An increase of microRNA-16-1 is associated with the high proliferation of squamous intraepithelial lesions in the presence of the integrated state of HR-HPV in liquid cytology samples

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Abstract. Studies of cervical cancer (CC) have reported that microRNA-16-1 (miR-16-1), which is an oncomiR, is increased in the tissues and cell lines of CC. The aim of the present study was to investigate the association of miRNA-16-1 expression level with squamous cell carcinoma (SCC), the presence of squamous intraepithelial lesions (SIL) and the integration of high-risk human papillomavirus (HR-HPV) DNA. The current study analyzed 80 samples obtained from women by liquid-based cytology, which revealed that 20 were negative for SIL (NSIL) and without HPV, 20 were low-grade SIL (LSIL), 20 were high-grade SIL (HSIL), and 20 were diagnosed as SCC with HR-HPV. The genotyping of the viral DNA was conducted via an INNO-LiPA-HPV array, the expression of miR-16-1 was determined by reverse transcription-quantitative PCR, and the physical state of the HR-HPV was ascertained by *in situ* hybridization with amplification with tyramide. A total of eight HR-HPV genotypes were distinguished; the most frequent of these being HPV16, followed by multiple infection with HR-HPV (including HPV16). The mixed state of the HR-HPV was observed in 60 and 65% of LSIL and HSIL cases, respectively, while an integrated HR-HPV state was identified in 90% of cases with SCC. The expression level of miR-16-1 increased according to the grade of SIL, and cases with HSIL exhibited a significantly higher miR-16-1 expression level compared with women with NSIL ($P<0.001$; Table II). It can therefore be determined that the expression of miR-16-1 effects cellular proliferation, due to the viral integration of various HR-HPV genotypes in unique infection or in multiple infection. Thus, the overexpression of miR-16-1 could be monitored in women with LSIL, in order to discard a major lesion.

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Introduction

Cervical cancer (CC) represents the fourth highest cause of mortality in the female population worldwide, with ~569,847 newly diagnosed CC cases and ~311,365 CC-associated deaths in 2018 (1). The most prevalent histological subtypes are squamous cell carcinoma (SCC) and adenocarcinoma (AC), with SCC representing ~70% of CC cases (2). Persistent infection associated with high-risk human papillomavirus (HR-HPV) is considered to be a key risk factor in the development of CC, particularly HPV types 16 and 18 (3). Additionally, the integration of HR-HPV DNA in fragile host-genome sites, favors the overexpression of oncoproteins E6 and E7. This, as a result, promotes the progression and transformation of malignant cells, inducing genetic and epigenetic instability (4).

From the cytological point of view, the Bethesda system classifies precursor lesions of CC as low-grade squamous intraepithelial lesions (LSIL) and high-grade lesions (HSIL). LSIL are characterized by the presence of cells with karyomegaly, perinuclear halo and binucleation (koilocytes), while HSIL is associated with the presence of intense binucleation and dyskaryosis in basal and parabasal cells with little cytoplasm, hyperchromatic and big nuclei. Furthermore, SCC is characterized by large undifferentiated and multinucleated cells lacking cytoplasm, pleomorphic nuclei and irregular distribution of chromatin (5).

Integration of HR-HPV, in addition to altering significant transcription patterns and regulating the expression of E6 and E7, also regulates the expression of host genes in fragile

integration sites (6). It is important to note that a number of these genes have an oncogenic function, for example as oncogenic microRNAs (miRNAs/miRs) (7). miRNAs suppress genetic translation by binding to the 3'-untranslated region of their target genes (8). Previous studies have shown that numerous miRNAs are implicated in CC, such as miR-16-1 (9), miR-21 (10), miR-22-3p (11) and miR-486-5p (12). These miRNAs contribute to a number of cellular processes, such as cell proliferation (9-12), invasion (9,10,12), migration (12) and apoptosis (11).

miR-16-1 is a regulator of gene expression at the post-transcriptional level. Studies have reported that miRNAs are often increased in certain cancer types, including breast cancer (13), hepatocellular carcinoma (14) and CC (9,15-20). miR-15a and miR-16-1 form part of a cluster in an intron region of the deleted in lymphocytic leukemia 2 (DLEU2) transcript on chromosome 13q14.3, which is frequently deleted in chronic lymphocytic leukemia. Both are implicated in cellular invasion, survival and proliferation (21).

It has been reported that the expression of miR-16-1 is decreased in CC cell lines that are positive for HPV16 and HPV18 following transfection with a siRNA for oncoprotein E7, suggesting that the increase in miR-16-1 expression may be due to the E7/E2F/miR-16-1 pathway (22). Furthermore, other studies have indicated that miR-16-1 possesses an oncomiR function in CC (15-18,20). The overexpression of miR-16-1 is associated with activation of genes implicated in the cell cycle, including CDK6, CDC27, CARD10, C10orf46 (23) and CCNE1 (9), which promote the proliferation of cancerous cells. The aim of the present study was to investigate the association of the miR-16-1 expression level with squamous intraepithelial lesions (SIL) and with the integration of HR-HPV DNA.

