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UNIDAD ACADÉMICA DE CIENCIAS QUÍMICO BIOLÓGICAS

UNIDAD DE INVESTIGACIÓN ESPECIALIZADA EN MICROBIOLOGÍA

DOCTORADO EN CIENCIAS BIOMÉDICAS

"METILACIÓN DE PROMOTORES DE GENES DE miRNAs Y SU EXPRESIÓN EN LA CARCINOGÉNESIS CERVICAL ASOCIADA A VPH 16"

T E S I S

QUE PARA OBTENER EL GRADO DE

DOCTOR EN CIENCIAS BIOMÉDICAS

P R E S E N T A

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ACTA DE APROBACIÓN DE TESIS

En la ciudad de Chilpancingo, Guerrero, siendo los 10 días del mes de julio del dos mil catorce, se reunieron los miembros del Comité Tutoral designado por la Academia de Posgrado del Doctorado en Ciencias Biomédicas, para examinar la tesis titulada **"Metilación de promotores de genes de miRNAs y su expresión en la carcinogénesis cervical asociada a VPH 16"**, presentada por la alumna Hilda Jiménez Wences, 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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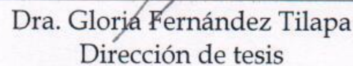

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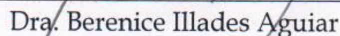

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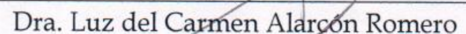

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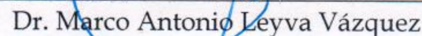

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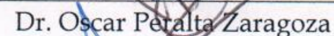

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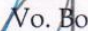

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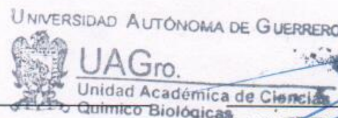

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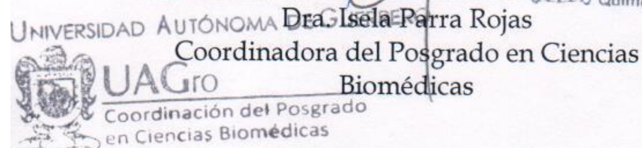

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Metilación de promotores de genes de miRNAs y su expresión en la carcinogénesis cervical asociada a VPH 16

El proyecto fue realizado en el Laboratorio de Investigación Clínica de la Unidad Académica de Ciencias Químico Biológica de la Universidad Autónoma de Guerrero (UACQB-UAGro) en Chilpancingo, Guerrero.

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DEDICATORIAS

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Resumen

La progresión de las lesiones precursoras a cáncer cervical invasor está influida entre otros factores por la infección persistente por virus del papiloma humano de alto riesgo (VPH-AR), los cuales inducen cambios en el genoma y epigenóma del hospedero. Actualmente se considera que la metilación aberrante de promotores de microRNAs (miRNAs) en cáncer cervical puede estar implicada en la alteración de su expresión. **Objetivo:** Evaluar el papel de la metilación en la regulación de la expresión de miRNAs en líneas celulares de cáncer cervical y determinar el estado de metilación de promotores de genes de miRNAs y su expresión en la carcinogénesis cervical asociada a VPH 16. **Metodología:** Se incluyeron en el estudio 72 mujeres originarias del Estado de Guerrero, México; 19 mujeres con LEIBG, 7 con LEIAG y 28 con cáncer cervical todas con VPH 16 y 18 mujeres sin LEI con y sin VPH 16. La detección y tipificación del VPH se realizó con el kit INNO LiPA genotyping Extra kit (Innogenetics, Barcelona, España). La metilación del promotor de cada miRNA se determinó con el arreglo Human Cancer miRNA EpiTect® Methyl II PCR Array (Qiagen, Maryland, USA). La expresión de cada miRNA se determinó por qRT-PCR con ensayos TaqMan individuales (Applied Biosystem, Foster, CA, USA). Las células HeLa, SiHa, CaSki y C33A fueron tratadas con 5-Aza-2'-deoxicitidina (5'-Aza-CdR). Para la normalización de los datos se utilizó a miR-92a. **Resultados:** Diferencias significativas en la metilación de 5 genes de miRNAs entre las mujeres de cáncer cervical y las mujeres con LEIBG fueron observadas; miR-124-2, miR-218-1, miR-218-2 y miR-34b/c se encontraron metilados mientras que miR-193b se encontró desmetilado en mujeres con cáncer cervical. Por otra parte, el análisis de la expresión de los miRNAs determinó que miR-124 y miR-218 se encuentran disminuidos y miR-193b sobre-expresado en mujeres con cáncer cervical en comparación con mujeres con LEIBG. El análisis de correlación entre el estado de metilación de los promotores de miR-124 y miR-193b con sus niveles de expresión indicó una correlación inversa (no significativo). Finalmente el tratamiento con 5'-Aza-CdR incrementó los niveles de expresión de miR-23b y miR-218, en líneas celulares de CaCU. **Conclusión:** Los resultados indican que es posible que alteraciones en la metilación de promotores de genes de miRNAs sea un mecanismo epigenético implicado en su expresión aberrante en cáncer cervical.

