Int J Clin Exp Med 2015;8(9):15999-16006
www.ijcem.com /ISSN:1940-5901/IJCEM0012901

Original Article

MicroRNA miR-16-1 regulates CCNE1 (cyclin E1) gene expression in human cervical cancer cells

Ma Isabel Zubillaga-Guerrero[1], Luz del Carmen Alarcón-Romero[1], Berenice Illades-Aguiar[1], Eugenia Flores-Alfaro[1], Víctor Hugo Bermúdez-Morales[2], Jessica Deas[2], Oscar Peralta-Zaragoza[2]

[1]Academic Unit of Biological Chemical Sciences, Guerrero Autonomous University, Avenida Lázaro Cárdenas S/N, Col. Haciendita, Chilpancingo 39070, Guerrero, México; [2]Direction of Chronic Infections and Cancer, Research Center in Infection Diseases, National Institute of Public Health, Av. Universidad No. 655, Cerrada los Pinos y Caminera, Colonia Santa María Ahuacatitlán, Cuernavaca 62100, Morelos, México

Received July 14, 2015; Accepted September 1, 2015; Epub September 15, 2015; Published September 30, 2015

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 upregulated, and is an indicator for poor outcome in squamous cell carcinomas (SCCs). 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 (C-33 A 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. 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.

Keywords: 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 miR-16-1 expression in cervical cancer (CC) tissues compared to normal cervical tissues; however, the overexpression 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 an oncomir, as well as by the cell-type specificity of them miR-16-1 overexpression 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 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 in 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 upregulated 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 overexpression 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 C-33 A (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 downregulation of CCNE1 gene expression in SiHa (17%), CaSki (46%), HeLa (58%) cells and C-33 A (22%) cells. 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 (C-33 A 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 Pure Yield plasmid midiprep system (Promega, Madison, WI) and integrity was verified by DNA sequencing in Genetic Analyzer 3500xl equipment (Applied Biosystems, Foster, CA). The miRBase reference sequence of

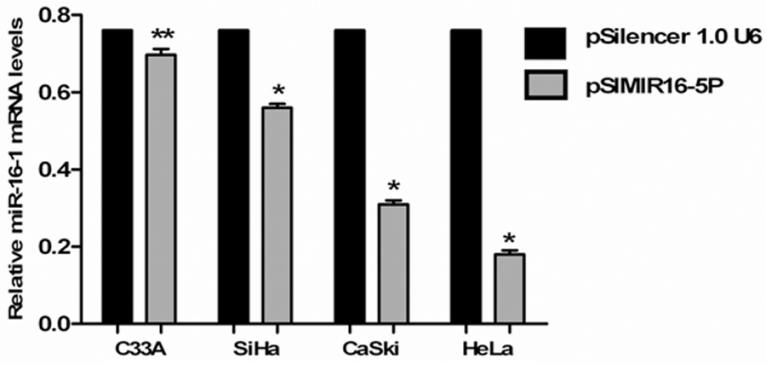

Figure 1. Silencing of human microRNA mir-16-1 expression by siRNAs. Quantitative real time RT-PCR analysis of miR-16-1 expression in C-33 A HPV-, SiHa HPV16+, CaSki HPV16+ and HeLa HPV18+ cells transfected with pSIMIR16-5P plasmid. Total RNA and cDNA synthesis were obtained from 1×10^5 cells per well 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 ($^*P<0.001$) ($^{**}P<0.05$).