Materials and methods

Participants and sample collection. The present study included 80 liquid-based cytology samples obtained from the squamous-column transformation zone (TZ) of the uterine cervix of female patients aged 18-71 years, who resided within the State of Guerrero, Mexico. The mean age was 42 years. Between March 2012 and September 2018, the patients presented at the Integral Diagnostic Service for the Timely Detection of Cervical Cancer and HPV of the Autonomous University of Guerrero (Chilpancingo, Mexico), to the Dysplasia Clinic of the General Hospital 'Raymundo Abarca Alarcón' (Chilpancingo, Mexico) and to the State Institute of Cancerology 'Arturo Beltrán Ortega' (Acapulco, Mexico).

The study was approved by the Ethics Committee of the Autonomous University of Guerrero, Guerrero, Mexico. All patients signed an informed consent for the use of their cervical samples and clinical information. This study was also performed according to the ethical guidelines of the Declaration of Helsinki 2008 (24).

Cytological examinations, HPV genotyping and measurements of miR-16-1 by reverse transcription-quantitative PCR (RT-qPCR) were performed in the present study. Three ectocervical and three endocervical samples were obtained from each patient, utilizing an Ayre spatula (ectocervix) and cytobrush (endocervix), ensuring cytological material was from the TZ of the uterine cervix. The cytological diagnosis was performed by a cytotechnologist (LdCA-R) who was accredited and certified by the Mexican Council of Technicians in Pathobiology, A.C., and the Mexican Council of Anatomopathological Physicians, A.C., with 29 years of experience, utilizing the criteria of the Bethesda System (5). The colposcopic diagnosis was performed by the colposcopist Dr Raúl Peralta-Catalán responsible for the Dysplasia Clinic of the General Hospital 'Raymundo Abarca Alarcón' (Chilpancingo, Mexico). The histopathological diagnosis, for confirmation of SIL and SCC, was performed by the pathologist Marco Antonio Jiménez-López at the State Institute of Cancerology 'Arturo Beltrán Ortega' (Acapulco, Mexico) (25).

Cytological examination. Slides with the cytological smears of the TZ for conventional cytology examination were fixed in ethanol for 10 min. The slides were then stained using the Papanicolaou kit (cat. no. 64294; Hycel, Chemical Reagents). Briefly, the slides were hydrated in a descending alcohol series and then incubated at room temperature for 45 sec with Harris hematoxylin to stain the nuclei. Additionally, Orange G colorant was added and incubated at room temperature for 80 sec, followed by EA-50 incubated at room temperature for 3 min, which stained the eosinophils and basophils cells, respectively. The slides were then cleared with Xylol reagent prior to microscopic observation (DM1000 LED; Leica Microsystems, Inc.; magnification, x10-x20).

Alternatively, the samples for liquid-based cytology were processed according to the manufacturer's protocol of liquid-PREP™ (LGM International, Inc.). Briefly, a clearing solution was added to each sample and then the samples were centrifuged at 1,000 x g for 5 min at room temperature. The supernatant was discarded after the addition of the cell base solution, which conserved the pellet. The samples were mixed and 10 μl was added to a slide, which was fixed at room temperature with ethanol for 10 min, following by staining using Papanicolaou kit and microscopic observation (DM1000 LED; Leica Microsystems, Inc.; magnification, x10-x20).

Genotyping and the physical state of HR-HPV. Using cervical cytology samples in PBS (pH 7.0), DNA was extracted by the standard method of phenol-chloroform extraction (26). For HPV genotyping, the reverse INNO-LiPA Genotyping Extra assay (Invitrogen; Thermo Fisher Scientific, Inc.) was used according to the manufacturer's instructions. This method allowed the simultaneous identification of 28 different HPV genotypes. Briefly, the L1 region of HPV was PCR-amplified with the SPF10 primers. The biotinylated amplicons were denatured and hybridized with specific and immobile oligonucleotides anchored to a membrane, and then Streptavidin conjugated with alkaline phosphatase was added, followed by Chromogen BCIP/NBT to reveal the reaction. The HLA-DPB1 gene was employed as a control for DNA amplification, and the L1 region of HPV6 was utilized as a positive control.

