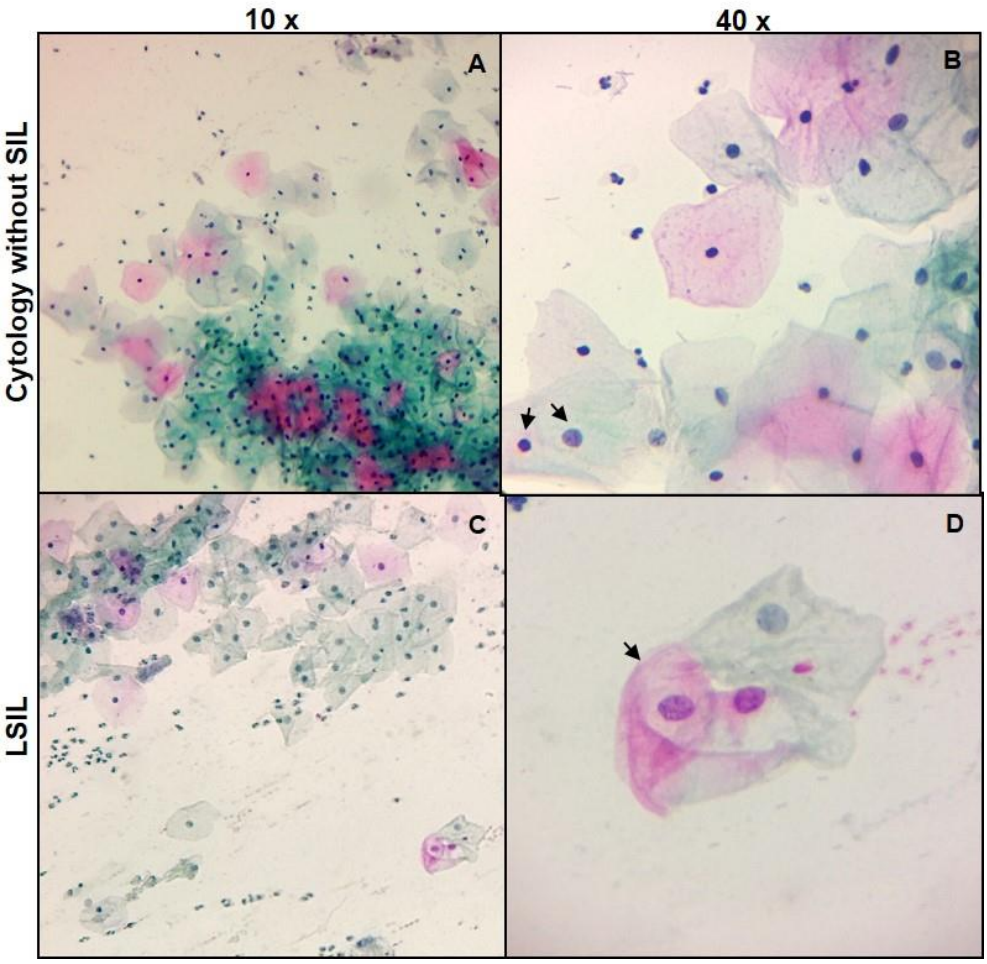

Fig S1 Cervical cytology without SIL and LSIL after one year of follow-up. Normal cells with discreet inflammatory atypical changes (black arrow) without SIL (A, B). In the LSIL, a coilocyte (black arrow) is observed, a cervical cell with karyomegaly compatible with the lesion (C, D). Papanicolaou stain. 10x and 40x objective.