Introducción

El cáncer se origina por alteraciones genéticas y epigenéticas que provocan cambios en la expresión de proteínas y RNAs pequeños y largos no codificantes que conllevan a la transformación celular maligna (Kang *et al*., 2014; Virani *et al*., 2012). Las modificaciones epigenéticas tales como, la metilación e hidroximetilación del DNA, la acetilación y metilación de histonas así, como la expresión de RNAs pequeños no codificantes como los miRNAs (miRNAs), tienen efectos en la expresión de genes sin alterar la secuencia primaria del DNA manteniendo así, la homeostasis celular (Rouhi *et al*., 2008; Virani *et al*., 2012; Whiteside *et al*., 2008). Alteraciones epigenéticas pueden ocurrir en etapas muy tempranas del desarrollo neoplásico y está ampliamente descrito que juegan un papel esencial en la progresión al cáncer (Virani *et al*., 2012).

Los miRNAs son moléculas pequeñas de RNA no codificantes de aproximadamente 22-25 nucleótidos (nt), a menudo conservados filogenéticamente que presentan un patrón de expresión tejido-tiempo-específico (Jost *et al*., 2011; Shivdasani, 2006). Existen más de 1,000 miRNAs que están involucrados en la regulación transcripcional y postranscripcional de más del 30% de todos los genes humanos (Deiters, 2009; Jost *et al*., 2011; Siomi & Siomi, 2010). La regulación a nivel pos-transcripcional de la expresión de genes por los miRNAs se lleva a cabo cuando el miRNA a través de una secuencia de 2-8 nt ubicada en su extremo 5´ se une a sitios afines en la región 3´ no traducida (3´UTR) del RNAm blanco. La complementariedad perfecta miRNA/RNAm induce la degradación del mRNA blanco y la complementariedad parcial de 1 o más bases inhibe la traducción del mRNA (Chu & Rana, 2007; Heneghan *et al*., 2010; Jost *et al*., 2011; Siomi & Siomi, 2010). Los miRNAs se han catalogado como importantes reguladores epigenéticos que controlan la expresión de genes y alteraciones en su expresión se ha asociado al desarrollo del cáncer (Bandres *et al*., 2009; Pavicic *et al*., 2011). Estudios de perfil de expresión en cáncer han revelado que los miRNAs actúan como supresores de tumor cuando sus blancos son transcritos de proto-oncogenes y como oncogenes (oncomirs), cuando sus blancos son transcritos de genes supresores de tumor (Bandres *et al*., 2009; Deiters, 2009; *Ng et al*., 2009; Nicoloso & Calin, 2008; Pavicic *et al*., 2011; Pereira *et al*., 2010).

La regulación de la expresión de los miRNAs es importante para mantener la homeostasis celular. La regulación de la expresión de los miRNAs se lleva a cabo por diversos mecanismos; 1) a nivel transcripcional, que comprende la transcripción del pri-miRNA por la RNA polimerasa II y III, 2) a nivel postranscripcional, que involucra la maduración del miRNA que comprende el procesamiento del pri-miRNA a pre-miRNA, su exportación al citoplasma y su incorporación al complejo RISC, y 3) a nivel de localización del miRNA en el genoma (Davis-Dusenbery & Hata, 2010; Rouhi *et al.*, 2008). Defectos en los mecanismos que regulan la expresión de un miRNA están implicados en la transformación celular (Bandres *et al.*, 2009; Ng *et al.*, 2009; Nicoloso & Calin, 2008; Pavicic *et al.*, 2011; Pereira *et al.*, 2010).