The determination of the physical state of viral DNA was performed by means of the Dako GenPoint™ Tyramide Signal Amplification System for Biotinylated Probes (Agilent Technologies, Inc.), according to the manufacturer's protocol. Briefly, liquid-based cytology smears were submitted to permeabilization for 30 min at 120˚C, followed by enzymatic digestion for 5 sec with K proteinase (1:1,000). The samples

were then added to 1 μl test reagent (Dako GentPoint™ HPV DNA Probe Cocktail, Biotinylated) for 13 HR-HPV genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68; Agilent Technologies, Inc.). The slides were subjected to DNA denaturation for 10 min at 95˚C and hybridization for 20 h at 37˚C on a Dako Hybridizer (Agilent Technologies, Inc.). Subsequently, the slides were placed in an astringent solution (1:20) for 20 min at 55˚C, followed by the addition of 30 μl primary streptavidin-HRP conjugate (1:50) for 1 h and incubation in a humidified chamber at room temperature. Subsequently, 30 μl biotinyl tyramide was added for 40 min and 30 μl secondary streptavidin-HRP conjugate was added for 1 h in a humidified chamber at room temperature. Diaminobenzidine (1:20) was then added for 10 sec, followed by counterstaining with Harris hematoxylin (Merck KGaA) for 10 sec, both incubations at room temperature. The positive reaction with the nucleus was identified by a brown color, which was classified as diffuse (episomal state), punctate (integrated state) or mixed (episomal and integrated state). As a positive control, the SiHa cervical cancer cell line (catalog no. HTB-35; American Type Culture Collection) was used and, as negative control, SiHa cells were used without the test reagent. The cell line was cultivated in DMEM (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Merck KGaA), 50 μg/ml penicillin/streptomycin (Merck KGaA), 2 mM L-glutamine (Merck KGaA) and 250 ng/ml fungizone (Merck KGaA), and placed at 37˚C in a humidified incubator containing 5% CO_2.

Analysis of miR-16-1 expression. Using the cervical cytology samples, total RNA was extracted using TRIzol® Reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Briefly, 1 ml TRIzol® Reagent was added to each sample and incubated for 5 min to permit the dissociation of the nucleus-protein complex. Subsequently, 200 μl chloroform for every 1 ml TRIzol® Reagent was added, followed by vigorous shaking for 15 sec and incubation for 3 min. Following incubation, the sample was centrifuged at 12,000 x g at 4˚C for 5 min. The aqueous phase, which contains the RNA, was transferred into a new tube. Next, 500 μl isopropanol per 1 ml TRIzol® Reagent was used for lysis for 10 min at -70˚C, followed by centrifugation at 14,000 x g at 4˚C for 15 min. The supernatant was then discarded and the sediment was re-suspended in 20 μl RNase-free water. The concentration of RNA was evaluated by UV absorbance at 260 nm (A260) using a Thermo Scientific NanoDrop 200c (Thermo Fisher Scientific, Inc.). Synthesis of the complementary DNA (cDNA) was carried out using the TaqMan MicroRNA Reverse Transcription kit (Thermo Fisher Scientific, Inc.), which possesses a high capacity for synthesizing cDNA from miRNA. In total, 5 ng total RNA was utilized for synthesizing the cDNA of the miR-16-1. Briefly, inverse transcription assays were prepared with 3 μl 5X RT primer, 5 μl RNA sample and 7 μl Master mix [100 mM dNTPs (with dTTP) (0.15 μl), MultiScribe™ Reverse Transcriptase (50 U/μl; 1 μl), 10X Reverse Transcription Buffer (1.50 μl), RNAse Inhibitor (20 U/μl; 0.19 μl) and Nuclease-Free water (4.16 μl) in a total volume of 15 μl]. The reactions were incubated in an Eppendorf thermocycler (Eppendorf Mastercycler EP Gradient Model 5341) for 30 min at 16˚C, 30 min at 42˚C, and 5 min at 85˚C, followed by storage at 4˚C until later use.

Table I. HR-HPV state according to cytological diagnosis.

	Diagnosis		
HR-HPV state	LSIL, n (%)	HSIL, n (%)	SCC, n (%)
Integrated	8 (40)	7 (35)	18 (90)
Mixed	12 (60)	13 (65)	2 (10)
Total	20 (100)	20 (100)	20 (100)

HR-HPV, high-risk human papillomavirus; LSIL, low-grade squamous intraepithelial lesions; HSIL, high-grade squamous intraepithelial lesions; SCC, squamous cell carcinoma.

The PCR reaction was conducted at 95˚C for 10 min followed by 40 cycles at 95˚C for 10 sec and at 60˚C for 60 sec using TaqMan™ MicroRNA Assays (Thermo Fisher Scientific, Inc.). Specific primers (Thermo Fisher Scientific, Inc.) were used for hsa-miR-16-1 (5'-UAGCAGCACGUAAAUAUUGGCG-3') and RNU44 (5'-CCTGGATGATGATAGCAAATGCTGACTGAA CATGAAGGTCTTAATTAGCTCTAACTGACT-3'), which was used for normalization. The reaction was incubated in PCR tubes and caps, RNase-free, 0.2 ml (catalog no. AM12230; Thermo Fisher Scientific, Inc.) in the CFX96 Touch™ Real-Time PCR Detection system supplied with analytical software (Bio-Rad Laboratories, Inc.). Each reaction was performed in triplicate. The $2^{-\Delta\Delta CT}$ method (27) was employed to evaluate the relative abundance of miR-16-1 compared with the expression of RNU44, which is a small nuclear RNA and is one of the 18 human endogenous controls identified as the most abundant in all tissues, based on C_T averages (22-28.9), good linearity test (R^2>0.96) and its relatively stable expression (28).

