UNIVERSIDAD AUTÓNOMA DE GUERRERO

UNIDAD ACADÉMICA DE CIENCIAS QUÍMICO BIOLÓGICAS

DOCTORADO EN CIENCIAS BIOMÉDICAS

EXPRESIÓN DEL MICRORNA-16-1 Y CCNE1 Y SU RELACIÓN CON LA INTEGRACIÓN DE LOS VPH-AR EN LA CARCINOGÉNESIS CERVICAL

T E S I S

QUE PARA OBTENER EL GRADO DE

DOCTORADO EN CIENCIAS BIOMÉDICAS

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UNIVERSIDAD AUTÓNOMA DE GUERRERO
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En la ciudad de Chilpancingo, Guerrero, siendo los 09 días del mes de julio del dos mil quince, se reunieron los miembros del Comité Tutoral designado por la Academia de Posgrado del Doctorado en Ciencias Biomédicas, para examinar la tesis titulada **"Expresión del microRNA-16-1 y CCNE1 y su relación con la integración de los VPH-AR en la carcinogénesis cervical"**, presentada por la alumna Ma. Isabel Zubillaga Guerrero, para obtener el Grado de Doctora 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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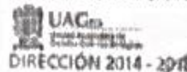

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Este trabajo se realizó en el Laboratorio de Investigación en Citopatología e Histoquímica en la Unidad Académica de Ciencias Químico-Biológicas de la Universidad Autónoma de Guerrero, en la ciudad de Chilpancingo, Guerrero, México.

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RESUMEN

Introducción. La integración de los VPH-AR altera la expresión de oncogenes como Ciclina E1 (CCNE1). El incremento en la expresión de E6 y E7 de los VPH-AR se ha encontrado relacionado con la expresión incrementada del microRNA-16-1 (miR-16-1) en carcinoma cervical (CC). En otros tipos de cáncer CCNE1 es blanco del miR-16-1, pero en CC esto no ha sido reportado. **Objetivos:** Evaluar la participación de miR-16-1 en la expresión del RNAm CCNE1 en cultivos de células C33A (VPH-), SiHa (VPH16), CaSki (VPH-16) y HeLa (VPH-18) tratadas con un plásmido de expresión de siRNAs y determinar el patrón de expresión de Ciclina E1 en lesiones escamosas intraepiteliales (LEI) de bajo (LEIBG) y alto grado (LEIAG)y CC relacionarlo con la integración de los VPH-AR. **Materiales y Métodos:** Experimentalmente se diseñaron y generaron 3 siRNAs para miR-16-1 quefueron transfectados en células C33A, SiHa, CaSki y HeLa con X-tremeGENE 9 DNA por triplicado, posteriormente se extrajo el ARNpara realizar los ensayos de RT-PCR en tiempo real para el gen CCNE1 y miR-16-1. Por otra parte, se realizaron tomas de muestra de la zona escamocilíndrica del cérvix uterino, a 200 mujeres incluidas en el estudio. Se realizó la tinción de Papanicolaou y diagnóstico citológico. También se realizó citología en base liquida (LiquiPREP) para realizar la técnica de Inmunocitoquímica para Ciclina E1 e Hibridación *in situ* con amplificación con tiramida para determinar el estado físico de los VPH-AR. La tipificación de los VPH-AR fue mediante INNO-LiPA. **Resultados:** Se validaron por secuenciación los 3 plásmidos generados (pSIMIR-16-3P, pSIMIR-16-5P y pSIMIR-16-5PP). Se encontró que el plásmido pSIMIR16-5P inhibe la expresión endógena de miR-16-1 y al silenciar esta expresión observamos disminución en la expresión del RNAm de CCNE1 a las 12 hrs de transfección en las células VPH+. Por otra parte diagnosticaron 48 citologías sin LEI, Sin VPH, 32 Sin LEI con VPH-AR, 67 LEIBG con VPH-AR, 23 LEIAG y 30 CC ambas con VPH-AR, encontrando queel 75% de citologías sin LEI y el 55.2% de casos de LEIBG ambos grupos con VPH-AR, presentaron el patrón mixto, mientras que el 60.8% de las LEIAG y el 100% de los casos de CC presentaron el patrón integrado de los VPH-AR, mientras que las citologías sin LEI y sin VPH, no expresaron Ciclina E1. El 100% de las citologías

positivas a VPH-AR expresaron Ciclina E1 a nivel nuclear, observándose un incremento en el porcentaje de núcleos positivos eintensidad de la inmunotinción de acuerdo al grado de LEI y los casos de CC. **Conclusiones:** La expresión de Ciclina E1 se relacionó con el grado de LEI y conla integración de los VPH-AR sólo o con infección múltiple ($p<0.001$), estos resultados sugieren que Ciclina E1 puedeser un biomarcadorútil en la detección de LEI con VPH-AR integrado. Además, se encontró que CCNE1 es blanco de miR-16-1 a nivel posttranscripcional, lo cual sugiere que en CC miR-16-1 podría tener un papel oncogénico, representando así un posible blanco terapéutico en CC.

Abstract

Introduction. Integration of HR-HPV alters the expression of oncogenes such as Cyclin E1 (CCNE1). The increased expression of E6 and E7 of HR-HPV has been found associated with increased expression of microRNA-16-1 (miR-16-1) in cervical carcinoma (CC). In other cancers CCNE1 is target of miR-16-1 but in CC this has not been reported. **Objectives:** 1) To assess the involvement of miR-16-1 CCNE1 mRNA expression in cultured cells C33A (HPV-), SiHa (HPV 16), CaSki (HPV-16) and HeLa (HPV-18) treated with an expression plasmid siRNAs and determine the pattern of expression of cyclin E1 squamous intraepithelial lesions (SIL) low (LSIL) and high grade (HSIL) and CC relate to the integration of HR-HPV. **Materials and Methods:** In the experimental part, of this work we designed and generated three siRNAs to miR-16-1, they were transfected into C33A, SiHa, CaSki and HeLa cells with X-tremeGENE 9 DNA by triplicate, then RNA was extracted for the RT-PCR in real time for CCNE1 and miR-16-1 gene. Moreover, samplings of the squamocolumnar area of the uterine cervix, 200 women included in the study were performed. Papanicolaou staining and cytological diagnosis was made. Cytology was also performed on liquid based (LiquiPREP) for immunocytochemistry for cyclin E1 and *in situ* hybridization tyramide amplification to determine the state physical of the HR-HPV. The classification of HR-HPV was by INNO-LiPA. **Results:** 3 validated sequencing plasmids generated (pSIMIR-16-3P, pSIMIR-16-5P and pSIMIR-16-5PP). It was found that the plasmid pSIMIR16-5P inhibits expression of endogenous miR-16-1 and to silence the expression observed decrease in mRNA CCNE1expression at 12 hrs transfection in HPV+ cell. Moreover, diagnosed 48 smears without SIL, without HPV; 32 without SIL with HR-HPV; 67 LSIL with HR-HPV, 23 HSIL HR-HPV and 30 CC with HR-HPV, finding that 75% of smears without SIL and 55.2% of LSIL cases of both groups with HR-HPV, showed mixed pattern, while 60.8% of HSIL and 100% of CC cases showed the integrated pattern HR-HPV, while without SIL smears and without HPV not expressed Cyclin E1. 100% of positive cytology HR-HPV expressed cyclin E1 at the nuclear level, with an increase in the percentage of positive nuclei and intensity of immunostaining according to the degree of SIL and CC. **Conclusions**: The expression of Cyclin E1 related to the degree of SIL and

integrating with HR-HPV alone or with multiple infection ($p<0.001$), these results suggest that cyclin E1 can be a useful biomarker for detecting SIL with integrated HR-HPV. In addition, it was found that CCNE1 is target of miR-16-1 at post-transcriptional level, suggesting that miR-16-1 in CC could have an oncogenic role, representing animportant therapeutic target in CC.

INTRODUCCIÓN

La causa subyacente primaria del carcinoma cervical invasor (CCI) es la infección por uno o varios tipos de virus del papiloma humano de alto riesgo oncogénico (VPH-AR). En 11 estudios caso control desarrollados en 9 países coordinados por la agencia internacional de investigación en cáncer identificó al DNA del VPH en el 96.6% de los casos de CCI y en 15.6% de las pacientes control (Moscicki *et al*., 2012). Estudios epidemiológicos sugieren que al menos 14 tipos de VPH-AR están asociados con la progresión a CCI (de Sanjose *et al*., 2010). La mayoría de estos está filogenéticamente relacionados a los tipos VPH-16 (31, 33, 35, 52 y 58) o VPH-18 (39, 45, 59 y 68) (Zuna *et al*., 2011). La mayoría de las infecciones por VPH ceden espontáneamente, sin embargo si la infección es persistente, pueden desarrollarse lesiones premalignas conocidas citológicamente como lesiones escamosas intraepiteliales (LEI) o Neoplasias intraepiteliales cervicales (NIC) y las LEI sobre todo las de alto grado, sin un tratamiento adecuado, pueden transformarse en CCI. En el Estado de Guerrero, México, este tipo de cáncer es un problema de salud pública y se ha reportado una prevalencia del 68.1% del VPH-16 en los casos de CCI, además de que el 27.4% y 10.6% de las LEI de alto (LEIAG) y bajo grado (LEIBG) respectivamente, presentan este tipo viral (Illades-Aguiar B *et al*., 2010).

La infección persistente de los VPH-AR es un factor importante que favorece el desarrollo de CCI. Los genes E6 y E7 del VPH-16 son capaces de inactivar a los genes supresores de tumores (p53 y pRB respectivamente) e inducir la sobreexpresión de oncogenes (Chen *et al*., 2014). La incapacidad para discriminar entre una infección persistente o transitoria ha llevado a la necesidad de buscar nuevos parámetros adicionales que permitan la identificación de mujeres positivas a

VPH-AR que tengan mayor riesgo de desarrollar una LEIAG. En este contexto, diversos estudios han investigado el valor de la integración del DNA del VPH-16 como posible marcador de diagnóstico de las LEI (Ramanakumar*et al.*, 2010, Guan *et al.*, 2012). La integración de los VPH-AR además de modificar los patrones de transcripción importantes para la sobre regulación de la expresión de los oncogenes virales (E6 y E7), también sobre regula la expresión de genes del huésped en sitios vulnerables de integración (sitios frágiles), muchos de estos genes con función oncogénica como los oncomirs (Dal *et al.,* 2008; Schmitz *et al.*, 2012).

Los microRNAs (miRNAs) son pequeñas moléculas de RNA, endógenas, de cadena sencilla, no codificantes, que pueden regular la proliferación celular, diferenciación y apoptosis a través de la unión con genes blanco. Diversos miRNAs se han reportado implicados en la carcinogénesis. El incremento en la expresión de miR-16-1 ha sido observada en líneas celulares HeLa y casos de NIC II y NIC III positivos a VPH-AR (Wang *et al.*, 2014) y se ha descrito que este incremento puede estar relacionado con el desarrollo de CCI (Zheng*et al.*, 2011). Se ha reportado que Ciclina E1 (CCNE1) es blanco de miR-16-1 (Wang *et al.*, 2009). Al respecto hemos reportado en Mujeres del estado de Guerrero, México que el 40% de las muestras citológicas negativas a LEI más VPH-AR y el 100% de los casos de LEIBG con VPH-AR expresan Ciclina E. Estos resultados se relacionaron con el estado físico de los VPH-AR encontrando que las citologías sin LEI más VPH-AR presentaron en un 23.3% el patrón integrado y las LEIBG el patrón fue mixto (90%) y 10% integrado, lo cual se sugiere que en las LEIBG la expresión de Ciclina E ocurre predominantemente en presencia del estado mixto que incluye copias integradas de los VPH-AR, por lo que es importante seguir investigando esta relación para seguir contribuyendo en el entendimiento de la patogénesis cervical y búsqueda de biomarcadores útiles en el diagnóstico temprano del CCI (Zubillaga-Guerrero *et al.*, 2013) **Anexo 1**.

Se conoce que CCNE1, es una Ciclina esencial en la activación de CDK2, además regula la transición de la fase G1-S de células mamíferas en división celular (Sauer K y Lehner CF., 2005). En diversos tipos de cáncer CCNE1 se ha reportado incrementada y se ha sugerido que uno de los mecanismos de regulación de la

expresión de CCNE1 podría ser a nivel posttranscripcional mediante miRNAs como el miR-16-1 (Ofir *et al.*, 2011), sin embargo esto no es claro en el CC. Por lo que los objetivos de este trabajo fueron evaluar la participación de miR-16-1 en la expresión del RNAm del gen CCNE1 en cultivos de células C33A (VPH-), SiHa (VPH16), CaSki (VPH-16) y HeLa (VPH-18) tratadas con un plásmido de expresión de siRNAs y además, nos pareció importante determinar el patrón de expresión de Ciclina E1 en la carcinogénesis cervical incluyendo las lesiones premalignas y malignas del cérvix uterino y su relación con la integración de los VPH-AR, para seguir contribuyendo en el entendimiento de la patogénesis cervical y la búsqueda de biomarcadores celulares, que permitan ayudar en el diagnóstico y predicción de mujeres con riesgo de progresar a un CCI.

Para abordar estos planteamientos, el trabajo se ha dividido en dos capítulos que se describen a continuación:

CAPÍTULO I

El objetivo de este capítulo fue: Evaluar la participación de miR-16-1 en la expresión del RNAm del gen CCNE1 en cultivos de células C33A (VPH-), SiHa (VPH16), CaSki (VPH16) y HeLa (VPH18) tratadas con un plásmido de expresión de siRNAs capaz de silenciar la expresión de este miRNA.

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MICRORNA MIR-16-1 REGULATES CCNE1 (CYCLIN E1) GENE EXPRESSION IN HUMAN CERVICAL CANCER CELLS

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RUNNING HEAD: Target CCNE1 gene of miR-16-1 in cervical cancer cells.

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Manuscript Type:	Brief Communication
Abstract	MicroRNAs are involved in diverse biological processes through regulation of gene expression. The microRNA profile has been shown to be altered in cervical cancer (CC). MiR-16-1 belongs to the mi R-16 cluster and has been implicated in various aspects of carcinogenesis including cell prolifera tion and regulation of apoptosis; however, its function and molecular mechanism in CC is not clea r. Cyclin E1 (CCNE1) is a positive regulator of the cell cycle that controls the transition of cel ls from G1 to S phase. In CC, CCNE1 expression is frequently upregulated, and is an indicator for poor outcome in squamous cell carcinomas (SCCs). Thus, in the present brief communication, we dete rmine whether the CCNE1 gene is regulated by miR-16-1 in CC cells. To identify the downstream cell ular target genes for upstream miR-16-1, we silenced endogenous miR-16-1 expression in cell lines derived from CC (C33A HPV-, CaSki HPV16+, SiHa HPV16+, and HeLa HPV18+ cells), using siRNAs expres sed in plasmids. Using a combined bioinformatic analysis and RT-qPCR, we determined that the CCNE1 gene is targeted by miR-16-1 in CC cells. SiHa, CaSki, and HeLa cells demonstrated an inverse corr elation between miR-16-1 expression and CCNE1 mRNA level. Thus, miR-16-1 post-transcriptionally do wn-regulates CCNE1 gene expression. These results, suggest that miR-16-1 plays a vital role in mod ulating cell cycle processes in CC.
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ABSTRACT

MicroRNAs are involved in diverse biological processes through regulation of gene expression. The microRNA profile has been shown to be altered in cervical cancer (CC). MiR-16-1 belongs to the miR-16 cluster and has been implicated in various aspects of carcinogenesis including cell proliferation and regulation of apoptosis; however, its function and molecular mechanism in CC is not clear. Cyclin E1 (CCNE1) is a positive regulator of the cell cycle that controls the transition of cells from G1 to S phase. In CC, CCNE1 expression is frequently up regulated, and is an indicator for poor outcome in squamous cell carcinomas (SCCs). The mechanism leading to its dysregulation remains incompletely defined. Thus, in the present brief communication, we determine whether the CCNE1 gene is regulated by miR-16-1 in CC cells. To identify the downstream cellular target genes for upstream miR-16-1, we silenced endogenous miR-16-1 expression in cell lines derived from CC (C33A HPV-, CaSki HPV16+, SiHa HPV16+, and HeLa HPV18+ cells), using siRNAs expressed in plasmids. Using a combined bioinformatic analysis and RT-qPCR, we determined that the CCNE1 gene is targeted by miR-16-1 in CC cells. SiHa, CaSki, and HeLa cells demonstrated an inverse correlation between miR-16-1 expression and CCNE1 mRNA level, while CCNE1 expression in C33A cells was not affected. Thus, miR-16-1 post-transcriptionally down-regulates CCNE1 gene expression. These results suggest that miR-16-1 plays a vital role in modulating cell cycle processes in CC.

Key words: Cervical cancer, CCNE1, HPV, microRNAs, miR-16-1, siRNAs.

INTRODUCTION

MicroRNAs have an important role in the regulation of cellular differentiation, proliferation and apoptosis **[1]**. Furthermore, some microRNAs are considered to be oncogenes or tumor suppressor genes and have altered expression profiles in several tumors **[2]**. The tumor suppressor microRNAs miR-15a and miR-16-1 are expressed as a microRNA cluster from an intron region of the DLEU2 (Deleted in Lymphocytic Leukemia 2) transcript and influence cell proliferation, survival, and invasion. Also has been reported that higher levels of miR-15a and miR16-1 expression in cervical cancer (CC) tissues compared to normal cervical tissues; however, the over expression of this microRNA cluster does not appear to affect growth of CC cells **[3, 4]**. In addition, miR-16-1 has been shown to play a role in cell cycle regulation in tumor cell lines by targeting CDK6 **[5, 6]**, and CDC7 **[3]**. Recent data have shown that miR-16-1 regulates CCNE1 (cyclin E1) expression in HeLa, HEK293, MCF-7 and A549 cells **[7]** through the 3'-UTR regulatory region of the CCNE1 gene. Interestingly, apparent discrepancies have been reported between the known functions of miR-16-1 as a cell cycle inhibitor and its prognostic value in certain cancers, reflecting the complex role of miR-16-1 in tumorigenesis process. This complexity can be explained by its function like as tumor suppressor gene or its function like as oncomir, as well as by the cell-type specificity of them miR-16-1 over expression effects on cell cycle arrest and apoptosis regulation **[3, 8]**.

An important component of the genetic regulatory network of the cell cycle is the CCNE1 gene, which codes for an essential cyclin activating CDK2, which regulates the G1-S phase transition of normal mammalian cell division cycles **[9]**. CCNE1 levels begin to rise in mid G1, peak during late G1, and drop off around the G1/S transition. Thus, the timing of its expression plays a direct role in initiation of DNA replication as well as chromatin remodeling during tumorigenesis **[10, 11]**. Different studies suggest that CCNE1 is expressed at significantly higher than physiologic levels in many types of human tumors. However, there is evidence indicating that CCNE1 expression is uncoupled from cell cycle progression **[12, 13]**.