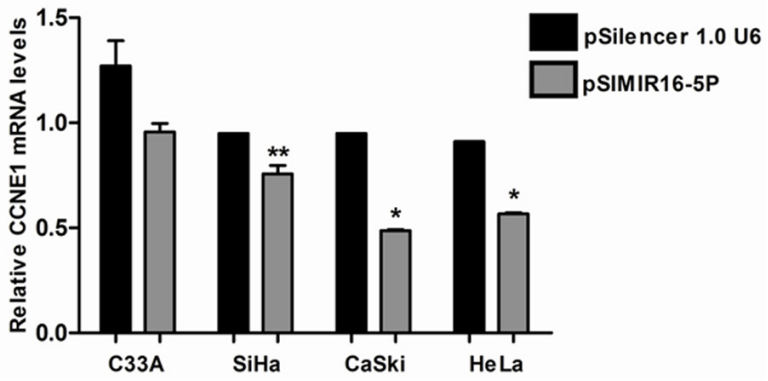

Figure 2. Analysis of CCNE1 gene expression by RT-qPCR after miR-16-1 silencing. Quantitative real time RT-PCR analysis of miR-16-1 expression in C-33 A HPV-, CaSki HPV16+, SiHa HPV16+, and HeLa HPV18+ cells transfected with pSIMIR16-5P plasmid. Total RNA and cDNA synthesis were obtained from 1×10^5 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 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 ($^*P<0.001$) ($^{**}P<0.05$).

GenBank to the sequence of hsa-miR-16-1 is: MI0000070.

Transfection assays with sirna expression plasmids

C-33 A, 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 1×10^5 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 3 μg 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 Tri-Pure 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 10 sec and 60°C for 60 sec. 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 Taq-Man one-step RT-PCR master mix reagent kit (Life Technologies, Foster City, CA: 430-9169). The PCR reaction was conducted at 48°C for 30 min followed by an AmpliTaq gold activation at 95°C for 10 min followed of 40 cycles of 95°C for 15 sec 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.

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 C-33 A (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.

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. **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 C-33 A 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.

Discussion

In the present study, we demonstrate that in CC cells, the over expression 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 encod-

ing 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 C-33 A (HPV-) cells (**Figure 1**). These findings are relevant because we silenced the expression of miR-16-1 which is over expressed 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 C-33 A cells despite treatment with pSIMIR16-5P plasmid. The C-33 A 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 that miR-16-1 could have a role similar to an oncomir in human CC cells, since it has been reported upregulated 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 included 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 (459-492 nt), MRE-3 (1641-1676) and MRE-4 (1878-1914) [7, 29].

Our results show a decrease in CCNE1 differential between HPV16+ and HPV18+ cells com-

pared to HPV- cells. C-33 A 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 HPV16 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 overexpression of HPV18 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 increased expression of CCNE1 unlike they 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, 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 over expressed in a subset of human cancers, and thus manipulation of this pathway may represent a therapeutic strategy for cancer therapy in the future.

Acknowledgements

This article was carried-out at the National Institute of Public Health from México (INSP) and it received funding sources from INSP, as well as from the National Council of Science and Technology (CONACYT) with file numbers: SALUD-2008-01-87130, SALUD-2009-01-111-892 and CB-2011-01-169209. We also thank the strengthening program graduate high with file number: I010/455/2013 C-677/2013. Zubillaga-Guerrero MI was recipient of a Doctoral fellowship with file number 231250 from CONACYT, México. We thank Crisalde Ramirez Celis for their assistance in reviewing the English grammar of the manuscript.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Oscar Peralta-Zaragoza, Direction of Chronic Infections and Cancer, Research Center in Infection Diseases, National Institute of Public Health. Av. Universidad No. 655, Cerrada los Pinosy Caminera. Colonia Santa María Ahuacatitlán, Cuernavaca 62100, Morelos, México. Tel: +527773293000; Fax: (+52)-777-1012925; E-mail: operalta@insp.mx

References

[1] Bartel PD. MicroRNAs: Genomics, Biogenesis, Mechanism, and Function. Cell 2004; 116: 281-297.

[2] Chen CZ. MicroRNAs as oncogenes and tumor suppressors. N Engl J Med 2005; 353: 1768-1771.