Statistical analysis. Data are presented as frequencies for the qualitative variables and as the mean ± standard error for quantitative variables. One-way analysis of variance followed by Bonferroni's post hoc test was used to compare the expression level of miR-16-1 between study groups. The association of cytological diagnosis or the physical state of the HPV with the expression level of miR-16-1 was evaluated through linear regression models. This obtained the regression coefficients (β), as the average change in the expression of miR-16-1 by cytological diagnosis or physical state of the HPV, in comparison with the reference category (NSIL). P<0.05 was considered to indicate a statistically significant difference. Statistical analysis was performed using STATA V.13 statistical software (StataCorp LLC).

Results

Genotypes and physical state of the HPV. Once the cytological results were obtained, the following cytological samples of women were selected as follows: 20 samples with negative diagnosis for SIL and without HPV infection; 20 samples with LSIL; 20 samples with HSIL (of which 45% were diagnosed with carcinoma *in situ*); and 20 samples with SCC. Women with SIL or SCC had HR-HPV infection. From the findings of the present study, eight types of HR-HPV could be identified;

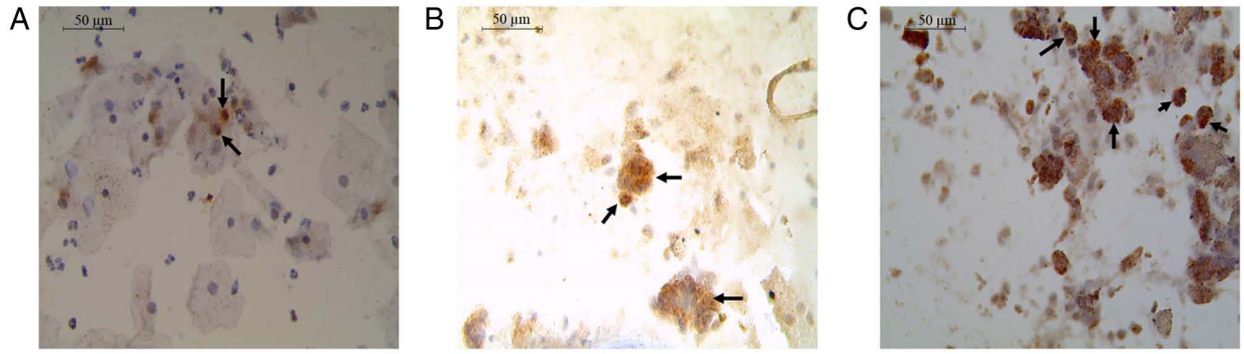

Figure 1. Physical state of the HR-HPV according to the grade of squamous intraepithelial lesions and SCC. (A) Integrated state (black arrows) of the genome of HPV16 in cells with karyomegaly with hyperchromatic nuclei in a case of low-grade squamous intraepithelial lesions. (B) Integrated state (black arrows) of HPV16 DNA in groups of cells with intense dyskaryosis, in basal and parabasal cells with big and hyperchromatic nuclei, compatible with a high-grade squamous intraepithelial lesions. (C) Integrated state (black arrows) of HPV16 DNA in a case of SCC in undifferentiated cells with irregular chromatin distribution in pleomorphic nuclei (Magnification, x40). HR-HPV, high-risk human papillomavirus; SCC, squamous cell carcinoma.