Better understanding of the uncoupling of CCNE1 expression from cell cycle control is critical to understanding the development of cancer and elucidation of the CCNE1 regulatory mechanism could explain in part such dysregulation. The expression pattern of CCNE1 in biopsy samples of cervical carcinoma at different stages has been studied and the data suggest that the pattern of expression is an indicator for poor outcome in CC **[14]**. In addition, the miR-15a/16-1 cluster expression has been reported to be up regulated in High-grade intraepithelial lesions (Cervical intraepithelial neoplasia (CIN 2-3)) as well as in CC cell lines in comparison with normal tissues **[15-18]**.

The over expression of CCNE1 and miR-16-1 has been shown to have effects on cell cycle control and these molecules may play a role in cervical carcinogenesis. Thus, analysis of the regulatory molecular network involving miR-16-1 and CCNE1 represents a relevant area of study in CC. To understand the molecular mechanism of CCNE1 gene expression mediated by miR-16-1 and test its trans-regulation abilities, we investigated the effect of miR-16-1 regulation on CCNE1 gene expression in cell lines derived from CC. With a bioinformatics approach, we identified four microRNA response elements (MREs) to miR-16-1 in CCNE1 3'-UTR regulatory region. Thus, we decided to evaluate whether expression of the CCNE1 gene can be regulated by miR-16-1. The cell lines C33A (HPV-), SiHa (HPV16+), CaSki (HPV16+) and HeLa (HPV18+); were used as CC models to investigate whether silencing of miR-16-1 has an effect on CCNE1 gene expression. Toward this end, we generated siRNA expression plasmids for miR-16-1, which have nucleotide complementarity to the gene coding for pre-miR-16-1. We observed that silencing of miR-16-1 induced down regulation of CCNE1 gene expression in SiHa (17%), CaSki (46%), and HeLa (58%) cells, while its expression in C33A cells was not affected (22%). Silencing of miR-16-1 also induced inhibition of cell proliferation. These data suggest that CCNE1 may be targeted by miR-16-1 in CC cells.

MATERIALS AND METHODS

CELL LINES AND CULTURE CONDITIONS

Cell lines derived from CC (C33A HPV-, SiHa HPV16+, CaSki HPV16+, HeLa HPV18+ cells) were obtained from the American Type Culture Collection (ATCC) and were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin (50 µg/ml), 2 mM L-glutamine, 250 ng/ml fungizone, and maintained at 37°C in 5% CO_2. The cells were used in transfection assays and total RNA isolation was carried out with TriPure isolation reagent (Roche, Indianapolis, IN) for the RT-qPCR assays.

SIRNA EXPRESSION PLASMIDS FOR HUMAN MICRORNA MIR-16-1

DNA inserts of siRNAs specific for human microRNA hsa-miR-16-1 were designed using siRNA at Whitehead software (Applied Biosystems, Foster, CA) **[19]** and a genomic fragment spanning the miR-16-1 locus from human chromosome 13 was cloned in *Apa I* and *Eco RI* restriction sites in the pSilencer1.0-U6 siRNA expression plasmid (Applied Biosystems, Foster, CA), which contains the U6 RNA Pol-III promoter to generate small RNA transcripts, to generate the pSIMIR16-5P plasmid. The DNA insert was generated using the sense 5´-GCC-TTA-GCA-GCA-CGT-AAA-TAT-TGT-TCA-AGA-GAC-AAT-ATT-TAC-GTG-CTG-CTA-AGG-CTT-TTT-T-3´ and antisense 5´-AAT-TAA-AAA-AGC-CTT-AGC-AGC-ACG-TAA-ATA-TTG-TCT-CTT-GAA-CAA-TAT-TTA-CGT-GCT-GCT-AAG-GCG-GCC-3' primers. The primers were aligned using annealing buffer (300 mM HEPES pH 7.4, 100 mM potassium acetate, 2 mM magnesium acetate) at ratio of 100 molar and were incubated at 95°C for 5 min and 37°C for 1 hour. To decrease the probability of homology sequences with other human genes, the siRNAs-encoding sequences were analyzed by Blast. The plasmids were isolated for PureYield plasmid midiprep system (Promega, Madison, WI) and integrity was verified by DNA sequencingin Genetic Analyzer 3500xl equipment (Applied Biosystems, Foster, CA) (supplementary

information). The miRBase reference sequence of GenBank to the sequence of hsa-miR-16-1 is: MI0000070.

TRANSFECTION ASSAYS WITH SIRNA EXPRESSION PLASMIDS

C33A, SiHa, CaSki, and HeLa cells were transiently transfected with the pSIMIR16-5P plasmid to silence the miR-16-1, using X-TremeGENE 9 DNA transfection reagent (Roche, Indianapolis, IN) according to the manufacturer's instructions. Briefly, one day before the transfection assay, the cells were plated at a density of $1X10^5$ cells per well in a six-well plate containing 2 ml of DMEM with 10% FBS and penicillin/streptomycin. At the time of transfection, the plasmids and X-TremeGENE 9 DNA reagent were diluted in DMEM and incubated for 20 min at room temperature. The plasmid DNA concentration and X-TremeGENE 9 DNA reagent were normalized and all assays were carried out with 3µg of plasmids. Cells were incubated with plasmids and X-TremeGENE 9 DNA reagent for 4 hours, rinsed and replenished with DMEM containing 10% FBS. Twelve hours after transfection, cells were harvested and RNA isolation was carried out for quantitative real-time RT-PCR assays. Transfection assays were carried out on three separate occasions; each time with three replicates.

RNA ISOLATION AND RT-qPCR ASSAYS

Total RNA was extracted from the cultured cells using TriPure isolation reagent (Roche, Indianapolis, IN) according to the manufacturer's protocol. RT-qPCR was performed to confirm the expression level of mRNAs. cDNA was produced with random primers and reverse transcription was carried out according to the protocol of TaqMan microRNA reverse transcription kit (Life Technologies, Foster City, CA), and qPCR was performed as described in the method TaqMan microRNA assays (Life Technologies, Foster City, CA: 4366596) with ViiA7 Real-Time PCR systems supplied with analytical software. The PCR reaction was conducted at 95°C for 10 min followed by 40 cycles of 95°C for 10sec and 60°C for 60sec. Specific primers to hsa-miR-16-1 (Life Technologies, Foster City, CA: 4427975) were used and RNU44 (Life

Technologies, Foster City, CA: 4427975) mRNA levels were used for normalization. The CCNE1 mRNA expression level was evaluated with the TaqMan one-step RT-PCR master mix reagent kit (Life Technologies, Foster City, CA: 4309169). The PCR reaction was conducted at 48°C for 30 min followed by an AmpliTaqgold activation at 95°C for 10 min followed of 40 cycles of 95°C for 15sec and 60°C for 1 min. Specific primers to CCNE1 (Life Technologies, Foster City, CA: 4331182) and GAPDH (Life Technologies, Foster City, CA: 402869) mRNA levels were used for normalization. The comparative threshold cycle (Ct) method was used to evaluate the relative abundance of miR-16 compared with RNU44 and CCNE1 compared with GAPDH expression. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to the expression of RNU44. The reaction was incubated in a 384 well plate in ViiA7 Real-Time PCR systems supplied with analytical software. All experimental treatments were carried out on three separate occasions; each time with three replicates.The NCBI reference sequence of GenBank to the sequence of CCNE1 gene is: NM_001238.2

STATISTICAL ANALYSIS

Results were expressed as means ± SD unless otherwise indicated. Differences between groups were assessed by unpaired, two-tailed Student's t-test, $p<0.05$ was considered significant. All data were plotted using the GraphPad Prism 5.0 program.

SUPPLEMENTARY INFORMATION

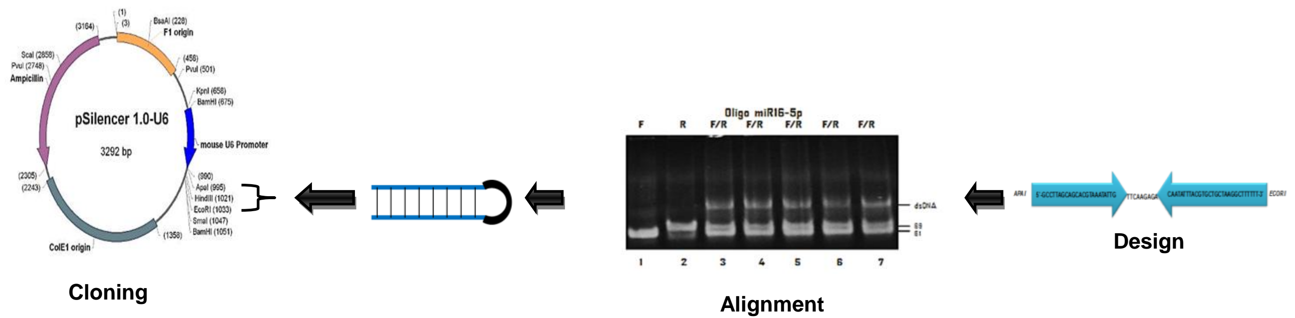

Figure 3. Design, alignment and cloning of the plasmid pSIMIR16-5P.

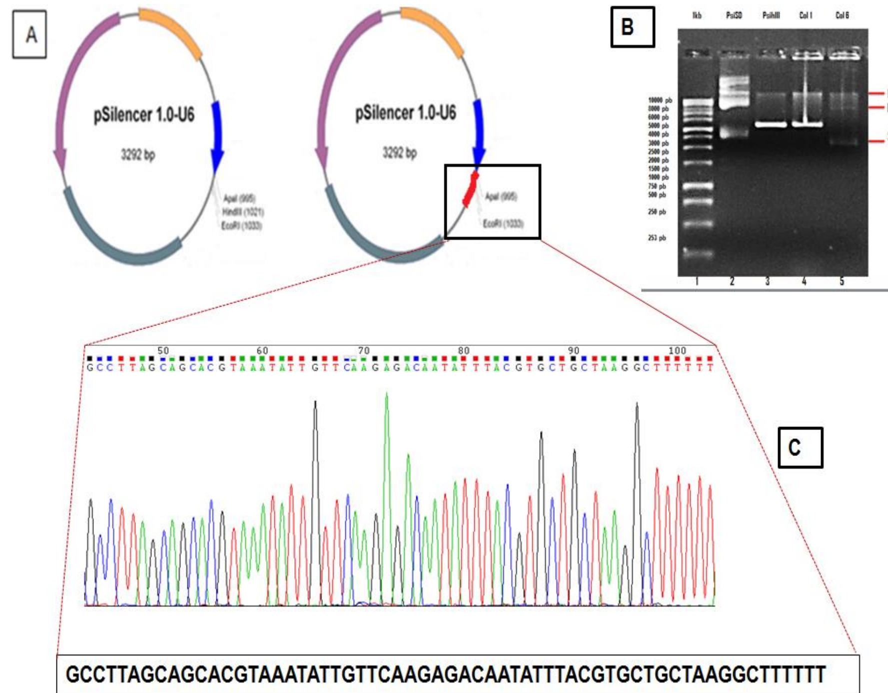

Figure 4. Cloning, integrity and sequencing of the plasmid pSIMIR16-5P

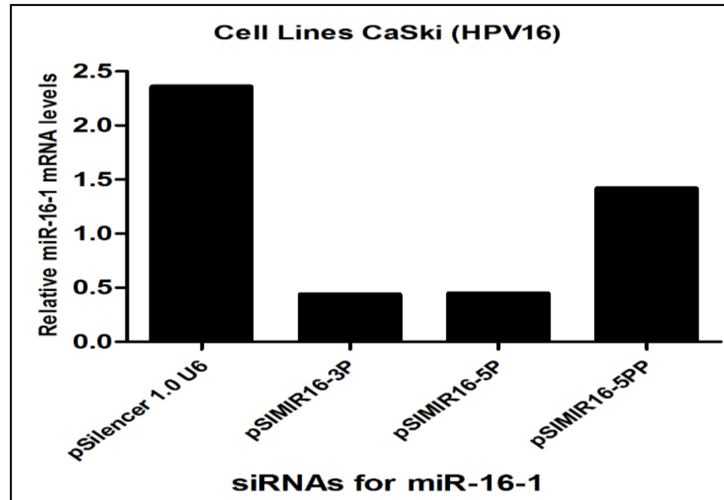

Figure 5. The pSIMIR16-5P and pSIMIR16-3P plasmids inhibit the expression of miR-16-1 endogenously.

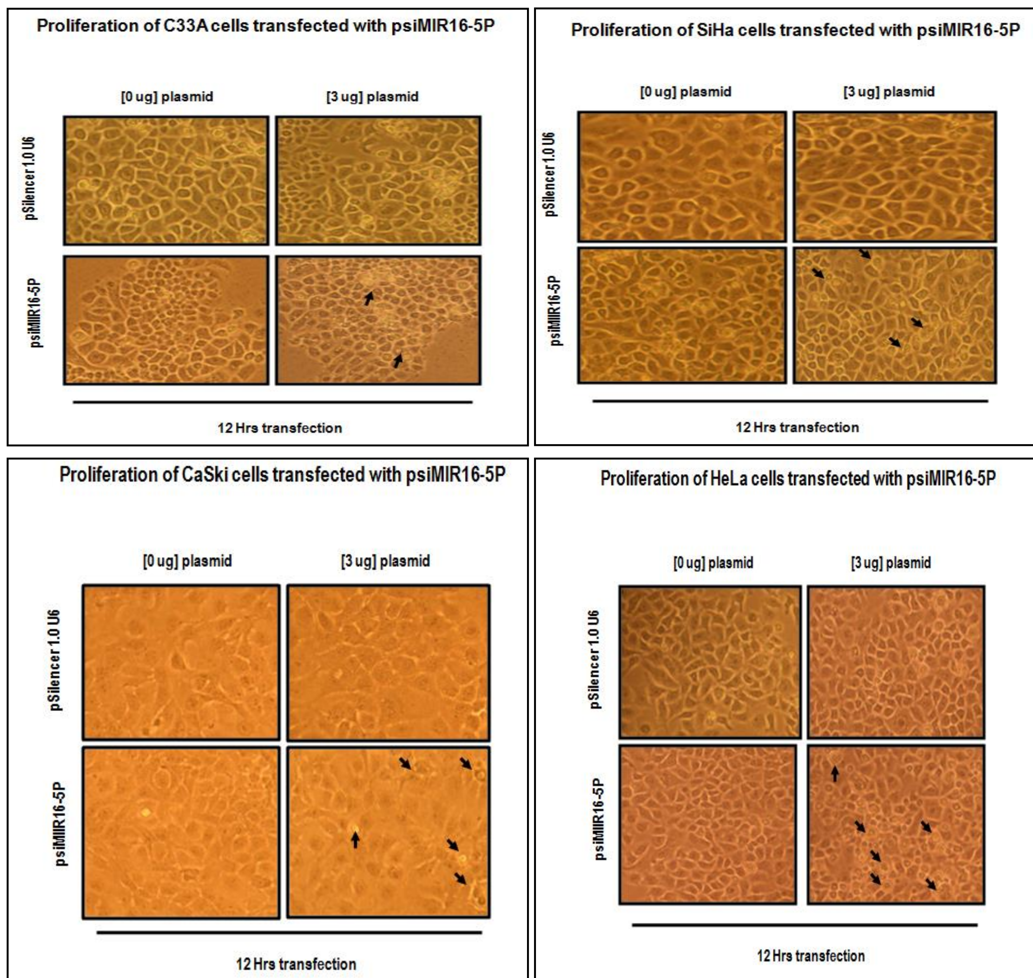

Figure 6. Inhibition of proliferation in HPV+ cell with the plasmid pSIMIR16-5P

RESULTS

SIRNA EXPRESSION PLASMIDS FOR MIR-16-1 INDUCE SILENCING OF HUMAN MICRORNA MIR-16-1

The effect of siRNA on miR-16-1 is influenced by the secondary structure and positioning of the cognate sequence within the pre-miR-16-1 molecule. To analyze the effect of the pSIMIR16-5P plasmid, we first determined whether if the siRNAs could induce a specific silencing effect on miR-16-1 expression with transient transfection of the pSIMIR16-5P plasmid. As shown in **Figure 1**, siRNAs to miR-16-1 expressed in plasmids, had effects on the miR-16-1 expression level and we identified a significant decrease in C33A (6%), SiHa (20%), CaSki (45%) and HeLa cells (58%), compared with cells transfected with pSilencer1.0-U6 plasmid (empty vector).The RNU44 RNA expression level did not show any changes in these same conditions.

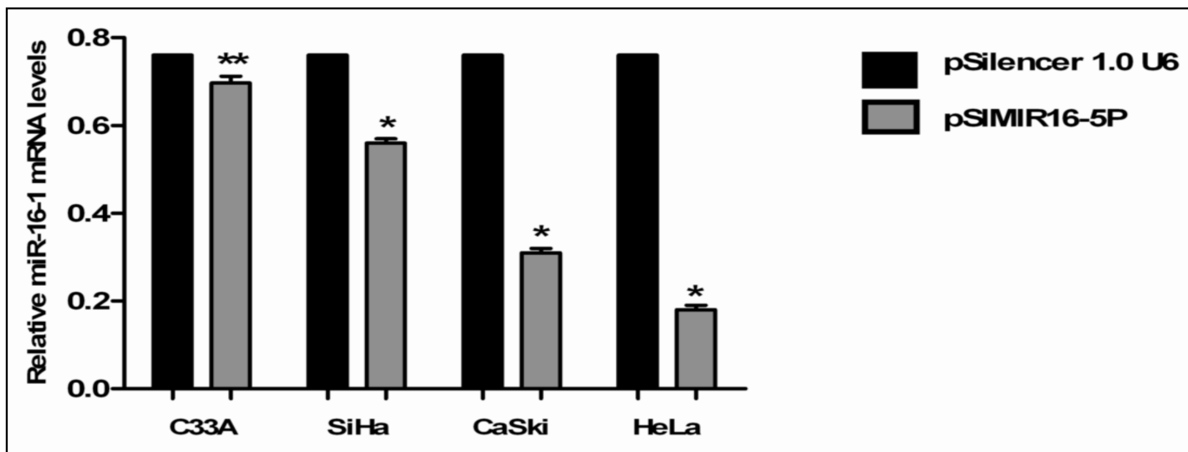

Figure 1.Silencing of human microRNA mir-16-1 expression by siRNAs. Quantitative real time RT-PCR analysis of miR-16-1 expression in C33A HPV-, SiHa HPV16+, CaSki HPV16+ and HeLa HPV18+ cells transfected with pSIMIR16-5P plasmid. Total RNA and cDNA synthesis were obtained from $1X10^5$ cells per well in a six-well plate containing DMEM at 37°C with 5% CO_2 after 12 h transfection with pSIMIR16-5P plasmid (3 µg). Relative expression by real-time RT-qPCR analysis of miR-16-1 was calculated using the $2^{-\Delta\Delta Ct}$ method and was normalized by miR-16-1/RNU44 ratio relative expression units. The Ct values were analyzed with pSilencer1.0-U6 empty vector transfection and pSIMIR16-5P plasmid and values are presented as mean ± SD. The *p* values are indicated with asterisks (*$p<0.001$) (**$p<0.05$).