[3] Calin GA, Cimmino A, Fabbri M, Ferracin M, Wojcik SE, Shimizu M, Taccioli C, Zanesi N, Garzon R, Aqeilan RI, Alder H, Volinia S, Rassenti L, Liu X, Liu CG, Kipps TJ, Negrini M and Croce CM. MiR-15a and miR-16-1 cluster functions in human leukemia. Proc Natl Acad Sci U S A 2008; 105: 5166-5171.

[4] Zheng ZM and Wang X. Regulation of cellular miRNA expression by human papillomaviruses. Biochim Biophys Acta 2011; 1809: 668-677.

[5] Linsley PS, Schelter J, Burchard J, Kibukawa M, Martin MM, Bartz SR, Johnson JM, Cummins JM, Raymond CK, Dai H, Chau N, Cleary M, Jackson AL, Carlton M and Lim L. Transcripts targeted by the microRNA-16 family cooperatively regulate cell cycle progression. Mol Cell Biol 2007; 27: 2240-2252.

[6] Chen RW, Bemis LT, Amato CM, Myint H, Tran H, Birks DK, Eckhardt SG and Robinson WA. Truncation in CCND1 mRNA alters miR-16-1 regulation in mantle cell lymphoma. Blood 2008; 112: 822-829.

[7] Wang F, Xiang D, Zhou Y and Zhang Y. Down-regulation of the cyclin E1 oncogene expression by microRNA-16-1 induces cell cycle arrest in human cancer cells. BMB Rep 2009; 42: 725-730.

[8] Wang S, Tang S, Le Y, Lu R, Rader JS, Meyers C and Zheng ZM. Aberrant expression of oncogenic and tumor-suppressive microRNAs in cervical cancer is required for cancer cell growth. PLoS One 2008; 3: e2557.

[9] Sauer K and Lehner CF. The role of cyclin E in the regulation of entry into S phase. Prog Cell Cycle Res 1995; 1: 125-139.

[10] Hu W, Nevzorova YA, Haas U, Moro N, Sicinski P, Geng Y, Barbacid M, Trautwein C and Liedtke C. Concurrent deletion of cyclin E1 and cyclin-dependent kinase 2 in hepatocytes inhibits DNA replication and liver regeneration in mice. Hepatology 2014; 59: 651-660.

[11] Sheu JJ, Choi JH, Guan B, Tsai FJ, Hua CH, Lai MT, Wang TL and Shih IeM. Rsf-1, a chromatin remodelling protein, interacts with cyclin E1 and promotes tumour development. J Pathol 2013; 229: 559-568.

[12] Nakayama N, Nakayama K, Shamima Y, Ishikawa M, Katagiri A, Iida K and Miyazaki K. Gene amplification CCNE1 is related to poor survival and potential therapeutic target in ovarian cancer. Cancer 2010; 116: 2621-2634.

[13] Coradini D, Boracchi P, Oriana S, Biganzolo E and Ambrogi G. Differential expression of genes involved in the epigenetic regulation of cell identity in normal human mammary cell commitment and differentiation. Chin J Cancer 2014; 33: 501-510.

[14] Erlandsson F, Martinsson-Ahlzen H, Wallin K, Hellström AC, Andersson S and Zetterberg A. Parallel cyclin E and cyclinA expression in neoplastic lesions of the uterine cervix. Br J Cancer 2006; 94: 1045-1050.

[15] Lui WO, Pourmand N, Patterson BK and Fire A. Patterns of known and novel small RNAs in human cervical cancer. Cancer Res 2007; 67: 6031-6043.

[16] Lee JW, Choi CH, Choi JJ, Park YA, Kim SJ, HwangSY, Kim WY, Kim TJ, Lee JH, Kim BG and Baue DS. Altered microRNAs expression in cervical carcinomas. Cancer Res 2008; 14: 2535-2542.

[17] Pereira PM, Marques JP, Soares AR, Carreto L and Santos MA. MicroRNA expression variability in human cervical tissues. PLoS One 2010; 5: e11780-e11780.