Defectos en la expresión de miRNAs en cáncer se han relacionado con 1) alteraciones genéticas como deleciones, amplificaciones y mutaciones puntuales debido a que más de la mitad de los miRNAs se encuentran en sitios frágiles o en regiones relacionadas con el cáncer y, 2) alteraciones epigenéticas como modificación de histonas y la metilación aberrante del DNA (Cho, 2007; Ferreira *et al.*, 2012; So *et al.*, 2011; Yang *et al.*, 2009; Zhang *et al.*, 2008). Además varias proteínas claves en la biogénesis de los miRNAs pueden estar disfuncionales o desreguladas en cáncer (Zhang *et al.*, 2008). Actualmente las investigaciones se han enfocado en revelar el papel de las modificaciones epigenéticas en la desregulación de los miRNAs en cáncer ya que al poseer promotores con una estructura similar a la estructura de los promotores de los genes codificantes (elementos TATA, elementos de reconocimiento a TFIIB, elementos de inicio de la transcripción marcas de histonas e Islas CpG de aproximadamente 500 pb), pueden estar sujetos a regulación epigenética (Bandres *et al.*, 2009; Hata & Davis, 2009; Ozsolak *et al.*, 2008).

Uno de los mecanismos epigenéticos mas estudiado es la metilación del DNA proceso que ocurre después de la replicación del DNA como una reacción enzimática. La metilación es un tipo de modificación covalente en el cual un grupo metilo es adicionado en el carbono 5 de citosinas adyacentes a guanina (dinucleótidos CpG) vía S-adenosilmetionina (Weber *et al.,* 2007). La metilación del DNA es catalizada por tres

tipos de enzimas DNA-metiltransferasas (DNMTs); la DNMT1, una DNA-metiltransferasa de mantenimiento que preserva el patrón de metilación en cada división celular y la DNMT3a y DNMT3b que son DNA-metiltransferasas *de novo*. Las DNMTs son las responsables de adicionar el grupo metilo en el carbono 5 de las citosinas adyacentes a guanina provocando el silenciamiento génico (Bandres *et al*., 2009). La metilación del DNA es una reacción reversible que ocurre en áreas específicas del genoma llamadas islas CpG que comprenden alrededor de 0.5-3 kb y se encuentran en promedio cada 100 kb en el genoma (Li *et al*., 2012). Aproximadamente la mitad de los genes humanos contienen islas CpG que están ubicadas principalmente en su región promotora (Li *et al*., 2012; Valeri *et al*., 2009; Yang *et al*., 2008; Yan *et al*., 2011).

El silenciamiento génico por metilación de islas CpG se lleva a cabo de forma directa e indirecta. La forma directa se debe a que la adición del grupo metilo en las CpG interfiere con la unión de los factores de transcripción y la forma indirecta se lleva a cabo por el reclutamiento de proteínas con dominio de unión a dinucleótidos CpG metilados (MeCP ó MBD) que inducen cambios en la estructura de la cromatina (Weber *et al*., 2009; Virani *et al*., 2012). La represión de la transcripción por metilación puede depender de la concentración local de CpGs en el promotor ya que promotores ricos en dinucleótidos CpG metilados son incompatibles con la actividad génica (Weber *et al*., 2007). Aunque la metilación de dinucleótidos CpG en regiones promotoras de genes es un mecanismo importante en la regulación de la expresión génica, la metilación aberrante de genes supresores de tumor tienen un papel significativo en el desarrollo tumoral (Bandres *et al*., 2009). De hecho, la disminución en la expresión de genes supresores de tumor en cáncer ha sido fuertemente relacionada con la hipermetilación de islas CpG en sus regiones promotoras (Bandres *et al*., 2009). A pesar de que existen pocos estudios sobre la regulación de la expresión de miRNAs por mecanismos epigenéticos, se ha propuesto que la metilación aberrante podría ser responsable de la desregulación de la expresión de miRNA en cáncer (Yao *et al*., 2013).