SIRNA-INDUCED SILENCING OF MIR-16-1 EXPRESSION HAS EFFECTS ON CCNE1 GENE EXPRESSION

To explore miR-16-1 target genes, we focused on CCNE1 gene which regulates the G1-S phase transition of the normal mammalian cell division cycles. The **Figure 2** shows that siRNAs to miR-16-1 have effects over the CCNE1 mRNA expression level. In particular, we identified a significant decrease in mRNA expression of CCNE1 when cancer cells HPV+ were treated with siRNAs to miR-16-1.Twelve hours after transfection, the CCNE1 gene expression level decreased 22% in C33A cells, 17% in SiHa cells ($p<0.05$), 46% in CaSki cells ($p<0.001$) and 58% in HeLa cells ($p<0.001$), compared with cells transfected with pSilencer 1.0-U6 empty vector. The GAPDH mRNA expression level did not show any changes.

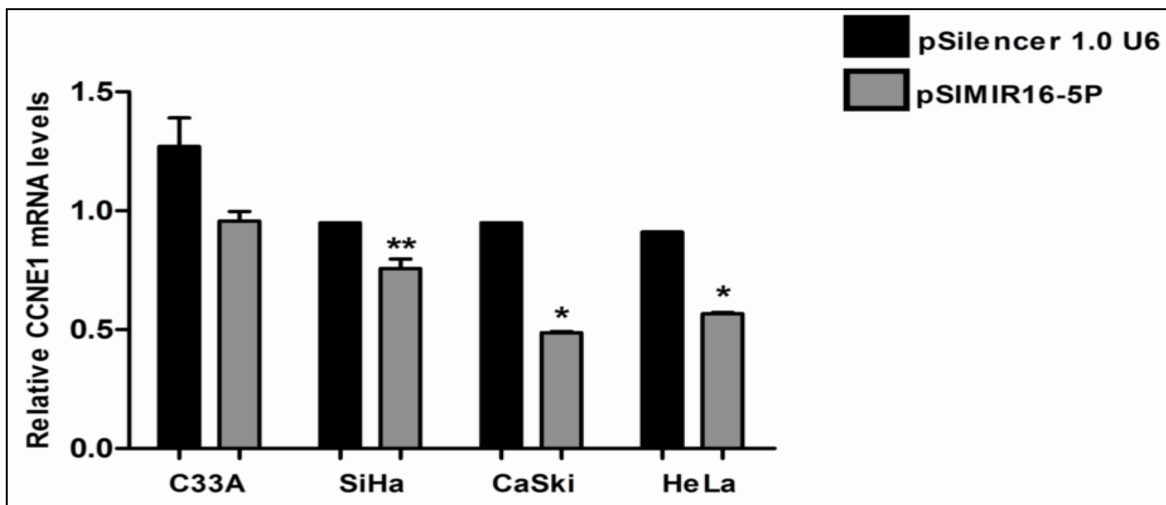

Figure 2.Analysis of CCNE1 gene expression by RT-qPCR after miR-16-1 silencing. Quantitative real time RT-PCR analysis of miR-16-1 expression in C33A HPV-, CaSki HPV16+, SiHa HPV16+, and HeLa HPV18+ cells transfected with pSIMIR16-5P plasmid. Total RNA and cDNA synthesis were obtained from $1X10^5$ cells per well in a six-well plate containing DMEM at 37°C with 5% CO_2 after 12 h transfection with pSIMIR16-5P plasmid (3µg). Relative expression by real-time RT-qPCR analysis of CCNE1 was calculated using the $2^{-\Delta\Delta Ct}$ method and was normalized by CCNE1/GAPDH ratio relative expression units. The Ct values were analyzed with pSilencer1.0-U6 empty vector transfection and pSIMIR16-5P plasmid and values are presented as mean ± SD. The *p* values are indicated with asterisks (*$p<0.001$) (**$p<0.05$).

DISCUSSION

In present study, we demonstrate that in CC cells, the overexpression of miR-16-1 contributes to the process of carcinogenesis by altering the expression of cellular genes involved in checkpoint regulation, including CCNE1, which is an essential cyclin activating Cdk2 that regulates the G1-S phase transition of the normal mammalian cell division cycles, and whose deregulation is implicated in tumor progression. Here we report that miR-16-1 post-transcriptionally down-regulates the expression of CCNE1 in cell lines derived from CC.MicroRNAs are increasingly recognized as important post-transcriptional regulators of gene expression. They have been shown to play a significant role in carcinogenesis by altering the expression of oncogenes and tumor suppressor genes **[20]**. It has previously been reported that CCNE1 and miR-16-1 are over expressed in CC **[14, 16-18]**. CCNE1 has been proposed as a regulatory target of miR-16-1 **[7]**. SiRNAs expressed in plasmids have recently been used to silence the expression of viral oncogenes or genes encoding tumor antigens, for the purpose of suppressing proliferation of specific cancer cells **[21, 22]**. Accordingly, gene silencing by siRNAs represents a strategy to silence the expression of oncogenes activated by high-risk HPV (HR-HPV) in CC, including the expression of microRNAs.

In this study, we generated siRNAs expressed in plasmids specific to microRNA miR-16-1. The pSIMIR16-5P plasmid induced specific silencing of miR-16-1 in SiHa, CaSki cells transformed with HPV16 and HeLa cells transformed with HPV18, when compared to C33A (HPV-) cells (**Figure 1**). These findings are relevant because we silenced the expression of miR-16-1 which is overexpressed in CC cells transformed with HR-HPV. It is noteworthy that in HeLa cells further decrease was observed in the expression of miR-16-1 compared with CaSki cells. MiR-16-1 is located on chromosome 13q14 DLEU2 gene **[3]**, which has been reported that is one of the frequent sites of integration of the HPV16 **[23]**. The CaSki cells have up to 60-600 integrated copies of HPV16 in cellular genome, which favors an increase in the expression of E7 oncoprotein to interact with E2F transcription factor. This scenario

can promote the endogenous activation of miR-16-1 and this regulatory genetic network has been associated to CC development. The SiHa cells have integrated 1-2 copies and the silencing of miR-16-1 expression was lower compared with CaSki cells possibly by the low number of integrated copies **[24, 25]**. The molecular pathway between E7/E2F/miR-16-1 may explain why a total silencing of miR-16-1 is not observed when CC cells were treated with siRNA expression plasmids to miR-16-1. In contrast, HeLa cells that have integrated 50 copies of HPV18, one of the frequent sites of integration is on chromosome 8q23-24. Interestingly, on this chromosomal locus is localized the proto-oncogene c-Myc **[26]**. It has been reported that c-Myc when is overexpressed can join and negatively regulate the DLEU2 gene promoter region which generates a decreased expression of miR-16/miR-15 cluster **[27]**. Possibly having fewer copies of HPV integrated and down-regulation of c-Myc has been contributed to greater decrease in expression of miR-16-1 in HeLa cells. Moreover, in our study we observed that miR-16-1 expression levels were affected in C33A cells despite treatment with pSIMIR16-5P plasmid. The C33A cells have a mutation in the pRb gene (exon 20) and p53 (codon 273), which suggests that inactivation of such tumor suppressor proteins, through interaction with E6 and E7, may have an equivalent function to mutations specific for p53 and pRb. However, mutated tumor suppressor genes may result in a gain of functions, some of which cannot be performed by the E6/p53 and E7/pRB interaction **[27]**. Our results suggest thatmiR-16-1 could have a role similar to an oncomir in human CC cells, since it has been reported up regulated in patient samples with CIN 2-3 and CC compared to normal tissue **[15-18]**.

By combined bioinformatics analysis and RT-qPCR assays, we determined that miR-16-1 controls the expression of the positive cell cycle regulator CCNE1 oncogene, in CC cells transformed by HPV16 and HPV18 (**Figure 2**), probability by targeting the 3’ UTR regulatory region of CCNE1 mRNA. This finding describes a mechanism of post-transcriptional regulation of CCNE1 expression and connects the function of miR-16-1 with a cellular gene involved in cell cycle control. Nevertheless, we observed subtotal repression of CCNE1 mRNA expression level, possibly due to

HPV E7 oncoprotein expression can activates endogenously to CCNE1 **[28]**. However, further *in vitro* and *in vivo* investigations are needed to characterize the regulatory circuit between miR-16-1, CCNE1, and HPV E7.

We found a significant reduction in the mRNA expression level of CCNE1 oncogene following administration of siRNA expressed in plasmids against miR-16-1. Although multiple mechanisms including genetic mutation, promoter methylation and post-transcriptional modification may contribute to the deregulation of the CCNE1 gene. MicroRNA response elements (MRE) are binding sequences in the 3'-UTR of mRNA through which microRNAs suppress their target gene. Four CCNE1 MRE sequences for miR-16-1 have been reported in the online databases miRanda, Target Scan Human and miRBase; however, their functionality in CC cells has not been evaluated to date. MRE16-1 (229-254 nt), MRE16-2 (459-492nt), MRE-3 (1641-1676) and MRE-4 (1878-1914) **[7, 29]**.

Our results show a decrease in CCNE1 differential between HPV16+ and HPV18+ cells compared to HPV-cells. C33A cells expressing only CCNE1 decreased by 22% in the presence of pSIMIR16-5P; it has reported that E2F can directly activate transcription of CCNE1 or E2F may also participate in the transcription of miR-16-1 which could in these cells play a role in oncomir and induce transcription of CCNE1, coupled with the mutation p53 and pRB in these cells, both processes could be contributing to the proliferation and immortalization of cancer cells **[27].** In this study we observed a significant decrease in CCNE1 in HPV16 + cells compared to HPV-cells, suggesting that by silencing the expression of miR-16-1 are also reducing the levels of mRNA CCNE1, which supports the hypothesis that miR-16-1 could in this type of cancer have an oncogenic role **[17],** these findings are important because it has been reported in cervical carcinoma tissue to chromosome 19q12 represents one of the integration sites of HPV-16 and even more important on this site is the CCNE1 gene **[30].**

We observed a significant decrease of mRNA CCNE1 in HeLa cells (HPV18) this may be in addition to over expression of HPV-18 E7 on CCNE1, in these cells

exist an integration site in the region 11-13 chromosome 5, near there on chromosome 6 region 21.2 is the peroxisome proliferator-activated receptors δ (PPAR δ) which binds to the promoter region of miR-16-1 inducing transcription and possibly the increased expression of CCNE1 unlike that do not contain this integration site HPV16+ cells **[31].** In contrast has been reported to use an anti-miR-16-1 on MCF-7 and HeLa cells, mRNA levels increased significantly CCNE1 after 48 hrs of transfection **[7, 29]**. Nevertheless, we observed that siRNAs induce greatest inhibition of mRNA within 12 hours of transfection. These data highlight the complexity of the regulation of gene expression by miRNAs. However, further experiments are needed to identify the molecular mechanisms by which miR-16-1 targets the CCNE1 3'-UTR and the role of MREs as recognition sequences for miR-16-1-mediated regulation of CCNE1 oncogene expression.

A limitation of this study is that we did not evaluate CCNE1 protein expression to correlate with the CCNE1 mRNA expression levels determined. The achievement of these effects in mechanistic terms and the molecular activation pathways involved in CCNE1 gene regulation by miR-16-1, as well as their biological effects, are not yet clear but are being investigated by our group. Our data will help to further our understanding of the molecular pathways involved in the development and progression of CC, which may highlight new therapeutic strategies in the treatment of this disease. In summary, in this study we found that the CCNE1 gene is a target of microRNA miR-16-1 in human CC cells transformed by HPV16 and HPV18. This regulation most likely occurs via directly targeting of the CCNE1 3' UTR regulatory region by miR-16-1. MiR-16-1 may negatively regulate the CCNE1 oncogene, which overexpressed in a subset of human cancers, and thus manipulation of this pathway may represent a therapeutic strategy for cancer therapy in the future.

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CAPÍTULO II

El objetivo de este capítulo fue: Relacionar el estado integrado de los VPH-AR con la expresión de Ciclina E1 en la carcinogénesis cervical.

Clinical Cancer Research

La integración de los VPH-AR incrementa la expresión de Ciclina E1 en lesiones premalignas y carcinoma cervical de mujeres del Sur de México

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INTRODUCCIÓN

El carcinoma cervical invasor (CCI) es el segundo cáncer más común en las mujeres a nivel mundial y es causado por la infección persistente de los VPH oncogénicos **(Moscicki *et al.*, 2012).** Los VPH de alto riesgo (VPH-AR), como el VPH-16 y VPH-18 tienen una contribución mundial de ~70% de los casos con CCI **(de Sanjose *et al.*, 2010).** En el Estado de Guerrero, México, se ha reportado una prevalencia del 68.1% de los casos de CCI en presencia del VPH-16, además de que el 27.4% de las lesiones escamosas intraepiteliales (LEI) de alto grado (LEIAG) y el 10.6% de las LEI de bajo grado (LEIBG), presentan este tipo viral **(Illades-Aguiar *et al.*, 2010).**

La mayoría de las LEI regresan espontáneamente, pero un pequeño porcentaje persiste llevando al desarrollo de LEI que pueden progresar a CCI. Las oncoproteínas, E6 y E7, de los VPH-AR desestabilizan, a p53 y la proteína retinoblastoma (pRB), respectivamente. La elevada expresión de E6 y E7 de los VPH-AR en las células basales, alteran la regulación del ciclo celular normal, inhibe la diferenciación celular, induce daño cromosomal, conduciendo a la proliferación celular y evitando la muerte de la célula infectada y alterada **(Roman *et al.*, 2013).** La incapacidad para discriminar entre una infección persistente o transitoria ha llevado a la necesidad de buscar nuevos parámetros adicionales que permitan la identificación de mujeres positivas a VPH-AR que tengan mayor riesgo de desarrollar el CCI. En este contexto, diversos estudios han investigado la integración del DNA viral como posible marcador diagnóstico de las LEI. La integración dentro del genoma del huésped es considerado un evento clave en la carcinogénesis asociada al VPH-AR como el tipo 16, por lo tanto la evaluación del estado físico del DNA del VPH (episomal vs integrado) ha sido sugerida como una herramienta para identificar mujeres con riesgo de desarrollar CCI **(Ramanakumar *et al.*, 2010).** Además, se ha sugerido que la integración viral, es un evento temprano en la carcinogénesis cervical **(Kulmala *et al.*, 2006)**, al respecto, diferentes estudios por nuestro grupo de trabajo encontraron un 75% de estado integrado en casos de NIC I **(Vega-Peña *et al.*, 2013)** y en 19.6% de casos de LEIBG **(Jiménez-Tagle *et al.*, 2014).**

Por otro lado, la Ciclina E1 (CCNE1) es un regulador positivo del ciclo celular, su expresión está restringida a la fase G1-S en células normales en división **(Sauer *et al.*, 1995).** Ciclina E1 participa en el inicio de la replicación del DNA, control de la biosíntesis de histonas y el ciclo del centrosoma **(Ma *et al.*, 2000).** Sin embargo, se ha reportado expresión alta de Ciclina E1 en cáncer de mama, cáncer de pulmón de células no pequeñas y Leucemia **(Tarik*et al.*, 2004).** Además el locus genómico donde se encuentra el gen CCNE1 (19q12-q13) se ha encontrado frecuentemente amplificado en canceres humanos sugiriendo que Ciclina E1 tiene un papel oncogénico en cáncer **(Akama *et al.,* 1995).** Se ha reportado en CCI que la expresión de Ciclina E1 incrementa conforme el grado de neoplasia intraepitelial cervical (NIC) con VPH-16 **(Erlandsson *et al.*, 2006).** La expresión de Ciclina E1 es clave en el desarrollo de CCI, se ha sugerido que Ciclina E1 podría utilizarse como biomarcador en la carcinogénesis cervical. Al respectohemos encontradoque la expresión de Ciclina E1 está relacionada con la integración de los VPH-AR en LEIBG y se ha sugerido ampliar la muestra e incluir las LEIAG y casos de CCI para seguir explorando hipótesis en presencia de los VPH-AR **(Zubillaga-Guerrero *et al.,* 2013) (Anexo 1).** Por lo anterior, es necesario, seguir investigando la relación de la expresión de Ciclina E con la integración del DNA de los VPH-AR en lesionespremalignas y carcinoma cervical en mujeres del estado de Guerrero, México.

MATERIAL Y MÉTODOS

Se estudiaron 200 citologías de mujeres residentes en el estado de Guerrero, México. Este estudio fue aprobado por el Comité de Ética de la Universidad Autónoma de Guerrero. Todas las participantes firmaron consentimiento informado **(Anexo 3)** y respondieron a un cuestionario con la finalidad de obtener información socio demográfico, clínico y antecedentes obstétricos, de las variables de estudio **(Anexo 4)**.

Colección de la muestra y diagnóstico citológico

A las mujeres incluidas en este estudio, se les realizaron 2 tomas de muestra endo/exocervical asegurando la zona de transformación escamocilíndrica, la primera se utilizó para lacitología convencional y tinción de Papanicolaou para realizar el diagnóstico citológico utilizando el sistema de Bethesda **(Solomon *et al.*, 2003)**. El diagnóstico citológico fue realizado por una Citotecnóloga acreditada y recetificada por el Consejo Mexicano de técnicos en Patobiología A,C., y Consejo Mexicano de Médicos Anatomopatólogos A.C., con 24 años de experienciaen la detección oportuna de Cáncer cérvicouterino. Una segunda toma se depositó en un vial para citología en base líquida (LiquiPREP), para realizar la técnica de Inmunocitoquímica y determinar la expresión de Ciclina E1 y la Hibridación *in situ* con amplificación con tiramidapara la determinación del estado físico de los VPH-AR, además se realizó la detección y tipificación del VPH por INNO-LiPA. Se captaron 48 mujeres sin LEI y VPH (-), 32 fueron sin LEI con VPH-AR, 67 con LEIBG más VPH-AR, 23 LEIAG con VPH-AR y 30 mujeres con Carcinoma cervical con VPH-AR.