[18] Wang X, Wang HK, Li Y, Hafner M, Banerjee NS, Tang S, Briskin D, Meyers C, Chow LT, Vie X, Tuschl T and Zheng ZM. MicroRNAs are biomarkers of oncogenic human papillomavirus infections. Proc Natl Acad Sci U S A 2014; 111: 1-6.

[19] SiRNA at Whitehead. SiRNA target finder and design tool (2004). http://jura.wi.mit.edu/bioc/siRNAext/Accessed: March 26, 2014.

[20] Gregory RI and Shiekhattar R. MicroRNA Biogenesis and cancer. Cancer Res 2005; 65: 3509-3512.

[21] Salazar-León J, Reyes-Román F, Meneses-Acosta A, Merchant H, Lagunas-Martínez A, Meda-Monzón E, Pita-López ML, Gómez-Cerón C, Bermúdez-Morales VH, Madrid-Marina V and Peralta-Zaragoza O. Silencing of HPV16 E6 and E7 oncogenic activities by small interference RNA induces autophagy and apoptosis in human cervical cancer cells. J Nucleic Acids Investig 2011; 2: 59-69.

[22] Peralta-Zaragoza O, De-la-O-Gómez F, Deas J, Fernández-Tilapa G, Fierros-Zarate G del S, Gómez-Cerón C, Burguete-García A, Torres-Poveda VH, Rodríguez-Dorantes M, Pérez-Plasencia C and Madrid-Marina V. Selective silencing of gene target expression by siRNA expression plasmids in human cervical cancer cells. Methods Mol Biol 2015; 1249: 153-171.

[23] Thorland EC, Myers SL, Gostout BS and Smith DI. Common fragile sites are preferential targets for HPV16 integrations in cervical tumors. Oncogene 2003; 22: 1225-1237.

[24] Ofir M, Hacohen D and Ginsberg D. miR-15 and miR-16 are direct transcriptional targets of E2F1 that limit E2F-induced proliferation by targeting cyclin E. Mol Cancer Res 2011; 9: 440-447.

[25] Schmitz M, Driesch C, Jansen L, Runnebaum IB and Dürst M. Non-Random Integration of the HPV Genome in Cervical Cancer. PLoS One 2012; 7: e39632.

[26] Ferber MJ, Thorland EC, Brink AA, Rapp AK, Phillips LA, McGovern R, Gostout BS, Cheung TH, Chung TKH, Fu WY and Smith DI. Preferential integration of human papillomavirus type 18 near the c-myc locus in cervical carcinoma. Oncogene 2003; 22: 7233-7472.

[27] Chang TC, Yu D, Lee YS, Wentzel EA, Arking DE, West KM, Dang CV, Thomas-Tikhonenko A and Mendell JT. Widespread microRNA repression by Myc contributes to tumorigenesis. Nat Genet 2008; 40: 43-50.

[28] Nguyen C and Münger K. Direct association of the HPV16 E7 oncoprotein with cyclin A/CDK2 and cyclin E/CDK2 complexes. Virology 2008; 380: 21-25.

[29] Kaddar T, Roualult JP, Chien WW, Chebel A, Gadoux M, Salles G, Ffrench M and Mgaud JP. Two new miR-16 targets: caprin-1 and HMGA1, proteins implicated in cell proliferation. Biol Cell 2009; 101: 511-524.

[30] Lu X, Lin Q, Lin M, Duan P, Ye L, Chen J, Chen X, Zhang L and Xue X. Multiple-Integrations of HPV16 Genome and Altered Transcription of Viral Oncogenes and Cellular Genes Are Associated with the Development of Cervical Cancer. PLoS One 2014; 9: e97588-e97597.

[31] Popescu NC, DiPaolo JA and Amsbaugh SC. Integration sites of human papillomavirus 18 DNA sequences on HeLa cell chromosomes. Cytogenet Cell Genet 1987; 44: 58-62.