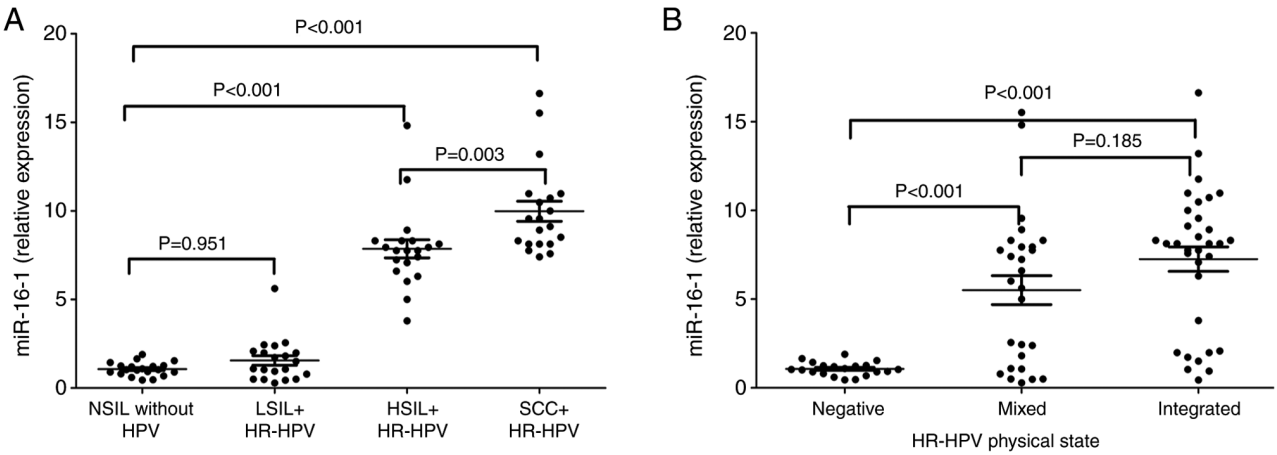

Figure 2. RT-qPCR of the expression of miR-16-1. The relative expression of miR-16-1 was assessed by RT-qPCR and the $2^{-\Delta\Delta CT}$ method. The relative expression of miR-16-1 was compared between the study groups and the different physical states of HR-HPV using the ANOVA. (A) Significant increase in miR-16-1 expression is identified in HSIL and SCC compared with NSIL (P<0.001) and an increase in SCC compared to HSIL (P=0.003). (B) Expression of miR-16-1 was significantly increased in the mixed (P<0.001) and integrated (P<0.001) physical state compared with the negative samples. RT-qPCR, reverse transcription-quantitative PCR; miR-16-1, microRNA-16-1; HR-HPV, high-risk human papillomavirus; NSIL, negative for squamous intraepithelial lesions; LSIL, low-grade squamous intraepithelial lesions; HSIL, high-grade squamous intraepithelial lesions; SCC, squamous cell carcinoma.

the most frequent being 16, 18, 31, 33, 45, 51, 52 and 58. The frequency of HPV16, in relation to the cytological diagnosis, was 20% in LSIL, 30% in HSIL and 40% in SCC. Notably, 15% of LSIL, 20% of HSIL, and 40% of SCC presented with multiple infection (MI) with the genotypes of HR-HPV, including HPV16 (data not shown). On the other hand, it is important to note that when analyzing the physical state of the HR-HPV DNA, an integrated state was identified in 40% of women with LSIL, in 35% of women with HSIL and in 90% of women with SCC (Table I). Cytologically in the LSIL cases, some intermediate cells with karyomegaly, binucleation, perinuclear halo and hyperchromatic nuclei were observed. These are considered to be characteristics of the HPV infection. The intermediate cells with karyomegaly presented 1-2 viral copies integrated (Fig. 1A). While in cytologies with HSIL, small groups of cells with moderate to intense dyskaryosis and binucleation were observed, in immature basal and parabasal cells with little cytoplasm, big and hyperchromatic nuclei. These cells presented multiple integrated copies (Fig. 1B). Finally, in the cases of SCC, multiple integrated copies were observed in large undifferentiated cells, multinucleated, devoid of cytoplasm, pleomorphic nuclei and irregular distribution of chromatin (Fig. 1C).

Expression of miR-16-1. It was demonstrated that the mean expression level of miR-16-1 was increased significantly in women with HSIL (7.9±0.5) and SCC (10±0.6), in comparison with women with NSIL or LSIL (P<0.001). Although a small increase was identified in the expression of miR-16-1 in patients with LSIL (1.6±0.3) in comparison with women with NSIL (1.1±0.1), this was not statistically significant (P=0.95; Fig. 2A). Linear regression analysis revealed a significant increase in the expression of miR-16-1 in women with HSIL (β=6.8; P<0.001) and in women with SCC (β=8.9; P<0.001) in comparison with women NSIL, with an explanation percentage of 83% (Table II).

Additionally, significant differences were identified for the expression level of miR-16-1 in the samples with a mixed or integrated HPV physical state compared with the samples that did not present with HPV infection (P<0.001; Fig. 2B). In

Table II. Associations of the cytological diagnosis or physical state of HPV with the expression level of miR-16-1.

Variable	Relative miR-16-1 expression, REU[a]	β (95% CI)[b]	P-value	r^2
Diagnosis				
NSIL	1.1±0.1	Ref.		0.83
LSIL	1.6±0.3	0.5 (-0.7-1.6)	0.395	
HSIL	7.9±0.5	6.8 (5.6-7.9)	<0.001	
SCC	10.0±0.6	8.9 (7.8-10.0)	<0.001	
Physical HPV state				
Negative	1.1±0.1	Ref.		0.33
Mixed	5.5±0.8	4.4 (2.3-6.5)	<0.001	
Integrated	7.3±0.7	6.2 (4.2-8.2)	<0.001	
Mixed				
Integrated[c]		Ref. 1.7 (-0.4-3.9)	0.105	0.04

[a]Data are reported as mean ± standard error. [b]According to Linear regression analysis. [c]Negative values were excluded. REU, relative expression unit; HR-HPV, high-risk human papillomavirus; LSIL, low-grade squamous intraepithelial lesions; HSIL, high-grade squamous intraepithelial lesions; SCC, squamous cell carcinoma; miR-16-1, microRNA-16-1; NSIL, negative for squamous intraepithelial lesions; CI, confidence interval; HPV, human papillomavirus.