Detección y tipificación del VPH

El DNAfue extraído de las citologías cervicales de acuerdo al método estándar SDS-proteinasa K-fenol-cloroformo **(Leornard *et al.*, 1994).** La tipificación del VPH se realizó mediante el método de hibridación reversa (INNO-LIPA HPV Genotyping Extra), en el Laboratorio de Biomedicina Molecular, brevemente, 10 ml del producto de PCR, que contiene restos de biotina en el extremo 59 de los primers, fueron desnaturalizados mediante la adición de una solución de NaOH. Después de 10 min, una tira de INNO-LIPA secolocó en la bandeja. Dos mililitros del buffer de hibridización precalentado (37 °C) (33 SSC [13 SSC en 15 mM de citrato sódico y 150 mM NaCl], 0,1% de Dodecil sulfato de sodio), se agregó y se incubó a 50°C durante 1 h. Todas las incubaciones y los pasos de lavado se realizaron de forma automática en un Auto-LiPA. Las tiras se lavaron dos veces durante 30s y una vez cada 30 min a 50 °C con 2 ml de solución de hibridación. Después de este lavado riguroso, las tiras se incubaron con 2 ml de fosfatasa alcalina-estreptavidina

conjugada durante 30 min a temperatura ambiente. Las tiras se lavaron dos veces con 2 ml de solución de enjuague y una vez con 2 ml de buffer sustrato. Dos mililitros de sustrato (5-bromo-4-cloro-3-indolilfosfato y nitroazul de tetrazolio) fue agregado y se incubó durante 30 min a temperatura ambiente. La reacción se detuvomediante la aspiración de la solución de sustrato y la adición de 2 ml de agua destilada. Después del secado, la hibridación y la reacción de color, se observó un precipitado color púrpura. Los resultados de la hibridación se evaluaron visualmente comparando el patrón de hibridación de plantillas estándar tipo específicas **(Kleter *et al.*, 1999).**

Determinación de la integración viral mediante Hibridación *in situ* con amplificación con tiramida (HIS)

La detección del genoma viral se realizó con el sistema de amplificación de señal con tiramida (Gen Point Dako Cytomation, Carpintería, CA, USA). Los extendidos en monocapa se sometieron a permeabilización por 6 minutos a 120 °C, posteriormente se sometieron a digestión por 5 segundos con proteinasa K (1:1000), posteriormente se aplicó 0.5µl del reactivo de prueba (DNA viral biotilinado) con sondas para 13 genotipos de VPH-AR, tipo 16, 18, 31, 33, 39, 45, 51, 52, 56, 58, 59 y 68.Las laminillas se desnaturalizaron por 10 minutos a 95°C y se sometieron a hibridación durante 20 horas (Hybridizer Dako, Carpentry CA, USA), posteriormente se colocaron en solución astringente, se agregó estreptavidina peroxidasa primaria, posteriormente biotinil tiramida, luego estreptavidina secundaria, se agregó DAB y finalmente Hematoxilina de Mayer (Merck). La reacción positiva se visualizó con un color marrón dentro del núcleo, y de acuerdo al tipo de señal, se clasificó en difusa (estado episomal), puntiforme (estado integrado) o mixto (difusa y puntiforme) **(Kalof y Cooper., 2006).**Se utilizaron como control positivo líneas inmortalizadas de cáncer cervical CaSki (VPH-16) que contienen hasta 500 copias integradas y como control negativo las mismas líneas celulares omitiendo la sonda.

Expresión de Ciclina E1 por Inmunocitoquímica

La expresión de Ciclina E1 se determinó por la técnica de inmunocitoquímica de Streptavidina biotina peroxidasa, utilizando el sistema de detección Cytoscan

HRP/DAB Cell (Cell Marque Corporation, Hot Springs, AR). El anticuerpo monoclonal utilizado fue: Ciclina E (13A3; 1:100; Novocastra, Newcastle-Upon-Tyne, UK). Las laminillas de citología en base liquida se sometieron a recuperación antigénica (InmunoDNA Retriever con citratos; BioSB, Inc, Santa Bárbara, CA, EUA) durante 6 minutos a 120°C. Posteriormente, se adicionóel anticuerpo primario durante 1 hora, luego el anticuerpo secundario acoplado a biotina, y se agregó estreptavidina peroxidasa. Para el revelado se utilizó el cromógeno DAB y Hematoxilina de Mayer (Merck) como tinción de contraste. Se utilizó como control positivo líneas celulares CaSki (VPH-16) que sobre expresa Ciclina E1 y las mismas líneas celulares omitiendo el anticuerpo primario, como control negativo.

La expresión se determinó por el porcentaje de núcleos positivos: 0%, 1-10%, 11-50% y >50%.La intensidad de la inmunotinción como: Leve, Moderada e intensa **(Halloush *et al.*, 2008).**Finalmente la evaluación nuclear se llevó a cabo bajo los criterios de **Wentzensen *et al.*, 2005** (Cuadro 1).

Puntuación 0	Puntuación 1	Puntuación 2	Puntuación 3	Puntuación 4
Ciclina E1 Negativa	Ciclina E1 positiva en células sin aberraciones nucleares	Ciclina E1 positiva en células con incremento radio núcleo-citoplasma o cromatina alterada o forma nuclear alterada o anisonucleosis	Ciclina E1 positiva en células con incremento radio núcleo-citoplasma y cromatina alterada o forma nuclear alterada o anisonucleosis	Ciclina E1 positiva en células con incremento radio núcleo-citoplasma y más de uno de los siguientes criterios: cromatina alterada y/o forma nuclear alterada y/o anisonucleosis

Análisis estadístico

Se utilizó la prueba exacta de Fisher para comparar frecuencias. Para determinar la relación entre la expresión de Ciclina E1 y la lesión cervical o el estado físico de los VPH-AR se utilizaron modelos de regresión logística multinominal. El análisis estadístico fue hecho con el software STATA versión 11.1. Un valor de $p<0.05$ fue considerado significativo. Todos los datos fueron ploteados usando el programa GraphPad Prism 5.0.

RESULTADOS

La edad de las mujeres fue entre 14 a 78 años, con una mediana de edad de 41. El grupo de mujeres con diagnóstico de LEIBG (80.6%), LEIAG (82.6%) y CC (100%) positivas a VPH-AR tuvieron una edad >25 años, además las mujeres con LEIBG (14.9%), LEIAG (56.5%) y CC (70%) iniciaron su vida sexual antes de los 18 años. Por otra parte en las mujeres con LEIBG (23.8%) y LEIAG (26.0%) positivas a VPH-AR refirieron tener >2 parejas sexuales. Es importante mencionar que las mujeres con LEIBG (35.8%), LEIAG (69.5%) y CC (100%) refirió tener >2 embarazos (Datos no mostrados).

Frecuencia de tipos de VPH-AR y estado físico del DNA viral

Un total de 8 tipos de VPH-AR fueron identificados: 16, 18, 31, 45, 51, 52, 56 y 58. El VPH-16 fue el más frecuente en un 37.5%. Además se identificaron 47 mujeres con infección múltiple con VPH-AR incluido el VPH-16 (23.5%). La frecuencia delVPH-16 en relación al diagnóstico citológico fue en sin LEI (34.3%), LEIBG (59.7%), LEIAG (17.3%) y CC (66.6%) (Figura 1). Es importante mencionar que dentro de las mujeres con diagnóstico de LEIAG encontramos que el 30.4% (7) de estas presentaron displasia severa/Carcinoma *in situ,* por otra parte dentro de las mujeres diagnosticadas con CC el 10% (3) son Adenocarcinomas.

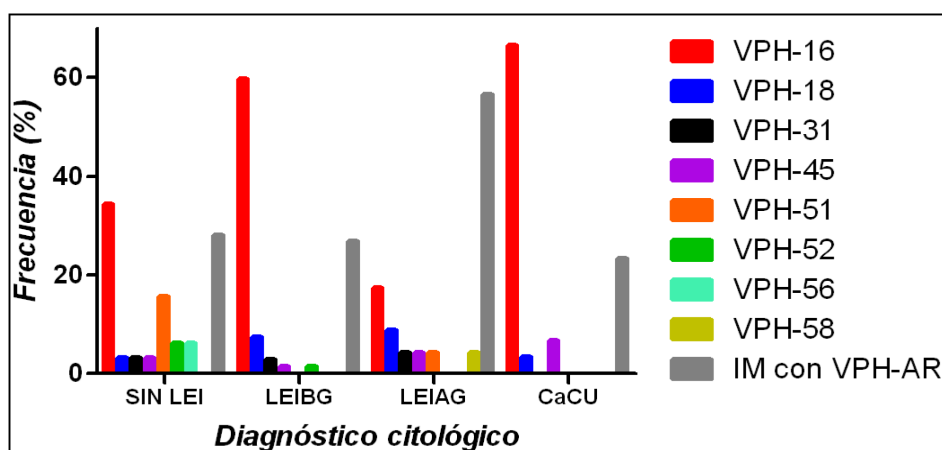

Figura 1. Frecuencia de VPH-AR en relación al diagnóstico citológico

El estado físico del DNA del VPH-AR se localizó en el núcleo. Los resultados (Tabla 1) muestran que el 75% de las muestras sin LEI con VPH-AR, presentaron patrón mixto (episomal e integrado por separado) en 2-4 células intermedias (Figura 2a), mientras que el 55.2% de las LEIBG con VPH-AR presentaron el patrón mixto en 4-6 células intermedias con cariomegalia y binucleación (Figura 2b). Por otro lado el 60.8% de las mujeres con LEIAG mostraron patrón integrado en células con discariosis profunda (Figura 2c) en contraste con las mujeres con CC donde el 100% mostraron patrón integrado en grupos de células con anisocariosis, con escaso citoplasma y distribución irregular de la cromatina (Figura 2d). Es importante señalar que el patrón integrado fue el estado físico más frecuentemente relacionado con los tipos VPH-16, VPH-18 y VPH-58 (Datos no mostrados).

Tabla 1. Estado físico del DNA de los VPH-AR en relación al diagnóstico citológico

Grupo de estudio	**Estado físico de los VPH-AR**		
	Mixto n (%)	**Integrado n (%)**	***p***
Sin LEI con VPH-AR	24 (75)	8 (25)	
LEIBG y VPH-AR	37 (55.2)	30 (44.8)	0.08[a]
LEIAG y VPH-AR	9 (39.1)	14 (60.9)	0.23[b]
CaCU y VPH-AR	0	30 (100)	<0.001[c]

[a]LEIBG vs sin LEI+VPH-AR; [b]LEIAG vs LEIBG; [c]CC vs LEIAG

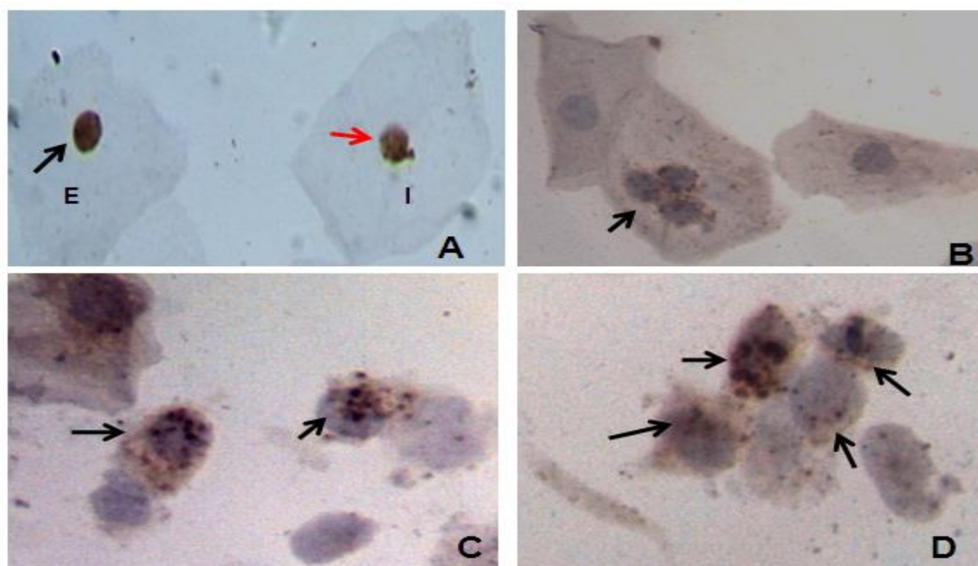

Figura 2. Estado físico del DNA de VPH-16. (A) Citología sin LEI que muestra estado mixto [Episomal (flecha negra) e integrado (flecha roja)] en células intermedias 40X, (B) Citología de LEIBG con patrón integrado en células con cariomegalia 40X (flecha negra) (C) Citologías de LEIAG con estado integrado en células con discariosis profunda 100X (flechas negras) (D) Citología con Carcinoma de células escamosas que muestra el estado integrado en células con anisocariosis y distribución irregular de la cromatina (Flecha negras) 100X. Técnicas: Citología en base liquida e Hibridación *in situ* con amplificación con tiramida.

Expresión de Ciclina E y su relación con el diagnóstico citológico

La expresión de Ciclina E1 se relacionó con el grado de LEI y CC ($p<0.001$) en comparación con las citologías sin LEI, sin VPH (Tabla 2).En las citologías sin LEI, sin VPH, la expresión de Ciclina E1 se observó 1-10% de células basales, cilíndricas y de metaplasia sin anormalidades nucleares, la inmunotinción fue leve por lo que fueron consideradas como negativas teniendo un puntaje de 0 (Figura 3a), el 65.6% de las citologías sin LEI con VPH-AR presentaron del 11-50% de núcleos positivos en células intermedias, cilíndricas y de metaplasia las cuales presentaron un puntaje de 1 con una intensidad de leve a moderada (Figura 3b), el 94% de las citologías de LEIBG con VPH-AR (puntaje 2) (Figura 3c) presentaron del 11-50% de núcleos positivos en células intermedias con cariomegalia, discreto halo perinuclear y binucleación con intensidad moderada en comparación con las LEIAG con VPH-AR (puntaje 3) los cuales tuvieron intensidad de moderada a intensa en células basales profundas con escaso citoplasma y anisocariosis ($p<0.001$) (Figura 3d), mientras que el 100% de las citologías con CC expresaron la proteína en >50% de núcleos

positivos en pequeños grupos de células malignas con anisocariosis profunda y pérdida o escaso citoplasma cuya inmunotinción fue intensa (Figura 3e) y el puntaje nuclear fue de 4 en comparación con las LEIAG (p<0.001) (Tabla 2).

Tabla 2. Expresión de Ciclina E1 y su relación con el diagnóstico citológico

Grupo de estudio	Expresión de Ciclina E1						
				% núcleos positivos			
	Negativo n (%)	Positivo n (%)	*p*	0% n (%)	1-10% n (%)	11-50% n (%)	>50% n (%)
Sin LEI sin VPH	48	0		48 (100)	0	0	0
Sin LEI con VPH-AR	0	32 (100)		0	11 (34.4)[a]	21 (65.6)[a]	0
LEIBG y VPH-AR	0	67 (100)	<0.001	0	4 (6.0)[b]	63 (94.0)[b]	0
LEIAG y VPH-AR	0	23 (100)		0	0[c]	23 (100)[c]	0
CC y VPH-AR	0	30 (100)		0	0	0	30 (100)[d]

Significancia entre grupos: [a]p=0.05; [b,c]p<0.001; [c,d]p<0.001

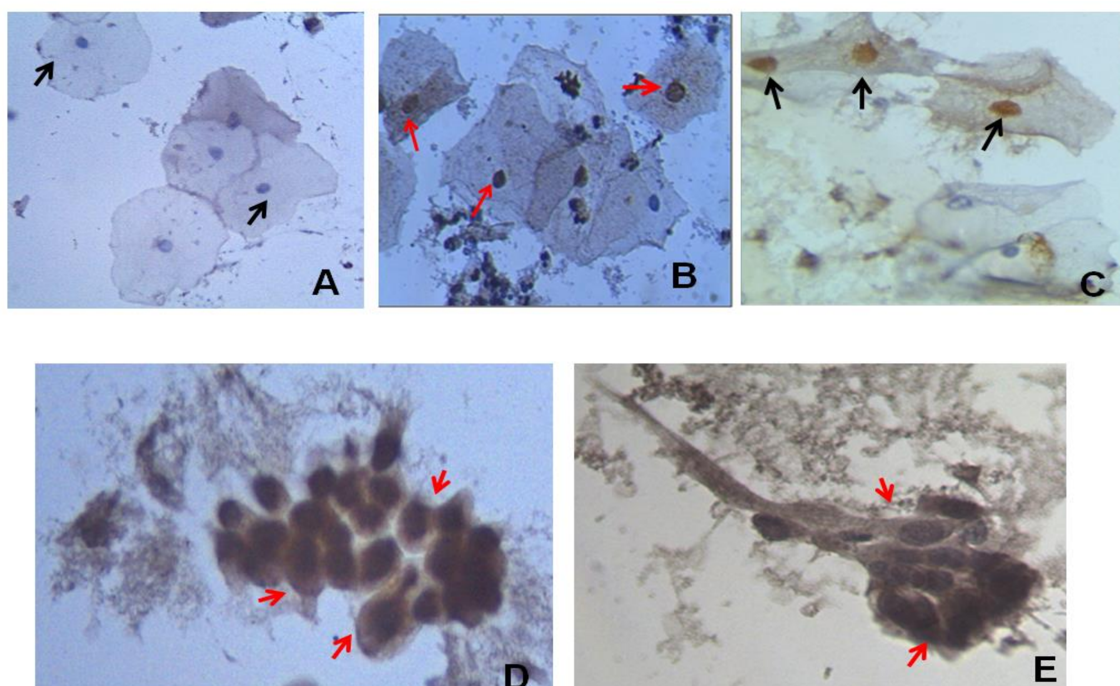

Figura 6. Expresión de Ciclina E1. A) Expresión negativa en citologías sin LEI, negativas a VPH (Flechas negras) puntaje 0 40X. B) Expresión positiva en células intermedias sin LEI con VPH-16 (Flechas negras) puntaje 1 40X. C) Expresión positiva en células intermedias con cariomegalia y halo perinuclear discreto (Flechas rojas) puntaje 2 40X. D) Expresión positiva en células basales profundas con intensa discariosis, núcleos grandes e hipercromáticos compatibles con la LEIAG con VPH-16 (Flechas rojas) puntaje 3 40X. E) Expresión positiva en un caso de Carcinoma de células escamosas positivo a VPH-16 (Flechas rojas) puntaje 4 40X. Método: Citología en base liquida. Técnica Streptavidina Biotina Peroxidasa.