En la carcinogénesis cervical se considera que uno de los eventos tempranos y frecuentes son las modificaciones en el estado de metilación del DNA celular (Yang, 2013), observándose una hipometilación global del DNA que incrementa progresivamente con el grado de la displasia que favorece la sobreexpresión de oncogenes y también ocurre la hipermetilación de genes supresores de tumor (Kim *et al.*, 1994; Missaoui *et al.*, 2010; Yang, 2013). Ambos eventos se relaciona con la severidad de la displasia cervical asociada a la infección por virus del papiloma humano de alto riesgo (VPH-AR) (Kim *et al.*, 1994; Missaoui *et al.*, 2010; Yang, 2013).

El involucramiento del VPH en la alteración de los patrones de metilación se ha reportado tanto *in vitro* como *in vivo*, sin embargo, los mecanismos y sus implicaciones aún no son conocidos (Burgers *et al.*, 2007). Reportes indican que el VPH interfiere con la maquinaria de metilación del DNA, resultando en la trans-activación y trans-represión tanto del DNA celular como del DNA viral (Leonard *et al.*, 2012; Missaoui *et al.*, 2010; Yang, 2013). También, se sabe que la integración viral (evento esencial durante la transformación maligna), induce la acumulación de cambios epigenéticos tanto en el DNA celular como viral (Chaiwongkot *et al.*, 2013; Kalantari *et al.*, 2004). Por otra parte, se especula que la regulación de la expresión de genes tempranos y tardíos durante la replicación del VPH se lleva a cabo por la metilación del DNA viral (Chaiwongkot *et al.*, 2013; Kalantari *et al.*, 2004). Además es posible que los cambios en la metilación del DNA celular surja como un mecanismo de defensa de la célula por la presencia de DNA extraño (Chaiwongkot *et al.*, 2013; Kalantari *et al.*, 2004).

Poco se sabe sobre la participación de los virus oncogénicos en la modificación de los patrones de metilación del DNA celular, sin embargo se ha observado que después de una infección por VPH 16 se producen alteraciones epigenéticas en el DNA celular inducidas por las oncoproteínas E6 y E7 (Burgers *et al.*, 2007; Au Yeung *et al.*, 2010; Leonard *et al.*, 2012). También se ha reportado que las oncoproteínas E6 y E7 del VPH-AR incrementan la actividad y la expresión de la DNMT1 (Au Yeung *et al.*, 2010; Leonard *et al.*, 2012). E6 lo hace a través de la degradación de p53 quien regula negativamente la expresión de la DNMT1, a través de la formación de un complejo

represor con la proteína de especificidad 1 (Sp1) y con proteínas modificadoras de la cromatina sobre el promotor de la DNMT1 (Li *et al*., 2010; Au Yeung *et al*., 2010; Leonard *et al*., 2012). La modulación de la expresión de la DNMT1 por E7, se puede llevar a cabo por dos vías; a) por vía indirecta, a través de la unión de E7 a pRb, provocando la liberación del factor de transcripción E2F que regula la actividad del promotor de la DNMT1, y b) por vía directa, por unión de E7 a la DNMT1, induciendo un cambio conformacional que expone el sitio activo de la DNMT1 promoviendo la unión DNMT1/DNA/S-adenosil-L-metionina (AdoMet) (Burgers *et al*., 2007). El aumento de la actividad enzimática de la DNMT1 puede conducir a la metilación aberrante de genes codificantes y no codificantes como los miRNAs (Burgers *et al*., 2007; McCabe *et al*., 2006).

En pacientes y líneas celulares de cáncer cervical se ha observado que el silenciamiento de miRNAs supresores de tumor por metilación aberrante de sus promotores favorece la carcinogénesis cervical por lo que en este estudio se valoró si la metilación aberrante de promotores de miRNAs reportados como desregulados en cáncer (anexo 1), está implicada en la alteración de su expresión en cáncer cervical.