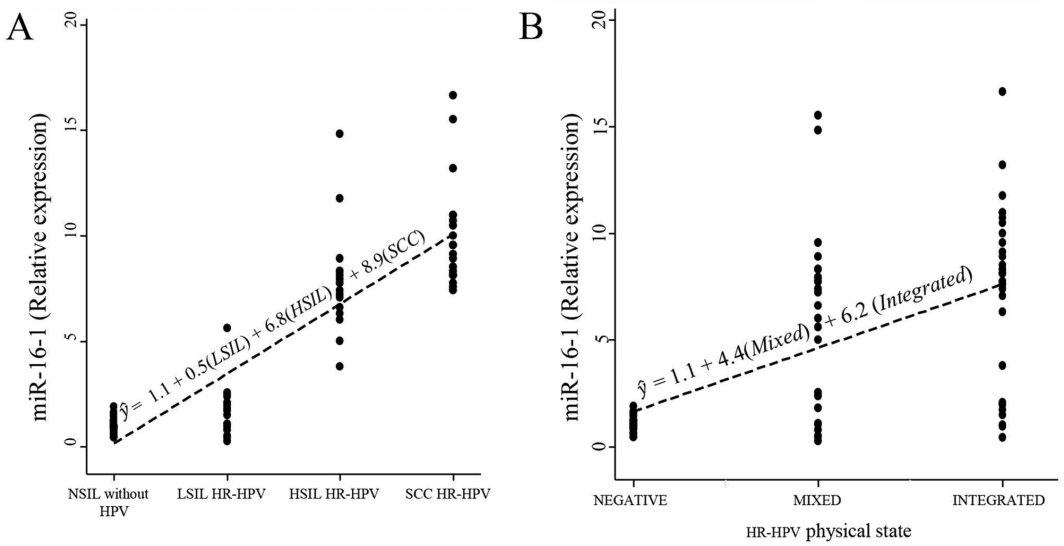

Figure 3. Effect of squamous intraepithelial lesions, squamous cell carcinoma and the physical state of HPV on the increase in the expression levels of miR-16-1. (A) The increase in the expression of miR-16-1 is associated with the progression of SIL to SCC. (B) There is no different effect on miR-16-1 overexpression between mixed and integrated HPV physical state. miR-16-1, microRNA-16-1; HR-HPV, high-risk human papillomavirus; NSIL, negative for squamous intraepithelial lesions; LSIL, low-grade squamous intraepithelial lesions; HSIL, high-grade squamous intraepithelial lesions; SCC, squamous cell carcinoma.

addition, a significant influence of the mixed (β=4.4; P<0.001) or integrated (β=6.2; P<0.001) state on the expression level of miR-16-1 (P<0.001) was identified, compared with the samples negative for HPV infection; with an explanation percentage of 33%. It is also important to comment that any relevant changes were not identified in the level of expression of miR-16-1 between the integrated physical state and the mixed state (β=1.7; P=0.105; Table II; Fig. 3).

Discussion

CC represents a serious public-health problem. Despite it being a preventable disease, it has high rates of incidence and mortality in developing countries, and in Mexico, CC is the third most common cancer in women with 7,689 new cases reported in 2018 (1). In Mexico, a nationwide cytology-based cervical cancer screening program was implemented in 1974, but the subsequent decrease in incidence and mortality have been modest (29). In Guerrero, Mexico, from the 2000 to 2013, CC represented the second most common cancer in women, which therefore makes CC the fifth highest contributor to mortality rates nationally (30). In Guerrero, Mexico, HPV16 has been identified as the most frequent genotype in CC, followed by HPV18 (31). It is important to note that our group has previously reported that, in the state of Guerrero, there are five circulating variants of E6 of HPV16 (E-G350, AA-a,

AA-c, E-C188/G350 and E-A176/G350). These variants have been associated with the development of SCC, and the AA-a variant has the greatest association with the development of CC (odds ratio, 69.01; confidence interval, 7.57-628.96) in comparison with the E-prototype variant (32).

In the present study, women diagnosed with LSIL, as well as women diagnosed with HSIL, presented with HPV16 with greater frequency in unique infection or MI compared with other types of HR-HPV. It has been noted that genotypes 16 and 18 are present in >70% of SCC cases (3). A number of studies have reported that MI with HR-HPV can increase the risk of cervical intraepithelial neoplasia (CIN) progressing to SCC (33-35). However, other reports that HPV16, in itself, can increase the risk of developing a HSIL should also be considered (36).