Asociación de la expresión de Ciclina E1 con el diagnóstico citológico

Los resultados muestran que las mujeres que presentan del 11-50% de núcleos positivos a Ciclina E1 tienen 9.9 veces más riesgo de presentar una LEIBG en comparación con las citologías sin LEI mas VPH-AR (p<0.001), mientras que aquellas mujeres que tienen >50% de núcleos positivos a Ciclina E1 tienen 450 veces más el riesgo de presentar CC en comparación con las mujeres sin LEI con VPH-AR (p<0.001) (Tabla 3).

Tabla 3. Asociación entre la % de núcleos positivos de Ciclina E1 con el diagnóstico citológico e infección de VPH-AR

	Porcentaje de núcleos positivos a Ciclina E1				
	1-10%	11-50%		>50%	
Diagnóstico + VPH-AR		OR (IC95%)*	*p*	OR (IC95%)*	*p*
Sin LEI	Ref				
LEIBG		9.9 (3.0-32.3)	<0.001	16.3 (1.5-178)	0.022
LEIAG		21.7 (2.5-189.5)	0.005	18.6 (0.5-∞)	0.106
CC		1.0 (0.1-∞)	0.974	450 (21.5-9413)	<0.001

Modelo de regresión logística multinomial ajustado por edad e inicio de vida sexual activa

DISCUSIÓN

El CC continúa siendo una de las primeras causas de morbilidad y mortalidad en mujeres alrededor del mundo. Durante el proceso de la carcinogénesis cervical las oncoproteínas E6 y E7 de los VPH-AR juegan un papel importante en la inactivación de mecanismos celulares implicados en el control del ciclo celular. Sin embargo, también ha sido postulado que el VPH es necesario-pero no suficientemente-una causa para el desarrollo de CC; otros factores como los celulares, inmunológicos, genéticos, epigenéticos o el medio ambiente, pueden afectar el resultado final de la enfermedad **(Zur Hausen., 2002; Moscicki *et al.*, 2012).**

En nuestro estudio encontramos que el I.V.S.A (<18 años), el número de parejas sexuales (>2) y el número de embarazos (>2) representan cofactores de riesgo de

progresión a CC. Se ha reportado que las adolescentes son un grupo vulnerable con respecto a las enfermedades de transmisión sexual, esto debido a la relativa inmadurez del tracto genital, haciendo a este propenso a traumas e infecciones, particularmente debido al desarrollo de la zona de transformación del cérvix uterino **(Lee *et al.,* 2006).** Por otra parte el hecho de tener vida sexual activa, varias parejas sexuales y la multiparidad se han relacionado con el desarrollo de LEI premalignas y CC, además de la adquisición de nuevos tipos de VPH-AR **(Moscicki *et al.,* 2001).**

El nivel socioeconómico bajo, la presencia de grupos étnicos y la residencia son factores asociados con el desarrollo de CC **(Bradley *et al.,* 2004).** El Estado de Guerrero, localizado al sur de México, es el segundo estado más pobre de México y la mayor parte de los habitantes tienen un estrato social bajo. En esta región, el CC es el segundo tipo de cáncer más común en mujeres y ocupa el cuarto lugar en mortalidad en el país con 12.5 muertes por cada 100, 000 mujeres, comparada con la tasa de mortalidad nacional de 9.1 por cada 100, 000 habitantes en el 2008 **(Secretaria de salud, 2006).** Se ha reportado al VPH-16 como el genotipo más frecuente en LEIAG y CC en mujeres del estado de Guerrero **(Illades-Aguiar *et al.,* 2010).** En nuestro estudio encontramos al VPH-16 con mayor frecuencia en las citologías sin LEI en un 34.3%, LEIBG (59.7%), LEIAG (17.3%) y CC (66.6%). El potencial carcinogénico del VPH-16 puede explicar porque este genotipo viral contribuye más al desarrollo de LEI premalignas y CC. Se ha reportado que la infección persistente por VPH-16 es más carcinogénica y requiere sustancialmente menos tiempo para progresar a una LEI premaligna o CC comparado con otros genotipos de VPH **(Kjaer *et al.,* 2010; Castle *et al.,* 2011).** Las variantes de los genotipos de VPH-AR, principalmente el VPH-16 también confieren mayor riesgo de desarrollar CC, se ha reportado que en el Estado de Guerrero las variantes más frecuentes en CC son E-G350, AA-a, AA-c, E-C188/G350 y E-A176/G350, no obstante la variante AA-a es la que tiene más alta asociación con CC **(Ortiz-Ortiz *et al.,* 2015).** Es importante mencionar que la carga viral de los VPH-AR también representa un factor de riesgo de las LEI a CC**.** Se ha reportado que la carga viral incrementa conforme al grado de LEI **(Doorbar *et al.,* 2012; Del Rio-Ospina *et al.,* 2015).** La coinfección con VPH-AR fue detectada en un 47% de las mujeres VPH

positivas, se ha sugerido que las mujeres con múltiples genotipos de VPH-AR pueden ser más propensas a la infección por VPH-AR y que la interacción entre genotipos resulta en el desarrollo de displasias y CC **(Rousseau *et al.,* 2003; Trottier *et al.,* 2006).**

Por otra parte encontramos que las citologías sin LEI y LEIBG positivas a VPH-AR presentaron más frecuentemente el patrón mixto, mientras que las LEIAG y CC tuvieron patrón integrado. La integración del DNA de los VPH-AR ha sido considerada como un evento clave en el proceso de la carcinogénesis cervical. El hecho de encontrar el DNA de VPH-AR integrado en el genoma del huésped incrementa el riesgo de desarrollar una LEI premaligna o CC **(Li *et al.,* 2008).** La integración del DNA viral interrumpe la función reguladora de E1 y E2, la perdida de E2 genera la sobre regulación de la transcripción de E6 y E7, promoviendo laproliferación e inmortalización continua uniéndose a sus principales blancos (p53 y pRb, respetivamente), lo cual lleva al desarrollo de LEI premalignas y CC **(Nishimura *et al.,* 2000).**

La integración de los VPH-AR no solo modifica los patrones de transcripción relevantes para la desregulación de la expresión de los oncogenes virales E6 y E7, también sobre regula la expresión de genes del huésped en sitios vulnerables de integración (sitios frágiles), muchos de estos genes con función oncogénica **(Dal *et al.,* 2008; Schmitz *et al.*, 2012).** Nosotros encontramos una expresión anormal de Ciclina E1 en todas aquellas mujeres con VPH-AR, observando que la expresión, el puntaje nuclear y la intensidad de la inmunotinción fue diferencial, encontrándose que las aberraciones nucleares, el número de células positivas y la intensidad de la tinción incremento conforme al grado de LEI. En diversos estudios se ha reportado una expresión incrementada de Ciclina E1 en citologías con LEIBG (96.7%) y LEIAG (91.6%) relacionadas con la integración de los VPH-AR **(Keating *et al.,* 2001).** Ciclina E1 es un importante regulador del ciclo celular en la fase G1-S, se le ha reportado incrementada en Cáncer de pulmón de células no pequeñas, Cáncer de mama y Leucemia **(Tarik *et al.*, 2004),** favoreciendo el desarrollo de cáncer y su expresión incrementada está relacionada a un mal pronóstico, por todo esto Ciclina

E1 juega un papel oncogénico en cáncer**.** La integración del VPH-16 lleva a la sobre regulación de E7, se ha reportado que E7 puede directamente interactuar con Ciclina E1 y favorecer su transcripción, además se ha reportado que E7 al inhibir la función de pRb e interactuar con el factor de transcripción E2F este último puede inducir la expresión de Ciclina E1 de manera endógena **(Nguyen C *et al.,* 2008; Ofir *et al.,* 2011).** Por otra parte el locus genómico de Ciclina E1 está localizado en el 19q12-q13 y se ha reportado amplificado en canceres humanos **(Akama *et al.,* 1995; Demetrick *et al.,* 1995),** se ha reportado que el VPH-16 y VPH-18 poseen un sitio de integración en el locus 19q13 en el sitio frágil FRA19A, muy cerca de la localización del gen de Ciclina E1 favoreciendo su transcripción **(Ferber *et al.,* 2003; Wentzensen *et al.,* 2002).** Se ha reportado que la integración de los VPH-AR sobre regula la expresión de miRNAs como el miR-16-1, el cual se encuentra sobre expresado en neoplasia intraepitelial cervical (NIC) 3 y CC en comparación con tejido normal relacionado con la expresión de E7 **(Zheng *et al.,*2011),** recientemente encontramos que miR-16-1 juega un papel oncogénico en CC con VPH-16 y VPH-18, esto debido a que alsilenciar la expresión endógena del miR-16-1 observamos una disminución en el RNAm de Ciclina E1, además de inhibición de la proliferación celular, lo cual sugiere que Ciclina E1 es blanco de miR-16 a nivel posttranscripcional posiblemente a través de la vía E7/E2F/miR-16-1 **(Zubillaga-guerrero *et al.,*2015).** E6 contribuye en la desregulación del ciclo celular degradando a p53 inhibiendo la posibilidad de que este active a inhibidores dependientes de cinasas como $p21^{CIP1}$ y $p27^{KIP1}$ los cuales entonces no pueden inhibir la formación del complejo CDK2/Ciclina E1, favoreciendo la proliferación e inmortalización continua **(Nguyen C *et al.,* 2008)**. No obstante múltiples mecanismos como mutaciones genéticas y metilación del promotor pueden contribuir en la desregulación del gen Ciclina E1 **(Wang *et al.,* 2009).**

En conclusión nuestros resultados sugieren que la expresión de Ciclina E1 es un evento temprano relacionado a la integración de los VPH-AR, la expresión de Ciclina E se relacionó conlas anormalidades nuclearesy fueron incrementando diferencialmente de acuerdo al grado de la LEI y los casos de CC relacionadas con la integración de los VPH-AR, principalmente el VPH-16 y VPH-18 ($p<0.001$); estos

resultados sugieren que Ciclina E1 podría utilizarse como biomarcador celular en la detección de LEIBG con riesgo de una lesión mayor, por lo que se requiere realizar estudios de seguimiento a gran escala para evaluar la utilidad clínica predictiva y el valor pronósticode la expresión de Ciclina E1 en mujeres con LEIBG.

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DISCUSIÓN Y CONCLUSIONES

El CC continúa siendo una causa de morbilidad y mortalidad en las mujeres a nivel mundial. Los miRNAs controlan la expresión de genes a nivel posttranscripcional. Sin embargo, la desregulación de la expresión de miRNAs ha sido reportada en una gran variedad de tumores y ha sido considerado que esto puede contribuir al desarrollo y progresión de cáncer (Chen *et al.*, 2014). La infección persistente, la integración y carga viral alta de los VPH-AR como el VPH-16 y VPH-18 contribuyen a la progresión del CC a través de la acción de las oncoproteínas E6 y E7, que interfieren con reguladores del ciclo celular, p53 y pRb, respectivamente (Wang *et al.*, 2014). En

nuestro estudio encontramos por primera vez que en líneas celulares de CC miR-16-1 juega un papel oncogénico regulando positivamente al oncogén CCNE1, ya que al inhibir su expresión endógena también se disminuyó el RNAm de CCNE1 lo cual sugiere que CCNE1 es blanco de miR-16-1 a nivel posttranscripcional (Zubillaga-Guerrero *et al.*, 2015). Se ha reportado que miR-16-1 es blanco de E7 y ha sido sugerido que este miRNA tiene un papel oncogénico debido a la activación de otros genes implicados en la progresión del ciclo celular en CC, como Ciclina E1 (Zheng *et al.*, 2011). Fuertes estudios *in vitro/in vivo* son necesarios para establecer un link entre E7/E2F/miR-16-1/Ciclina E1 en CC con el fin de investigar los mecanismos por los cuales miR-16-1 y CCNE1 regulan los eventos celulares en CC que puedan dar una mejor información sobre su rol como biomarcadores, así como también en el pronóstico y terapia de CC.

Por otra parte se ha reportado que Ciclina E1 se encuentra incrementada en su expresión en líneas celulares VPH+ y tejidos con NIC III y CC (Erlandsson *et al.*, 2006). No obstante recientemente en nuestro grupo de trabajo encontramos expresión incrementada de esta proteína en el 100% de LEIBG y citologías sin LEI en presencia del estado integrado de los VPH-AR, estos resultados sugerían que al igual que la expresión anormal de Ciclina E1 en estas LEIBG podría ser un reflejo de la integración viral en estas lesiones (Zubillaga-Guerrero *et al.*, 2013). En este estudio analizamos todas las etapas del CC, encontrando que la expresión de Ciclina E1 se encontró en todas aquellas mujeres con algún tipo de VPH-AR, no obstante observamos que el porcentaje de núcleos positivos, intensidad de la inmunotinción y el incremento en las aberraciones nucleares incremento con el grado de LEI. Estudios previos han reportado que la integración viral es un evento temprano en la carcinogénesis cervical (Kulmala *et al.*, 2006; Peitsaro *et al.*, 2008) y también se ha reportado que la integración da lugar al incremento de la oncoproteina E7 la cual se considera puede activar de manera endógena a Ciclina E1 alterando el patrón de expresión normal de la misma en CC (Quade *et al.*, 2008; Nguyen *et al.*, 2008). Es importante mencionar que el estado integrado de los VPH-AR se relacionó con el incremento en la expresión de Ciclina E1, lo cual sugiere CCNE1 puede utilizarse como marcadorcelular en la detección de LEIBG con riesgo de una LEIAG, no

obstante se requieren estudios de seguimiento a gran escala para evaluar el valor pronóstico de esta proteína en las LEIBG.

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ANEXO 1

The integration of HR-HPV increases the expression of cyclins A and E in cytologies with and without low-grade lesions

Original Article

The integration of HR-HPV increases the expression of cyclins A and E in cytologies with and without low-grade lesions

ABSTRACT

Background: Cyclin-A and cyclin-E are regulators of G1–S phase of normal cell cycle. Integration of human papilloma virus high-risk (HR-HPV) could alter this mechanism, and its overexpression has been associated with poor prognosis in cervical cancer.

Aim: To determine the expression of cyclin-A and cyclin-E, types of HR-HPV and physical state of DNA in cytologies with the diagnosis of low-grade squamous intraepithelial lesion (LSIL).

Materials and Methods: 115 cytological specimens in liquid base (liquid-PREP™) were analyzed. 25 specimens were with no signs of SIL (NSIL) and without HPV; 30 with NSIL with low-risk HPV (LR-HPV); 30 with NSIL with HR-HPV; and 30 with both LSIL and HR-HPV. The expression of cyclins 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. The physical state of HPV was evaluated by *in situ* hybridization with amplification with tyramide.

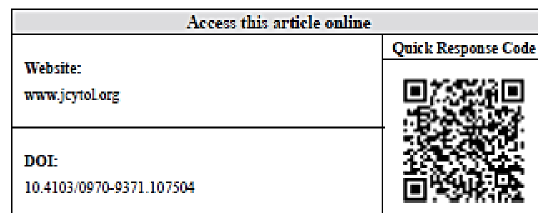
Results: In the cytologies NSIL with LR-HPV, the expression of cyclin-A and cyclin-E was found respectively in 23.3% and 33.3% of the specimens. Among the specimens of NSIL with HR-HPV, 33.3% expressed cyclin-A and 40% cyclin-E, while 100% of the LSILs expressed the 2 cyclins. On the other hand, 100% of the samples NSIL with LR-HPV presented an episomal pattern. Of the specimens of NSIL with HR-HPV, 56.6% exhibited an episomal pattern, 23.3% integrated and 20%, mixed. Among the LSILs, 90% were mixed and 10% integrated.

Conclusions: The cyclins A and E are present in the LSILs that occur predominantly in mixed state in the presence of HR-HPV.

Key words: Cyclin-A; cyclin-E; high-risk human papillomavirus; *in situ* hybridization; low-grade squamous intraepithelial lesion.

Introduction

Invasive carcinoma of the uterine cervix involves precursory stages known as squamous intraepithelial lesions (SIL).[1] Cytologically, SILs are divided into low-grade SIL (LSIL) and high-grade SIL (HSIL). LSIL represents an earlier diagnosis in cervical carcinogenesis. 80% of these lesions are associated with high-risk human papillomavirus (HR-HPV).[2] In the state of Guerrero, Mexico, 10 different types have been encountered: 16, 18, 31, 33, 35, 39, 45, 52, 58 and 59. HPV-16 is the most frequently found in cervical carcinoma (68.1%) and in HSIL (27.4%).[3]



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SIL emerges after a long period of viral persistence, as a result of viral genome integration into the host cell's genome, provoking E2 function loss and overexpression of E6 and E7, prerequisites for development of HSIL and invasive carcinoma.[4] It has been proposed that *in situ* hybridization (ISH) may detect the presence and physical state of HR-HPV

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DNA. The diffuse signal of viral DNA indicates an episomal state while the punctate signal indicates integration into the cellular genome.[5,6] In LSIL with HPV-16, the episomal state has been encountered in 15.4% of the cases; the integrated state in 7.7%; and the mixed state in 76.9%.[7]

Cyclins participate in various phases of the cellular cycle. Cyclin-E is synthesized in the late G_1 phase and is indispensable for moving into phase S. In normal cells, the cyclin-E expression diminishes rapidly as the cell enters into phase S. In premalignant and malignant lesions of the uterine cervix with expression of HR-HPV E7, the levels of cyclin E/cdk2 have been found to be increased.[8] It has been reported that expression of HPV-16 E7 could induce transcription of the promoter of cyclin-A through the binding site to E2F. This observation could suggest that activation is implicated in cyclin-A levels and this association could be necessary for cellular transformation.[9] It has been reported that cyclin-E and cyclin-A expression is an indicator for poor outcome in cervical carcinomas associated with HR-HPV.[10]

The aim of this study was to determine the immunoexpression of cyclins A and E, the physical state of HR-HPV DNA, in cytologies with and without LSIL, and to identify possible biomarkers of early cervical lesions.

Materials and Methods

Study subjects

115 female residents of the state of Guerrero, Mexico, were participants in this study approved by the Ethics Committee of the Autonomous University of Guerrero in the period 2010-2012. Each one of the participants signed informed consent and responded to a questionnaire with the purpose of obtaining sociodemographic, clinical and obstetrical information.

Specimen collection

All women included in this study provided exo-endocervical exfoliated cell samples collected by sampling the ectocervix with an Ayre spatula and endocervix with a cytobrush, making sure that cytologic material from the transformation zone was taken. Smears were used for cytomorphological examination using conventional Papanicolaou and cytological specimens in liquid base liquid-PREP™ (LPT) and read by an experienced cytopathologist and classified according to the Bethesda System.[11]

Subsequently, the specimens were classified into four groups for the cytological study: (a) no signs of SIL (NSIL) and HPV (i.e., NSIL without HPV) (25), (b) NSIL with LR-HPV (30), (c) NSIL with HR-HPV (30) and (d) with LSIL and HR-HPV (30).

HPV detection and typification

The DNA was extracted in accordance with the standard SDS-proteinase K-phenol-chloroform method.[12] DNA amplification was done in a 2400 GeneAmp polymerase chain reaction (PCR) system (Applied Biosystems, Foster City, CA, USA). The products of PCR were analyzed by electrophoresis and were displayed on 1.5% agarose gels dyed with ethidium bromide. 1 pg and 1 ng HPV-6 recombinant plasmid DNA were used as positive controls; sterile water was used as the negative control. The products of PCR were subjected to digestion with restriction enzymes *BamHI, DdeI, HaeIII, HinfI, PstI, RsaI y Sau3AI* (Invitrogen, Carlsbad, CA, USA). The viral type was determined by restriction fragment length polymorphism (RFLPs).[13] When specimens were being analyzed with the GP5+/6+ system, they were subjected to sequencing in an automated system (310 ABI-PRISM Genetic Analyzer, Applied Biosystems, Foster City, CA, USA). The obtained sequences were compared with HPV types' known sequences available on the NCBI website (http://www.ncbi.nim.nih.gov).[14]

Expression of cyclins A and E by immunocytochemistry

The expression of cyclins was determined by the streptavidin biotin peroxidase immunocytochemical method, utilizing the Cytoscan HRP/DAB Cell detection system (Cell Marque Corporation, Hot Springs, AR, USA). The monoclonal antibodies used were: cyclin-A (6E6; 1:100; Novocastra, Newcastle-Upon-Tyne, UK) and cyclin-E (13A3; 1:30; Novocastra, Newcastle-Upon-Tyne, UK). The cytology slides in liquid base were subjected to antigen retrieval (Immuno DNA Retriever with citrate, Bio SB Inc., Santa Barbara, CA, USA) for 6 minutes at 120°C. The primary antibody was added for 1 hour, and then the secondary antibody coupled with biotin was added. Streptavidin peroxidase was also added. For development, the chromogen DAB was used and Mayer's hematoxylin was used as a contrast dye. The cell line HeLa (HPV-18), which overexpresses cyclins, was used as positive controls. The same line, but omitting the primary antibody, was used as negative controls.

Expression of cyclins A and E

Expression was evaluated using the following criteria: *Weak*, if the colored reaction product did not obscure hematoxylin-counterstained chromatin; *moderate*, if the colored reaction product partially obscured counterstained chromatin; and *strong*, if the colored reaction product completely obscured counterstained chromatin.[15] In our experience, reproducibility of interpreting *weak* staining was poor, and we therefore scored cases with weak and no staining together as negative. *Moderate* and *strong* cyclins staining was scored as positive and was based on the presence of staining. We also evaluated the percentage of positive nuclei (0%, 1-10%, 11-50% and > 51%).[16]

In situ hybridization

Detection of the viral genome was done with a system of tyramide signal amplification (GenPoint Dako Cytomation, Carpinteria, CA, USA). The monolayer smears were submitted to digestion for 1 minute with proteinase K (1:1000). A drop of test reagent (biotinylated viral DNA) with probes for 13 HR-HPV genotypes (16, 18, 31, 33, 39, 45, 51, 52, 56, 58, 59 and 68) and with individual probes directed at HPV-6 and -11 was applied afterwards. The slides were denatured for 10 minutes and subjected to hybridization for 20 hours (Hybridizer Dako, Carpinteria, CA, USA). They were then placed in an astringent solution; primary streptavidin peroxidase was added, afterwards biotinyl–tyramide and then secondary streptavidin. DAB was added and finally Mayer's hematoxylin (Merck). Positive reaction was visualized with a brown color inside of the nucleus and according to the type of signal was classified as diffuse (episomal state), punctate (integrated state) or mixed (diffuse and punctate).[5] SiHa cell lines (HPV-16) were used as positive controls that showed an integrated state; the same cell lines without the probe were used as negative controls.

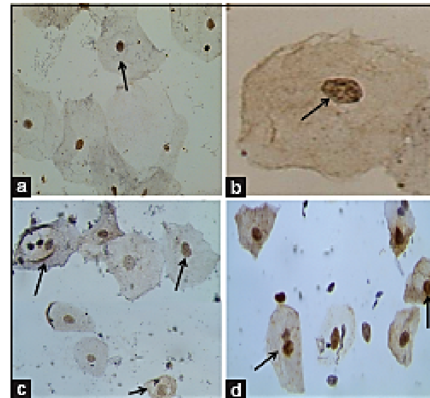

Figure 1: Physical state HPV-16 DNA and expression of cyclin A and E; (a) Specimen NSIL, showing normal cells that presented as an episomal pattern (black arrow); (b) Specimen LSIL showing cells with karyomegaly presented as an integrated pattern (black arrow); (c) Positive for cyclin A expression, cells intermediate with karyomegaly (black arrows); (d) Expression of cyclin E positive, cells intermediate with binucleation and karyomegaly (black arrows). (Technique: *In situ* hybridization with tyramide amplification, ×400 and ×1000; Streptavidin biotin peroxidase, ×400)

Statistical analysis

Fisher's exact test was used for comparison of frequencies. To determine the relationship of cyclin expression and cervical lesion or physical state of DNA, multinomial logistic regression models were evaluated. The statistical analysis was done with the software STATA, version 11.1. A value of $P < 0.05$ was considered significant.

Results

The age of the women was between 20 and 67 years old, with a median age of 44. The majority of women with LSIL and HR-HPV were between 51 and 60 years old (80%) and have had three or more sexual partners ($P < 0.001$) (data not shown).

Frequency of HPV genotypes and viral DNA physical States

A total of 9 HR-HPV genotypes were identified: 16, 18, 31, 35, 39, 45, 52, 58 and 59. HPV-16 was most frequent in NSIL (60%) and LSIL (53%) cytologies ($P < 0.001$). 90% of the LR-HPV specimens exhibited HPV-6 and only 10% corresponded to type 11 (data not shown). In 100% of the specimen with NSIL with LR-HPV, viral DNA was found in episomal form in a scant number of cells, while for the specimens NSIL with HR-HPV, the episomal state was observed in 56.6% of the cases [Figure 1a], mixed in 20% and integrated in 23.3%. For the specimens with LSIL and HR-HPV, 90% presented a mixed pattern and 10% an integrated pattern [Figure 1b].

Expression of cyclins A and E and their relationship with cytological diagnosis

Expression of cyclins A and E increased gradually within the study groups. In 100% of the LSIL specimens, these cyclins' expression was found ($P < 0.001$) [Table 1]. Also in the LSILs, the cyclin's expression was moderate to strong in some intermediate cells with koilocytosis, karyomegaly and discrete perinuclear halos [Figure 1c and d], compared with the cytologies NSIL with HR-HPV where the expression of these proteins was found weak to moderate for cyclin-A (33.3%) and cyclin-E (36.7%) in some normal intermediate cells (data not shown).

Association of expression of cyclins A and E with cytological diagnosis

Expression of cyclins A and E is strongly associated with LSIL (OR: 393 and 188.3 respectively, $P < 0.001$) in comparison with the women with NSIL and without HPV [Table 2] in the same comparison. In the specimens with NSIL with HR-HPV, this association was lesser for the cyclins A and E, with ORs of 5.9 and 5.8, respectively, in comparison with women with NSIL and without HPV [Table 2].

Analysis of HR-HPV DNA state and its relationship with expression of cyclins A and E for diagnosis

Specimens from those women who exhibited mixed (90%) and integrated (10%) patterns and LSIL were related to greater frequency with the expression of cyclins A and E in comparison with the cytologies NSIL with HR-HPV ($P < 0.001$) [Table 3].

Analysis of HR-HPV DNA state and its relationship with expression of cyclins A and E and HPV type

The mixed and integrated state and the presence of HPV-16 are related more frequently to the expression of cyclins A (72%) and E (76%) [Table 4] and HPV-6 and -11 with episomal

Table 1: Expression of cyclin A and E and their relationship with the cytological diagnosis

Expression of cyclins	Study groups				
	NSIL without HPV; *n*=25 (%) *n*	NSIL with LR-HPV; *n*=30 (%) *n*	NSIL with HR-HPV; *n*=30 (%) *n*	With both LSIL and HR-HPV; *n*=30 (%) *n*	*P* trend*
Cyclin A					
Positive	16 (4)	23.3 (7)	33.3 (10)	100 (30)†	<0.001
Negative	84 (21)	76.7 (23)	66.7 (20)	0	
Cyclin E					
Positive	16 (4)	33.3 (10)	40 (12)	100 (30)†	<0.001
Negative	84 (21)	66.7 (20)	60 (18)	0	

HR-HPV = Human papilloma virus risk-high; LR-HPV = Human papilloma virus risk-low; NSIL = No sign of intraepithelial lesion; LSIL = Low grade squamous intraepithelial lesion; *Rank sum test for trend; †P<0.001 compared with NSIL without HPV; NSIL with LR-HPV and NSIL with HR-HPV

Table 2: Expression of cyclin A and E with the cytological diagnosis

Diagnosis	Cyclin A OR (IC 95%)	Value *P*	Cyclin E OR (IC 95%)	Value *P*
NSIL without HPV*	1.0		1.0	
NSIL with LR-HPV	1.4 (0.3-6.9)	0.655	2.7 (0.7-10.8)	0.165
NSIL with HR-HPV	5.9 (1.2-28.3)	0.028	5.8 (1.4-23.8)	0.016
With both LSIL and HR-HPV	393 (28.1-∞)	<0.001	188.3 (17.8-∞)	<0.001

HR-HPV = Human papilloma virus risk-high; LR-HPV = Human papilloma virus risk-low; NSIL = No sign of intraepithelial lesion; LSIL = Low grade squamous intraepithelial lesion; OR = Odds ratio adjusted for age; CI = Confidence interval; *Reference category

Table 3: Status physical of HR-HPV DNA and its relationship with the expression of cyclin A and E for cytological diagnosis

Physical state	NSIL with LR-HPV % (*n*)	Value *P**	NSIL with HR-HPV % (*n*)	Value *P***	With both LSIL and HR-HPV % (*n*)
Cyclin A					
Episomal	100 (7)		70 (7)		0
Integrated	0	0.228	30 (3)	<0.001	10 (3)
Mixed	0		0		90 (27)
Cyclin E					
Episomal	100 (10)		58.3 (7)		0
Integrated	0	0.059	33.3 (4)	<0.001	10 (3)
Mixed	0		8.3 (1)		90 (27)

HR-HPV = Human papilloma virus risk-high; LR-HPV = Human papilloma virus risk-low; NSIL = No sign of intraepithelial lesion; LSIL = Low grade squamous intraepithelial lesion; *Compared NSIL with LR-HPV; **Compared NSIL with HR-HPV

Table 4: Analysis of the status physical of HPV DNA and its relationship with the expression of cyclin A and E and HPV genotypes

Genotypes HPV	16	18	31	35	39	45	52	58	59
Physical status % (*n*)									
Cyclin A									
Episomal	7 (28)	0	0	0	0	0	0	0	0
Integrated	2 (8)	1 (100)	1 (25)	1 (100)	0	1 (50)	0	0	0
Mixed	16 (64)	0	3 (75)	0	2 (100)	1 (50)	2 (100)	2 (100)	1 (100)
Cyclin E									
Episomal	6 (24)	0	0	0	0	0	0	0	1 (50)
Integrated	3 (12)	1 (100)	1 (25)	1 (100)	0	1 (50)	0	0	0
Mixed	16 (64)	0	3 (75)	0	2 (100)	1 (50)	2 (100)	3 (100)	1 (50)

*Data expressed in % (*n*)

pattern were related to expression of these proteins. Nevertheless, the expression of these proteins was found weak in some normal intermediate cells (data not shown).

Discussion

The prevalence and incidence of cervical cancer has decreased worldwide. This decrease is due to the introduction and improved penetration of cervical cancer screening programs,[17] the quality and reliability of which are further increased by HPV testing. However, several studies have shown that cytology has a limited sensitivity for detecting SIL because of subjectivity and interobserver and intraobserver variabilities;[18] in our study the cytological diagnosis was verified by PCR, RFLPs or sequencing.

Our data showed viral HR-HPV integration (10%) in LSIL. Furthermore, these lesions exhibited HPV-16 with greater frequency. In a cervical scraping study of 40 LSIL cases with HPV-16, the integrated pattern was found in 5% of them suggesting that viral integration is an early event in the progression of the disease; furthermore studies have demonstrated that cell populations with integrated HPV-16 posses a selective growth advantage compared to cells that maintain HPV-16 viral genomes as episomes.[19] We found mixed status viral DNA with greater frequency in LSIL (90%). This observation could indicate a low number of integrated and episomal copies that sustain the latent pattern of the virus and during the incubation period; the virus is first assumed to be episomal, after which a selection of integrated cell clones arises from a mixture of cells with both episomal and integrated HPV. These findings are similar to a study in which we found with greater frequency (76.9%) mixed forms in LSILs associated with HPV-16.[7]

It has been found that integration depends on several factors including the presence of HPV-AR (16 and 18) and also the presence of fragile sites genomic regions that are prone to chromosomal breaks that facilitate integration of foreign DNA;[20] it is suggested to further explore the relationship of the physical state of HR-HPV DNA with quantitative methods such as real-time PCR relative to the expression of cyclins A and E; this could be a complementary tool for cytological evaluation, as it could reduce the number of unnecessary colposcopy-guided biopsies in women with LSIL cytologic diagnosis and follow-up studies could be performed to identify patients who are at risk of developing HSIL and cervical cancer.[21] Moreover, in cytologies NSIL with HR-HPV, 23.3% showed the integrated pattern. This could suggest that these women have probability of progressing to LSILs. A rapid progression from NCIN to CINIII reported showed that the integrated and episomal forms of HPV-16 were found along with a heavy viral load.[19] These results approach reports which found the integrated pattern associated with HPV-16 in 11% and 20% of the cytologies NSIL,[7,22] unlike that reported in cases with LSIL and HPV-16 where the researchers did not observe viral integration.[23]

In our study, women with NSIL with LR-HPV presented only the viral episomal state. This agrees with another study in which only the episomal pattern in cases NSIL with HPV-6 was observed, as well as a low viral load, suggesting that the low number of copies of this virus could maintain E2 intact, by which the cellular cycle is not altered and morphological alterations are not observed.[24]

It is known that the HPV contributes to neoplastic progression predominantly through the action of two viral oncoproteins, E6 and E7, which interact with various host regulatory proteins such as cyclins A and E to influence the function or expression levels of host gene products, eventually leading to the disruption of the cell cycle.[25] In this regard, a number of evidence exists establishing that the cyclins are associated with the expression of oncoproteins and eventually also involved in methylation. Cyclin A promoter hypermethylation was found associated with the development of cervical cancer, but not in the LSILs,[26] though this relationship was not found in respect to cyclin E. It will be important to further explore the expression of cyclins and relate these methylation.

In a monitoring study on biopsies of premalignant and malignant uterine cervix lesions with HR-HPV, in which expression of cyclins A and E was evaluated, researchers observed expression of these proteins as the lesion grade increased, suggesting that upon these proteins being overexpressed in early lesions, they may be indicators of bad prognosis.[10] We encountered a significant relationship between LSILs and the expression of the cyclins A and E [Table 2].

In the women we studied with both LSIL and HR-HPV, 100% were positive for cyclins A and E. Besides, they had expression moderate to strong of some nuclei positives that related to the alterations characteristic of infection by HPV (koilocytosis, karyomegaly and discrete perinuclear halos), while the other study groups did not exhibit this characteristic. This results concur with the findings in LSILs in which 96.7% of the expression of cyclin-E was associated with HR-HPV.[8] This could be due to the fact that coupling of E7 with E2F results in prolongation of phase S, a condition which prevents inhibition of the cyclin-E/Cdk complex[9] and if you also add the presence of integrated DNA this could be associated with the fact to have found intermediate cells (undifferentiated) that they should not be in proliferation, which were the only ones that showed expression in positive nuclei with moderate to strong expression. With regard to the expression of cyclin-A this could be due to activation of cyclin-A by HR-HPV oncoproteins E6 and E7 seem altered by degradation of p53 by E6, a condition that prevents transcription of protein inhibitors of the Cdk-cyclin complex, such as p27^{Kip1}, being initiated, and consequently the increase in expression of cyclin A as well. On the other hand, E7 in conjunction with Cdk2/cyclin-A is important in cellular transformation.[9] The specific role of cyclins A and E as a biomarker of LSILs may be a bit controversial. Complementary studies are required to evaluate quantitatively the presence of these cyclins in LSIL.

We also observed that cytologies NSIL with HR-HPV were positive for cyclins A and E in a minor percentage in comparison with cases with LSIL and HR-HPV, a result that

has not been previously reported. These findings show that despite the fact that these cells do not present any morphological alterations, the elevated expression of these proteins could allow on a molecular level prior detection of deregulation of the cell cycle induced by HR-HPV. The immunoexpression could be due to the cyclin E/Cdk2 and cyclin A/Cdk2 complex being augmented in phase S. It is probable that cells found positive were encountered in this cell cycle phase stimulated by the presence of the HR-HPV oncoprotein E7. To date, there are no reports which have evaluated these proteins in women NSIL with LR-HPV. It has been shown that LR-HPV oncoproteins possess low affinity for cellular proteins pRb and p53. LR-HPV specimens having low immunoexpression of cyclins and therefore a low number of copies in episomal form.[27]

In our study, 16% of normal cytology without HPV showed weak expression of cyclins A and E in parabasal and basal cells. Similar results found that 8% of normal epithelial cells or metaplastic nuclear staining showed mild expression of cyclin-;[8] sporadic expression of cyclin-E in parabasal cells of normal squamous epithelium has also been observed,[28] while another study reported sporadic expression of cyclin-E and cyclin-A in <1% in the parabasal cell nucleus.[29]

Our study has limitations as to the quantitative evaluation of the expression of cyclins and in determining the number of copies integrated in the LSILs episomal; however, no studies have evaluated together where expression of cyclins A and E, HR-HPV types and physical state of the DNA in early stages of cervical carcinogenesis, so this study could be important for the explanation of the pathogenesis, recognizing that they have limitations as quantification, so it is suggested to consider this relationship.

In conclusion, the cyclins A and E were present in all cases of LSIL, this expression occurred predominately as mixed state in the presence of DNA of HR-HPV; these results may help understand the pathogenesis of HPV infection, so further large-scale studies are needed to examine the clinical usefulness of cyclins A and E immunoexpression and HR-HPV ISH signal patterns, alone or in combination, as markers for identifying LSIL.