Integration of viral DNA occurs because of chromosomal instability, which is induced by the aberrant expression of oncoproteins E6 and E7 (37). The biotinyl-tyramide-based *in situ* hybridization (ISH) amplification method has the advantage of allowing the *in situ* examination of the physical state of HPV DNA, preserving the morphology of the cells or tissues, as well as it being optimized to enable the reproducible detection of one to two integrated copies of the HPV-16 (38-40). It has been reported that in cervical scrapes or biopsy samples positive for HPV16 or 18 from 187 female patients without SIL, LSIL, HSIL and CC, ISH has a high concordance (96.1%) with qPCR to determine the physical state of HPV. These results suggest that ISH has good concordance with qPCR with regards to the detection of HPV integration. Therefore, this method can be used for determining the physical status of HPV (41). The present study identified that women with LSIL (60%), with HSIL (65%) and with SCC (10%) had principally a mixed state. In this regard, we reported previously that the mixed state of HR-HPV can be found in cytologies with LSIL with HR-HPV (42), while other investigators have reported it in HSIL (43). On the other hand, with respect to the integration of viral DNA, it was identified that 40% of LSIL, 35% of HSIL and 90% of women with SCC had viral integration. In our work group and with regards to previous studies, it was identified that in women with LSIL with HR-HPV, 10% had viral DNA integration (42), while some studies have reported that viral integration is an indicator of HSIL (44), and a predictive indicator that is markedly unfavorable for the survival of patients with primary CC, in comparison with mixed forms of HR-HPV (45). One possible reason why the percentage of women with viral integration was similar between LSIL and HSIL is that in both study groups, HPV16 and 18 were present in single or multiple infection with other HR-HPV (data not shown). However, differences in the number of copies integrated between both groups were observed (Fig. 1). It is important to note that in LSIL the number of altered cells is lower compared with HSIL. In addition, it has been reported that viral integration in SIL and CC is more frequently related to HPV16, 18 and 58 genotypes (41,46).

It must be considered that previous reports have demonstrated that viral integration is an early event in the progression of the disease (47,48). In addition, it has been reported that populations of cells with integrated HPV16 possess a selective advance in growth, compared with cells that maintain episomal HPV16 genomes (49). The present study identified that the number of cells and the integrated copies in them increased in cytologies with HSIL and SCC compared with cytologies with LSIL (Fig. 1A-C). These results are important since it has been reported that those cells with multiple integrated copies of HPV16 has an increase in methylation patterns in the upstream regulatory region (URR) region of the viral genome, compared with those with only 1-2 integrated copies or those that present only episomal copies. This suggests that methylation in E2 binding sites, in the URR region of HPV16, can lead to deregulation of E6 and E7 expression in early stages of cell transformation induced by HR-HPV (50).

Furthermore, a wide range of studies have reported that miRNAs serve an important role in the regulation of gene expression, and the deregulation of miRNAs plays an important role in the development of human cancers (51). The expression of miR-16-1 is of great interest for further analysis, as it has been reported to be increased in a variety of human cancers, in which its function has been described as an oncomiR (9,15-20,22). The present study did not identify significant differences in the expression of miR-16-1 between women who presented with NSIL and without HPV with those who presented with LSIL with HR-HPV (P=0.951). One limitation of the present study that must be considered when examining the results was the small sample size used to identify significant differences between these two groups. However, we found that HSIL, SCC or HPV physical state had an effect on the increase in miR-16-1 expression. The patients with HSIL and SCC with HR-HPV exhibited a significant increase in the expression of miR-16-1 in comparison with women with NSIL without HPV (P<0.001; Table II). There are studies that have evaluated this miRNA in cell lines and tissues with SCC, for example. miR-16-1 was increased in 19 SCC tissue samples, 7 adenocarcinomas, 2 adenosquamous cell carcinomas and 2 small-cell carcinomas, all of these with HR-HPV, in comparison with normal tissue (15). Furthermore, an increase was found in the expression of miR-16-1 in ten tissues with invasive SCC in comparison with normal tissue according to RT-qPCR (16). Through Northern blot analysis, it has also been demonstrated that the expression of miR-16-1 is increased in cell lines with HPV and in CC tissues in comparison with normal tissue (17). Through microarrays, the expression of diverse miRNAs has been studied, and results demonstrated that the expression of miR-16-1 increased according to the grade of CIN, with higher expression observed in cases with CIN III and SCC, in comparison with CIN I and normal tissue (18). By contrast, through RT-qPCR, it was identified that the expression of miR-16-1 was lower in ten normal tissues was compared with 18 cases of CIN II and CIN III, 9 cases of adenocarcinoma and 10 cases of SCC, in which the expression increased according to the grade of CIN (19). Similar results were confirmed in a study in which the expression of miR-16-1 was higher in CIN I, CIN II, CIN III and CC in comparison with normal tissue (20).

In the present study, a basal expression of miRNA-16-1 was found in samples without SIL that were negative for HPV. It has been reported that the normal function of miR-16-1 is to negatively regulate the progression of the cell cycle, by regulating cell targets, such as CDK1, CDK2, CDK6, cyclin D1, cyclin D3 and cyclin E1 (52). In addition, during the progression of the normal cell cycle, the endogenous inactivation of E2F leads to an increase in the basal expression of miR-16-1

and miR-15, and consequently the arrest of the cell cycle occurs in the G1 phase (53). The expression of miR-16-1 is increased in tissues of CIN I, CIN II, CIN III and CC compared with in normal tissue (20). Further studies have shown that when HR-HPV infection is present, the oncoprotein E7 dissociates the RB/E2F complex, resulting in the endogenous activation of E2F (54,55). By *in silico* analysis, it has been reported that the promoter of the human gene DLEU2 contains a binding site conserved for E2F in the position -4 to +4; therefore, the expression of miR-16-1 is endogenously regulated by E2F (53). These findings are important because cyclin E1 plays a crucial role in the transition of the G1/S phase, and it is known that this cyclin is transcriptionally regulated by E2F (56) and post-transcriptionally regulated by miR-16-1 (9,57). These two molecular alterations can cooperate during tumor development, maintaining an increased proliferation of transformed cells.