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ANEXO 2

MicroRNA-16-1 in the pathogenesis of cervical cancer

Review Article
Oncology Letters

MICRORNA-16-1 IN THE PATHOGENESIS OF CERVICAL CANCER

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Abstract

Cellular and viral microRNAs (miRNAs) are the transcriptional products of RNA polymerase II and are regulated by transcriptional factors for their differential expression. The altered expression of miRNAs in many cancer types has been explored as a marker for possible diagnosis and therapy.MiR-16-1 belongs to the miR-16 cluster and has been implicated in various aspects of carcinogenesis including cell proliferation and regulation of apoptosis; however, its function and molecular mechanism in cervical cancer (CC) is not clear. High-risk human papillomavirus (HR-HPV) E5, E6 and E7 oncoproteins have the ability to deregulate several cellular processes, mostly apoptosis, cell cycle control, migration, immune evasion, and induction of genetic instability, which promote the accumulation of mutations and aneuploidy. Recent evidence suggests miR-16-1 may have a function like as oncomir because it has been found that this miRNA to interact with the oncoprotein E7 of HR-HPV favors the development of CC. Thus, in the present review article, we show the most recent studies on the expression profile of miR-16-1 and the role of HR-HPV on the expression of miR-16-1 CC.

Keywords: miR-16-1, cervical cancer, E7, HR-HPV

1. Introduction

Cervical cancer (CC) is the second most common cancer in women worldwide and is caused by persistent infection of human papillomavirus high risk (HR-HPV) **(Moscicki AB, 2012).** Among the HR-HPV, HPV-16 and HPV-18 together contribute approximately 70% of cases worldwide CC **(de Sanjose S, 2010).** The productive life cycle of HPV is tightly linked to the differentiation of squamous cells **(Meyers C, 1992).** HPV infection is initiated when viral particles enter undifferentiated basal epithelial cells through abrasion or injury. Viruses help the expansion of the population of infected cells, and then the infection is established. The extrachromosomal viral DNA amplification and expression of the viral capsid proteins occurs sequentially in the intermediate and surface cells **(Wang HK, 2009).** Persistent infection of HR-HPV can lead to cervical intraepithelial neoplasia (CIN) grade 1, 2 or 3. Histologically, abnormal proliferative cells are restricted to the first third of the cervical epithelium are considered CIN1. CIN2 and CIN3 are characterized by the expansion of the second third neoplasia (CIN2) or more (CIN3) epithelium. CIN3 may include the entire thickness of the epithelium and are sometimes referred to as cervical carcinoma *in situ* **(Massad LS, 2012).**

The viral oncoproteins E6 and E7 of HR-HPV destabilize respectively to two major tumor suppressor proteins, p53 and retinoblastoma protein (pRb). Oncoproteins function is to support both the productive phase of infection in differentiated post-mitotic cells. However, high expression of E6 and E7 of HR-HPV in the basal epithelial cells disrupted with cell cycle regulation, inhibits cell differentiation, induce chromosomal damage, and prevents apoptosis, resulting in cell immortalization and transformation, bases HPV carcinogenesis **(Scheffner M, 1990; Roman A, 2013).**

MicroRNAs (miRNAs) are small non-coding RNAs of ~21nt, are derived from transcripts of RNA polymerase II or antisense gene, expression is subject to transcriptional and posttranscriptional regulation. MiRNAs act at mRNA by inhibiting translation or mRNA degradation target. Base perfect complementarity between miRNAs and mRNA degradation induced transcripts, while several base paired not induce arrest of translation **(Bartel DP, 2009; Doench JG, 2003).** MiRNAs are differentially expressed in many human cell types and some miRNAs are considered oncogenes or tumor suppressor genes **(Landgraf P, 2007; Chen CZ, 2004).** Tumor suppressor miRNAs, miR-16-1 and miR-15a are expressed as a miRNA cluster are located in the transcribed region an intron DLEU2 (Deletion chronic lymphocytic leukemia 2) influencing cell proliferation, survival and invasion. Recent studies have reported an increase in the expression levels of miR-15a and miR-16-1 in CC tissues compared with normal cervical tissues; however, overexpression of these miRNAs not seem to affect

the growth of cells CC **(Calin GA, 2008; Kaddar T, 2009; Zheng ZM, 2011).** Interestingly, apparent discrepancies have been reported between the known functions of miR-16-1 as an inhibitor of cell cycle and its prognostic value in certain cancers, reflecting the complex role of miR-16-1 in the tumorigenic process. This complexity can be explained by its function as a tumor suppressor gene or its function as oncomir as well as the cell type **(Calin GA, 2008; Wang S, 2008).** In this review, we describe the most recent studies on the expression profile of miR-16-1 and the role of HR-HPV on the expression of miR-16-1 in CC.

2. **Biogenesis and regulation of miRNAs**

MicroRNAs are a new class of endogenous small RNAs which have been associated with a variety of human diseases, including cancer. In recent years, miRNAs have been extensively studied and are known to play an important role in the regulation of important cellular processes such as cell proliferation, apoptosis, differentiation and cell migration **(Suzuki HI, 2011).** MiRNAs genes may be located in introns, exons and intergenic noncoding regions of genomes. The biogenesis of miRNA begins with the transcription of pri-miRNA in the nucleus; is initially transcribed by polymerase II into primary miRNAs (pri-miRNAs). The pri-miRNAs are subsequently cleaved in the nucleus by a microprocessor complex composed of Drosha and DGCR8 (Pasha) into a stem–loop precursor miRNA (pre-miRNA) which has a length of approximately 70 nt. Pre-miRNAs are in turn transported by exportin-5into the cytoplasm, where they are cleaved by a RNase III family member Dicer, into 22-nt mature miRNAs. The mature miRNAs are able to be incorporated into the RNA-induced silencing complex (RISC) that contains PACT, Dicer, Ago2, and TRBP. RISC functions to degrade mRNA or block protein translation by either binding mRNA through a perfect complementary match or through an imperfect match in the 3′ untranslated region (UTR) **(Lee Y, 2003; Newman MA, 2010).**

The biogenesis of miRNAs involves several steps which can be regulated by several mechanisms including: epigenetic regulation **(Cai Y, 2009)**, miRNA edition **(Yang W, 2006),** self-regulation in the way of miRNAs biogenesis **(Han J, 2003),** formation of the stem-loop structure **(Zhang X, 2010),** crosslinking between miRNAs biogenesis pathway and other cellular signaling pathways **(Suzuki HI, 2009),** regulation of feedback between miRNA and target gene **(Zhao C, 2009).** However, some miRNAs maturation does not follow the path of biogenesis **(Cheloufi S, 2010; Yang JS, 2010).**

3. miR-16-1/miR-15a cluster and Cancer

In contrast to oncogenic miRNAs, other miRNAs have activity and tumor suppressor genes are deregulated in cancer. Some studies have shown strong implications of miR-16-1 and miR-15a as tumor suppressor miRNAs. MiR-16-1/miR-15a cluster belongs to the family of miR-16; it was first described in patients with chronic lymphocytic leukemia (CLL) **(Calin GA, 2002)**. These miRNAs are located in chromosome 13q14 DLEU2 gene (deletion in chronic lymphocytic leukemia 2), a region that is frequently deregulated in B-cell CLL (B-CLL), two genes have been reported deregulated or inhibited in most CLL cases **(Calin GA, 2002, Calin GA, 2005).**

MiR-16-1 is considered as an important regulator of cell cycle **(Linsley, 2007; Liu O, 2008),** because it has been reported that this has important cellular targets genes involved in apoptosis, cell cycle, proliferation, angiogenesis, cell migration and differentiation **(Table 1).**

Table 1. Gene targets of miR-16-1 in cancer and cellular processes

Type of cancer	Gene targets	Cellular process	Chromosomal localization	Reference
Chronic lymphocytic leukemia (CLL)	BCL2	Apoptosis	18q21.3	**Cimmino A, 2005**
Mantle cell lymphoma (MCL)	CCND1	Cell cycle	11q13	**Chen RW, 2008**
Lymphoma cell lines MYC5	c-Myb	Cell cycle	6q22-q23	**ChungEY, 2008**
Hepatic stellate cells (HSCs)	CCND1	Cell cycle	11q13	**GuoCJ, 2009**
Breast cancer Cervical cancer (CC)	Caprin-1 HMGA1 CCNE1	Cell cycle	q1134-07-34.12 6p21 19q12	**Kaddar T, 2009**
Chronic lymphocyti leukemia (CLL)	CCND1 CCNE1	Cell cycle	11q13 19q12	**Lerner M, 2009**
Multiple myeloma (MM)	VEGF	Cell proliferation	6p12	**Roccaro AM, 2009**
Prostate cancer	Cyclin D3 CDK1 CDK2 Cks1 TACC1 TACC3	Cell cycle	6p21 10q21.2 12q13 1q21.2 8p11 4p16.3	**Takeshita F, 2009**
Breast cancer (MCF7) Lung cancer (A549) Cervical cancer (HeLa)	CCNE1	Cell cycle	19q12	**Wang F, 2009**
Bovine aortic endothelial cells and human	VEGFR2 FGFR1	Angiogenesis	4q11-q12 8p11.23-p11.22	**Chamorro-Jorganes, 2011**
Hepatoma cells (HepG2)	PCMT1	Apoptosis	6q25.1	**Sambri I, 2011**
Leukemic cells (K562, HL-60)	WT1	Apoptosis	11p13	**Sheng-Meng G, 2011**
Lung cancer (A549) Breast cancer (MCF7) Heart cancer (293a)	ARL2	Cell proliferation	11q13	**Wang, 2011**
Hepatoma cells (HepG2)	COX-2	Cell proliferation	1q25.2-q25.3	**Agra-Andrieu N, 2012**
Osteosarcoma cells (SOSP-9607, MG63)	CCND1	Cell cycle	11q13	**Cheng-Kui C, 2012**
Human monocyte (U937) and biliary epithelial cells (H69)	SMRT	Co-repressor of transcription	12q24	**Zhou R, 2012**
Osteosarcoma (SOSP-9607)	IGF1R	Progression and differentiation cell Cell migration	15q26.3	**Chen L, 2013**
Bladder cancer (TCHu-1)	CCND1	Cell cycle	11q13	**Qi-Quan J, 2013**

Cells of non-small lung cancer (NSCLC)	HDGF	Cell proliferation Angiogenesis	1q23.1	**Ke Y, 2013**
Multiple myeloma (MM)	VEGF-A	Angiogenesis	6p12	**Chun-Yan S, 2013**
Glioma cells (SHG44, U87, U373)	BCL2 NF-kβ1 MMP-9	Apoptosis Proliferation, migration and angiogenesis cell	18q21.3 4q24 20q13.12	**Tian-Quan Y, 2014**
Gastric adenocarcinoma (GAC)	YAP1	Cell proliferation	11q13	**Kang W, 2015**

In most cancers described above miR-16-1 or miR-15ahave a role of tumor suppressors, which are deregulated and encouraging the development and progression of cancer in these studies have found that by inducing expression of these miRNAs, some functions are reset target genes, which in most cases are tumor suppressor genes and this negatively regulates the development of cancer, so in these studies is considers these miRNAs as important therapeutic targets in cancer.

4. **Mechanisms of regulation of miR-16-1/miR-15a**

During development, the expression of miRNAs is tissue specific, it has been suggested that miRNAs may play a critical role in the establishment and maintenance of cell type and tissue identity **(Lagos-Quintana M, 2002; Giraldez AJ, 2005).** However different expression profiles analyzed, have shown that some miRNAs are regulated only post-transcriptional level, however, several studies have reported transcriptional regulation as the primary mechanism for regulating the expression of miRNAs **(Bartel DP, 2004).** Not only the factors transcription are key regulators of the genetic expression. Epigenetic mechanisms such as DNA methylation, histone modifications and translational nucleosomal remodeling, all can contribute to the modulation of gene expression and determination of cell-tissue specificity **(Jones PA, 2007).** However, the structure and transcriptional regulation of genes miRNAs are largely unknown. Some of these regulatory mechanisms known to miR-16-1/miR-15a they described below:

C-Myc

C-Myc is a potent transcriptional regulator expression of miRNAs **(O'Donnell KA, 2005) (Table 2).** Overexpression of c-Myc represses expression widely miRNAs. Assays chromatin immunoprecipitation revealed that this repression is due to direct binding of c-Myc promoters miRNAs, includinglet-7a-1/f-1/d, miR-15a/16-1, miR-22, miR-26a-2, miR-26b, miR-26b, miR-29a/b-1, miR-29b-2/c, miR-30e/30c-1, miR-34a, and miR-146a **(Chang TC, 2008) (Table 2).**

p53

The tumor suppressor p53 can interact with the Drosha/p68 complex to facilitate the processing of pri-miRNAs by Drosha mediated, consequently promotes expression of miR-15a/16-1, miR-103/107,miR-143/145, miR-203and miR-206 at the posttranscriptional level **(Suzuki HI, 2009) (Table 2).**

C-Myc, C-Myb, PPAR

Furthermore it has been reported that the expression of miR-16-1/miR-15a can be regulated by the union of c-Myc, c-Myb o PPAR (peroxisome proliferator-activated receptor δ) to the promoter region of the gene DLEU2 regulating positively (c-Myb and PPAR) or negatively (c-Myc) the transcription DLEU2 **(O'Donnell KA, 2005; Zhao OH, 2009; Yin KJ, 2010) (Table 2).**

E2F

E2F1 is a member of the E2F family, there are reports that this transcription factor in addition to mediate apoptosis and autophagy **(Polager S, 2008; Tracy K, 2007).** It has a role in up regulation of expression of certain miRNAs as miR-15a/miR-16-1 and miR-15b/miR-16-2 cluster. In addition to oncogene transcriptionally regulates Cyclin E1 (CCNE1) a positive regulator of the cell cycle at the G1-S phase, interestingly these miRNAs can repress expression of CCNE1 **(Ofir M, 2011) (Table 2) (Figure 1).**

Nucleolin

Recently it was found that the protein nucleolin is necessary for the proper processing of miR-16-1 / miR-15a, mainly in the processing of pri-miRNA complexed with Drosha and DGCR8 in the core. It was observed that in the absence of this protein extracts from HEK293 cell and MCF-7 cells are unable to process the miR-16-1/miR-15a *in vitro* **(Pickering BF, 2011) (Table 2).**

BRCA1

It has been reported that tumor suppressor gene for breast cancer 1 (BRCA1) increases the expression of both precursor and mature form of let-7a-1, miR-16-1, miR-145 and miR-34a; by direct association with Drosha microprocessor complex, suggesting that BRCA1 up regulates the biogenesis of miRNAs in interaction with the Drosha microprocessor complex and SMAD/p53/DHX9 **(Kawai S, 2012) (Table 2).**

HDACs

Another study shows that histone deacetylases (HDACs), which are overexpressed in CLL, may mediate the epigenetic silencing of tumor suppressor miR-15a, miR-16 and miR-29b. Consequently, patients with CLL exhibiting epigenetic silencing can benefit from a therapy based on inhibitors of HDACs **(Sampath D, 2012) (Table 2).**

MiR-709

Recent discoveries have shown that some miRNAs may regulate post-transcriptional level to other miRNAs has been reported that miR-709 mouse can join the pri-miR-16-1/miR-15a, preventing processing to pre-miR-16 -1/miR-15a, leading to suppression of the maturation of miR-16-1/miR-15a **(Tang R, 2012) (Table 2).**

Table 2. Regulatory mechanisms of miR-16-1/miR-15a

Regulatory Protein	**Type regulation**	**Up-regulation Or deregulation**	**References**
c-Myc	Transcriptional	Up regulation	**Chang TC, 2008**
P53/Drosha/p68	Posttranscriptional	Up regulation	**Suzuki HI, 2009**
c-Myb/PPAR/DLEU2	Transcriptional	Up regulation	**O'Donnell KA, 2005; Zhao OH, 2009**
c-Myc/DLEU2	Transcriptional	Deregulation	**Yin KJ, 2010**
E2F1	Transcriptional	Up regulation	**Ofir M, 2011**
Nucleolina/Drosha/DGCR8	Posttranscriptional	Deregulation	**Pickering BF, 2011**
BRCA1/Drosha /Smad/p53/DHX9	Posttranscriptional	Up regulation	**Kawai S, 2012**
HDACs	Epigenetic	Deregulation	**Sampath D, 2012**
miR-709	Posttranscripcional	Deregulation	**Tang R, 2012**

5. Expression of miR-16-1 in CC

The CC is one of the most common cancers and a leading cause of death in women worldwide (**Moscicki AB, 2012**). Epidemiological and experimental data have clearly demonstrated a causal role of HR-HPV in the initiation and progression of CC, affecting important cellular processes by inactivating p53 and pRB proteins. E5, E6 and E7oncoproteins have the ability to severely deregulated cellular processes such as apoptosis, cell cycle control, migration and induction of immune evasion genetic instability, processes which promote the accumulation of mutations and aneuploidy (**de Sanjose S, 2010, Scheffner M, 1990; Roman A, 2013**). In this scenario, the genomic profiles have shown that aberrant expression of tumor suppressors and oncomirs miRNAs play an important role in carcinogenesis of the CC. The HR-HPV infection and the expression of E6/E7 are essential but not sufficient for the development of CC; there are other genetic factors involved in this complex disease epigenetic. Recent evidence suggests a significant relationship between the levels of expression of E6/E7 of HR-HPV and cellular miRNAs and other non-coding RNAs **(Pedroza, 2014).**

In a study in CC cell lines, tissues of CC and normal tissue found that miR-16-1 has low expression in normal tissue compared to cell lines and CC cases where the expression was high **(Lui, 2007).** Similar results were obtained in tissue samples of squamous cell carcinomas compared with normal tissue **(Lee, 2008; Wang, 2008).** Interestingly it has been suggested that miR-16-1 may have an oncogenic role in promoting the growth of abnormal cells in the basal epithelial cells, since this miRNA is deregulated in NIC1/3 compared to normal tissue samples and overexpressed in CC **(Pereira, 2010).**

In CC these results suggested that the increased expression of miR-16-1 and miR-15a seemed to have an efficient tumor suppressors function in controlling cell growth CC. To see if these results were related to HR-HPV infection Northern Blot studies were made to compare expression levels of miR-16-1 on tissues derived from human keratinocytes with and without HPV-16 or HPV-18 infection, noting that there was an increase in miR-16-1 only in the presence of HPV-18. In addition to inducing expression of E6, E7 and E6/E7 it was found that increased expression levels of miR-16-1 was observed only in the presence of E7, this indicated that E7 was responsible for the overexpression of miR-16-1 in CC cells. This is corroborated when the expression of E7 silence by RNA interference (RNAi) in CaSki (HPV-16+) and HeLa (HPV-18+) cells, the results showed a decrease of miR-16-1 cells treated with the siRNA for E7 compared to control cells. These results suggested that E7 regulates the expression of miR-16-1/miR-15a in cells infected with HPV-16 and HPV-18 **(Zheng ZM, 2011).** Finally in keratinocyte cultures infected with HPV-16 and HPV-18, as well as in tissues with CIN 1/2/3 it was found that the expression of miR-16-1, miR-25, miR-92a and miR-378 increases in CC and CIN3 compared with normal tissue and CIN2 with HR-HPV **(Wang, 2014).** The differential expression of miR-16-1 in cervical samples and cell lines shown in **Table3.**

Table 3. Differential expression of miR-16-1 on normal cervical epithelium, CIN 1, CIN 2, CIN 3, CC and HPV + cell lines

MiRNA expression	**Normal tissue**	**CIN 1**	**CIN 2**	**CIN 3**	**CC**	**Cell lines HPV16+, HPV18+**	**References**
miR-16-1	Down regulated	-	-	-	Up regulated	Deregulated (SiHa, CaSki)	**Lui 2007**
miR-16-1	Down regulated	-	-	-	Up regulated	-	**Lee 2008**
miR-16-1	Down regulated	-	-	-	Up regulated	Up regulated (HeLa, SiHa y CaSki)	**Wang 2008**
miR-16-1	Down regulated	Down regulated	-	Down regulated	Up regulated	-	**Pereira 2010**
miR-16-1	Down regulated	-	-	-	-	Up regulated (HFK HPV16+ y HPV18+)	**Zheng, 2011**
miR-16-1	Down regulated	-	Down regulated	Up regulated	Up regulated	-	**Wilting, 2013**
miR-16-1	Down regulated	Down regulated	Down regulated	Up regulated	Up regulated	Up regulated (HFK HPV16+ y HPV18+)	**Wang, 2014**

6. Regulation of miR-16-1 in CC

So far we have described that in CC the miR-16-1 seems to have a role oncomir, since it has been demonstrated that its upregulation contributes to cervical carcinogenesis. Differences in expression profiles of miR-16-1 on normal CIN1-3, CC cervical tissue and CC cell lines, may be due to oncogenic potential of the HR-HPV mainly of HPV16 and HPV18 (E6/E7), viral load, viral DNA integration, variants and methylation.

E6 and E7 of HR-HPV

Few reports been made on the expression of miR-16-1 related to CC, however it has been reported that the integration of HR-HPV results in increased expression of E7 and has been considered to this oncoprotein as the main responsible the regulation of miR-16-1 **(Zheng, 2011).** Within the cellular targets of E7 have been reported to E2F transactivate to c-Myb, c-Myc can bind to the promoter of the DLEU2 gene and upregulate the expression of miR-16-1; it has been suggested that this could be the main route by which E7 can increase the expression of miR-16-1 in CC **(Ofir, 2011) (Figure 1).** It is noteworthy that one of the cellular targets of miR-16-1 on other cancers is CCNE1 oncogene (negatively regulates). In our working group Cyclin E1 protein has been reported increased in expression in squamous intraepithelial lesions (SIL) of low grade (LSIL) and positive cytology with integrated HR-HPV **(Zubillaga-Guerrero, 2013).** Currently our working group is investigating whether CCNE1 is target miR-16-1 CC cell lines.

Furthermore it has been reported that E6 oncoprotein not contribute to the regulation of miR-16-1, this could be due to one of the cellular targets of E6 is p53, and p53 have been reported to induce expression of miR-16-1 **(Zheng, 2011) (Figure 1).**

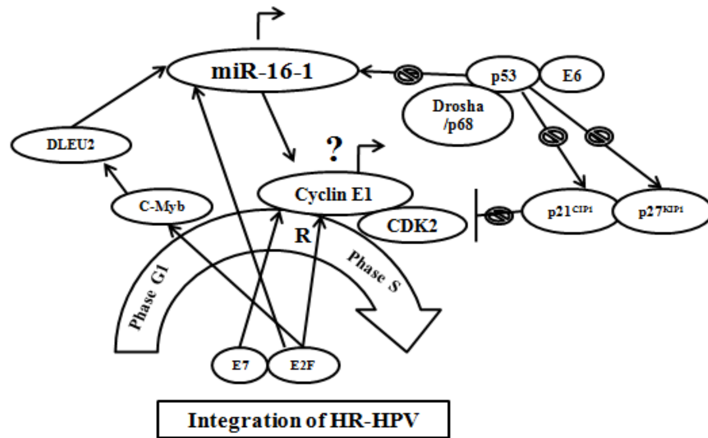

Figure 1. Regulation of miR-16-1 CC. Integration of HR-HPV results in an increased expression of the E6 and E7oncoprotein, E7 however pRb to degrade and leave free E2F transcription factor, this can transactivate other transcription factors such as c-Myb it binds to the promoter of the DLEU2 gene transcription inducing miR-16-1, can also directly activate E2F transcription of miR-16-1 binding to its promoter. E6 down regulates expression of miR-16-1 by p53 degradation.

It is known that HPV integration into the host cell genome can cause genetic alterations (such as deletions, amplifications, or complex rearrangements) and epigenetic alteration, thus intriguing to speculate that the expression of cellular miRNA genes at or near HPV integration sites may contribute to the tumor phenotype **(Lui, 2007).** MiR-16-1 is located on chromosome 13q14 DLEU2 gene **(Calin, 2008)**, which has been reported that is one of the frequent sites of integration of the HPV16 **(Thorland, 2003)**. The CaSki cells have up to 60-600 integrated copies of HPV16 in cellular genome, which favors an increase in the expression of E7 oncoprotein to interact with E2F transcription factor. This scenario can promote the endogenous activation of miR-16-1 and this regulatory genetic network has been associated to CC development **(Ofir, 2011, Schmitz, 2012).**

Notably, the E7 oncoprotein of HR-HPV by inducing activation of E2F favors continuous over expression of miR-16-1/miR-15a cluster (**Wilting, 2013).**Due to these properties of HPV *E7* oncogene, this has relevant attention because allow the understanding of the molecular mechanism of human viral oncogenesis and are considered suitable target for gene therapy strategy for CC treatment **(Salazar-Leon, 2013).** Several studies have analyzed the HPV *E7* mRNA expression inhibition using ribozymes and antisense oligonucleotides as treatment strategy **(Zheng YF, 2002; Choo CK, 2000).** Nevertheless, these approaches have low efficiency, short time of stability and high cost for design and administration. Alternatively, an effective strategy that has been used to knock-down gene expression

in a sequence specific way and that represses in considerable magnitude the viral oncogenes at posttranscriptional level, is by the RNAi **(Elbashir SM, 2001; Elbashir SM, 2001).**

The siRNAs are non-coding RNAs of 21-25 nucleotides in length that mimic endogenous microRNAs, which can effectively reduce the translation of target mRNAs by binding to their 3'-UTR. The process is dependent upon mRNA accessibility and within the target mRNA molecule, upon accessibility of the short internal nucleotide sequences that are homologous to the siRNA transcripts. Therefore, various factors can influence the vulnerability of a given mRNA to siRNA-mediated degradation, including secondary structures of the mRNA, and proteins which package mRNA for translocation within the cell. Other protein-mRNA interactions are also relevant, including proteins which can direct a given mRNA to a specific sub-cellular locus, and those mRNAs which can be bound by the proteins they encode. As a consequence, the generation of biofunctional siRNAs must be carefully and robustly designed to get highly efficient siRNAs for the silencing of specific target genes **(Salazar-Leon, 2010).**

Salazar-León *et al*. reported that siRNAs can silence the expression of oncogenic proteins *E6* and *E7* HPV16 by a decrease in mRNA expression level in human cervical cancer cells. To understand the mechanism by which siRNAs for *E6* and *E7* inhibit the growth of SiHa cells, they examined their effects on p53 and pRb protein expression, which are the most relevant cellular target molecules of HR-HPV *E6* and *E7* oncogenes. In your model, they demonstrated that psiRNAE6B and psiRNAE7B plasmids were able to cause a specific decrease of *E6* and *E7* oncoprotein expression as well as are establishing of p53 and pRb cellular proteins, and induction of cellular death **(Salazar-Leon, 2010).** These results are promising since under physiological circumstances remains attached pRb to E2F transcription factor, preventing this stimulate the expression of other genes involved in cell cycle progression **(Wise-Draper TM, 2008).**

Due to the potential role of miR-16-1 oncomir and its implications in the development of CC it would be important to use the RNAi mechanism for this miRNA and use it as a strategy in gene therapy of this disease, plus they could be used as a biomarker CC diagnosis.

7. **Conclusions and perspectives**

Cervical cancer remains as a leading cause of morbidity and mortality for women worldwide. MiRNAs control gene expression at posttranscriptional level. However, the deregulated expression of miRNAs has been found in a variety of tumors and is considered that this contributes to the development and progression of cancer. Persistent infection, integration and viral load of HR-HPV such as HPV-16, contributes to the progression to cervical cancer through the action of E6 and E7 oncoproteins, which interfere with critical cell cycle pathways, p53 and pRb, respectively. MiR-16-1 is target of E7 and it has been suggested that this miRNA has an oncogenic role activating other genes involved in cell cycle progression in CC. Further studies, in *vitro / in vivo* are needed to establish a link between E7/E2F/miR-16-1 CC. Review of the evidence regarding miR-16-1 expression in CC reveals that the expression pattern of miR-16-1 in cancer cell lines and cervical (pre)malignant lesions provides valuable information about the role of miR-16-1 in the different stages of cervical carcinogenesis. However, future studies are needed to dissect the function, transcriptional targets, and the mechanisms by which miR-16-1 regulate the cellular events within both normal and CC tissue that could give better information on their role as biomarker, as well as in prognosis and therapy of CC.

Conflicts of interest

The authors declare no conflict of interest.

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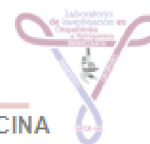
ANEXO 3

Consentimiento informado

UNIVERSIDAD AUTÓNOMA DE GUERRERO
UNIDAD ACADÉMICA DE CIENCIAS QUÍMICO BIOLÓGICAS

LABORATORIO DE INVESTIGACIÓN EN CITOPATOLOGÍA E HISTOQUIMICA Y BIOMEDICINA MOLECULAR EN COORDINACIÓN CON LA CLINICA DE DISPLASIAS DEL HOSPITAL GENERAL RAYMUNDO ABARCA ALARCÓN

Av. Lázaro Cárdenas, S/N. Ciudad Universitaria. Chilpancingo, Gro. 39090. Tel/Fax (747)4710901



No. De control____________

CONSENTIMIENTO INFORMADO

Chilpancingo, Gro. a ____________________

A quien corresponda.

Por medio del presente hago constar que he sido informada acerca del estudio que se pretende conducir en el Laboratorio de investigación en Citopatología e Histoquímica y Biomedicina Molecular de la Unidad Académica de Ciencias Químico Biológicas, de la Universidad Autónoma de Guerrero, en colaboración con el Hospital General Raymundo Abarca Alarcón., que bajo la dirección de la Dra. Berenice Illades Aguiar y la Dra. Luz del Carmen Alarcón Romero, que consiste en los proyectos de MicroRNA-16-1 y su relación con la expresión de Ciclina E1 en lesiones malignas del cérvix uterino con VPH-16 y Biomarcadores celulares, estrés oxidativo y su relación con la integración del VPH de alto riesgo en la carcinogénesis cervical, el cual incluye: Estudio de Glucosa, Tipo sanguíneo, Citología convencional de Papanicolaou, Estudio Microbiológico y la prueba molecular de INNO-LiPA, para la detección y tipificación del VPH. Se me ha pedido autorización para participar en el estudio y acceder a las entrevistas, toma de muestra de la zona de transformación exo/endocervical y biopsia dirigida, me aseguran no provocará daño físico, mental o social, además de que se tendrá ética profesional, confidencialidad en los resultados, los cuales me serán entregados por escrito y debidamente avalado por las responsables del proyecto.

Doy mi autorización para que me incluyan en el estudio.

Atentamente

____
Nombre y firma de la paciente

Atentamente Responsables del proyecto:

_________________________________   _________________________________
Dra. Berenice Illades Aguiar   Dra. Luz del Carmen Alarcón Romero

ANEXO 4

Formato de la encuesta

UNIVERSIDAD AUTÓNOMA DE GUERRERO
UNIDAD ACADÉMICA DE CIENCIAS QUÍMICO BIOLÓGICAS

LABORATORIO DE INVESTIGACIÓN EN CITOPATOLOGÍA E HISTOQUÍMICA
Servicio de Diagnóstico Integral en la detección oportuna de Cáncer Cérvico Uterino y VPH
Av. Lázaro Cárdenas, S/N. Ciudad Universitaria. Chilpancingo, Gro. 39090. Tel/Fax (747)4710901
Responsable: Dra. Luz del Carmen Alarcón Romero.
Citotecnóloga del área ginecológica acreditada y recertificada por el Consejo Mexicano de Médicos Anatomopatólogos A.C., y Técnicos en Patobiología A.C. No. de Registro: 0152.

E N C U E S T A

No. DE CONTROL: ________________ **FECHA DE TOMA:** ____________________________

Nombre de la paciente: ____ Edad: ______ Años

Dirección: ____ Teléfono: _____________

Correo electrónico: ________________________ Grado de escolaridad: ________________ Institución donde estudia: ___________________

Trabaja: __________________ Originaria de: ______________________________ Actualmente reside en: ______________________________

1.- ¿A qué edad empezó a reglar?: ______ Años. FUR: _________ X ___ días de sangrado menstrual. Edad de inicio de vida sexual: _______ Años.
3.- N° de compañeros sexuales que ha tenido o tiene: ________________ N° de compañeros sexuales que ha tenido su pareja: ________________
4- ¿Utiliza condón en sus relaciones sexuales? Sí___ No___ ¿Desde cuándo? ______________________________
5.- ¿Qué método de planificación usa? HO: _______ HI: ______ Ritmo menstrual: ______ Salpingoclasia: _______
Otros: ______________________________ ¿Desde cuándo? ______________________________
6.- N° de embarazos que ha tenido: ________ Partos: ________ Abortos: ________ Cesáreas: ________
7.- ¿Ha tenido alguna infección vaginal? Sí____ No ______ ¿Cuál fue esta? ______________________________
¿Hace cuánto tiempo la tuvo? ______________________ ¿Recibió tratamiento? ______________________________
8.- ¿Tiene el hábito de fumar? Sí____ No ______ ¿Desde cuándo? ______________________ Frecuencia: ________________
9.- ¿Tiene el hábito de consumir alcohol? Sí____ No ____ ¿Desde cuándo? ______________________ Frecuencia: ________________

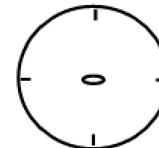
10.- ANTECEDENTES DE RESULTADOS PREVIOS: Estudios de Papanicolaou: Sí_________ No________ Primera vez _________________
Resultado: ____ Fecha de último estudio: _____________
Estudio de **PCR**: Sí____ No____ Resultado: ______________________________ Fecha de último estudio: _____________
Estudio **Colposcópico**: Sí____ No____ Resultado: ______________________________ Fecha de último estudio: _____________
Estudio Histopatológico (Biopsia): Si____ No____ Resultado: ______________________________ Fecha del último estudio: _____________
11.- Tratamientos previos: Sí____ No____ Cauterización: ____ Criocirugía: ____ Conización: ______ Radioterapia: _____ Quimioterapia: _____
Otros: ______________________________ Fecha del tratamiento: ______________________________
12.- ¿Conoce sobre la vacuna contra el VPH? Sí__ No__ ¿Se la ha aplicado? Sí__ No__ ¿Qué tipo de vacuna? ______________________
13.- ¿Presenta actualmente síntomas?: Sí__ No__ Comezón: ______ Ardor: ______ Flujo vaginal: ______________________
Sangrado anormal: ______________ Dolor al coito: ______________ Sangrado al coito: ______________ Otros: ______________
14.- ¿Presenta alguna enfermedad crónico degenerativa u hormonal? Sí____ No____ ¿Cuál (es)? ______________________________
15.- ¿Actualmente está sometida a estrés en su vida diaria? Sí____ No____ Escala del 1, 2 o 3: ______________________________
16.- ¿Ha consumido vitaminas antioxidantes? Sí____ No____ ¿Cuál (es)? ______________________ Hace cuanto tiempo__________
17.- IMC: ________________ Masa: ___________________ Estatura: __________________________
18.- ¿Cuántas comidas realiza al día? __________________________ ¿En qué lapso de tiempo? ______________________________
19.- ¿Consume frutas? Sí______ No______ ¿con que frecuencia (por día)? ______________________________
20.- ¿Consume carnes? Sí______ No______ ¿con que frecuencia (por día)? ______________________________
21.- ¿Consume café? Sí______ No______ ¿con que frecuencia (por día)? ______________________________
22.- ¿Consume vino? Sí______ No______ ¿con que frecuencia (por día)? ______________________________
23.- ¿Se encuentra bajo el régimen de una dieta? Sí______ No______ ¿De que tipo? ______________________________
24.- ¿Practica algún tipo de ejercicio? Sí______ No______ ¿De que tipo? ____________________ ¿Hace cuanto tiempo? ________________
25.- ¿Cuánto tiempo labora al día (horas)? ______________________________
26.- ¿Padece o ha padecido depresión? Sí______ No______ ¿Hace cuanto tiempo? ______________________________

ASPECTOS DEL CÉRVIX UTERINO:
Aparentemente normal: ________ Ectropión: _________ Quiste de Naboth ________ Erosión: ________ Úlcera: _________ Pólipo: ___________
Condilomas: ________ Neoplasia: __________ Flujo vaginal: ______________________________ Otras observaciones: ________________

NOTA IMPORTANTE: Los datos proporcionados son de carácter confidencial.

Indicar la localización de la lesión

FECHA DE ENTREGA DE RESULTADOS: ____



ESPECIFICAR MATERIAL COLECTADO:

____ EXO/ENDOCERVICAL
____ OTRO CUÁL: _______________

pH vaginal_________
Prueba de KOH: (+) (-)
No. de laminillas: ____
Sangrado a la toma endo: (Sí) (No)
Cantidad: (+) (++) (+++)
Prueba del Ácido acético: (+) (-)
La zona acetopositiva es leve ( ) moderada ( ) intensa ( )
Señalar su ubicación: ______________________________

Si hay lesión tomar la muestra de esa zona

Practicó encuesta

____
Nombre y firma

Practicó toma de muestra y extendido

____
Nombre y firma