It is noteworthy that the increased expression of miR-16-1 was mainly related to HSIL and SCC in 6.8 and 8.9 relative expression units, respectively, compared with NSIL (Table II). In this regard, the viral genome is replicated as episomal DNA during productive infections, while viral integration in the host chromosome by the HR-HPV has been associated with the progression of SIL to SCC (58). Deletion of the E2 gene results in the loss of negative regulation of the transcription of oncogenes E6 and E7, favoring dyscontrolled cellular proliferation and immortalization (59). Having found an increased expression in LSIL (1.6±0.3), in comparison with normal samples (1.1±0.1), it can be suggested that these cells possess a high proliferative capacity and that this could facilitate the detection of cells with potential transformation into an HSIL. These conclusions allow us to consider the importance of strict follow-up of patients with LSIL with HR-HPV alone or with MI, which also would allow the evaluation of the prognostic value of miR-16-1.

Furthermore, the present study identified that the mixed or integrated HPV states exhibited a significant effect on the expression level of miR-16-1, in comparison with samples negative for HPV ($P<0.001$); however, the variability in the expression level of miR-16-1 has a greater explanation by the changes induced by SIL and SCC (83%) than by the HPV physical state (33%). In addition, when comparing women who presented with the mixed state and those with the integrated state, no significant difference was observed ($P=0.105$). A limitation of the present study was that patients who presented only with the HPV episomal physical state were not included, which could have provided additional information on the expression level of miR-16-1 compared with those with an integrated physical state. Notably, to the best of our knowledge, no studies have analyzed this relationship before. However, it has been suggested that the increased expression of miR-16-1 in CC could be due to the molecular mechanism induced by the interaction of E7 of the HPV16 and E2F (53). To demonstrate whether E7 is directly associated with the increase in the expression of miR-16-1 but not E6, a study was performed with tissue samples derived from human keratinocytes, with and without HPV16 and HPV18. The results demonstrated that on inducing the expression of E6, E7 and E6/E7, the increase in the expression level of miR-16-1 was only observed in the presence of E7. This suggests that E7 was responsible for the overexpression of miR-16-1 in CC cells. In addition, by silencing the expression of E7 by small interfering RNA in CaSki (HPV16) and HeLa (HPV18) cell lines, it was demonstrated that E7-knockdown decreased the expression of miR-16-1 in comparison with the control cells (22).

It has been reported that HPV16 possesses an integration site in chromosome 13q14 (60), where the DLEU2 gene is localized, and this could activate the transcription of miR-16-1 (61). It has also been reported that HPV18 contains an integration site on chromosome 8q23-24, near the c-Myc gene (62,63), which is known to be able to activate the DLEU2 gene and induce the transcription of miR-16-1 (64). The overexpression of miR-16-1 has been found to be associated with the activation of genes implicated in cellular proliferation, such as CDK6, CDC27, CARD10, C10orf46 (23), CDC7 (21) and CCNE1 (9). Additionally, E6, on degrading into p53, inhibits the expression of kinase-inhibitor proteins (55), generating an uncontrolled environment for the proliferation and immortalization of cancerous cells.

In conclusion, the present results demonstrated that the increased expression of miR-16-1 was associated with increased cellular proliferation of HSIL and SCC in the presence of the integrated state of the HR-HPV DNA alone or in MI. This suggests that the expression level of miR-16-1 could serve as an additional tool in the diagnosis of HSIL that exhibits potential progression to SCC. Therefore, follow-up studies on a larger scale are required in order to examine the clinical usefulness of the expression of miR-16-1 as a prognostic biomarker of SIL, particularly in women with a diagnosis of LSIL and the integrated state of the HR-HPV, which can later progress to HSIL.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

LDCAR and EILBP designed and supervised the research. LDCAR performed the cytological diagnosis. BIA carried out the molecular diagnosis of the HPV. YCC and HJW collected

the samples and the survey data. MIZG conducted the extraction of the RNA for reverse transcription-quantitative PCR. KIGP carried out the liquid-based cytology for *in situ* hybridization with amplification with tyramide. EFA performed the statistical analysis. MIZG, LDCAR and EFA wrote and revised the manuscript. All authors read and approved the manuscript.

Ethics approval and consent to participate

All patients signed an informed consent for the use of their cervical samples and clinical information, and this study was approved by the Bioethics Committee at the Autonomous University of Guerrero, Guerrero, Mexico (approval no. CB-003/2018).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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