CAPÍTULO I

Human papilloma virus, DNA methylation and microRNA expression in cervical cancer (Review)

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Human papilloma virus, DNA methylation and microRNA expression in cervical cancer (Review)

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Abstract. Cancer is a complex disease caused by genetic and epigenetic abnormalities that affect gene expression. The progression from precursor lesions to invasive cervical cancer is influenced by persistent human papilloma virus (HPV) infection, which induces changes in the host genome and epigenome. Epigenetic alterations, such as aberrant miRNA expression and changes in DNA methylation status, favor the expression of oncogenes and the silencing of tumor-suppressor genes. Given that some miRNA genes can be regulated through epigenetic mechanisms, it has been proposed that alterations in the methylation status of miRNA promoters could be the driving mechanism behind their aberrant expression in cervical cancer. For these reasons, we assessed the relationship among HPV infection, cellular DNA methylation and miRNA expression. We conclude that alterations in the methylation status of protein-coding genes and various miRNA genes are influenced by HPV infection, the viral genotype, the physical state of the viral DNA, and viral oncogenic risk. Furthermore, HPV induces deregulation of miRNA expression, particularly at loci near fragile sites. This deregulation occurs through the E6 and E7 proteins, which target miRNA transcription factors such as p53.

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3. HPV and cervical cancer
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6. miRNAs and their deregulation in cancer
7. HPV and miRNA promoter methylation in cervical cancer
8. Conclusion

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Key words: miRNAs, methylation, human papilloma virus, cervical cancer

1. Introduction

Cervical cancer is one of the most frequently occurring malignant tumors in women worldwide, with ~470,000 new cases and 233,000 deaths per year (1). Squamous cell cervical carcinoma represents approximately 80% of cases. Cervical cancer develops through well-defined pre-malignant lesions, which are known as cervical intraepithelial neoplasia (CIN), ranging from grades I to III (2). Cervical adenocarcinomas represent 10-20% of cases, but the preceding stages are not well characterized (2). The high-risk HPV (HR-HPV), as well as environmental, immunological, genetic and epigenetic factors, are among the etiological causes contributing to cervical carcinogenesis; progression of precursor lesions to invasive cancer is influenced by HR-HPV infection (3,4). Although the mechanisms by which HR-HPV induces changes to the host's genome and epigenome are still unknown, it has been established that integration of the viral DNA into the cellular genome causes genetic (deletions, amplifications and DNA rearrangements) and epigenetic (modifications to the DNA methylation status and aberrant miRNA expression) alterations. These result in the silencing of tumor-suppressor genes and the overexpression of oncogenes favoring tumor progression (5-8).

Epigenetic modifications are just as important as genetic modifications in terms of regulating gene expression and controlling disease onset. It has been shown that epigenetic silencing of some miRNA genes is functionally involved in cervical carcinogenesis (2,5,7). The interaction between HR-HPV and miRNAs occurs at different times during carcinogenesis, given that: i) some miRNA loci localize to fragile sites, which are the sites where HR-HPV inserts its DNA; ii) proteins encoded by HR-HPV can influence miRNA expression within the host cell and iii) it has been observed that

the E6 and E7 proteins of HR-HPV modulate the expression of DNA methyltransferases, which are enzymes that regulate gene expression by methylating promoter regions (2,5,9,10).

Changes in the expression profile of miRNAs have been reported in cervical cancer cell lines, cervical cancer tissue and precursor lesions (1,11-13). Similarly, studies conducted in cell lines suggest that HPV participates in deregulating miRNA expression by modifying the expression profile of miRNAs associated with the presence of HPV and the viral genotype (1,5,11,14,15). Given that a considerable number of miRNAs are subject to epigenetic regulation, it has been proposed that aberrant methylation of miRNA promoters is one of the mechanisms responsible for deregulated miRNA expression in cervical cancer (2,12,16). Here, we analyzed the influence of methylation on miRNA expression as well as on the expression of proteins that regulate cellular processes and participate in carcinogenesis. We further discuss the likelihood of HPV inducing modifications in the methylation status of miRNA promoters in cervical cancer. Lastly, we also assess the relationship between HR-HPV infection, methylation and miRNA expression.

2. HPV and cancer

HPV is one of the most common sexually transmitted infections worldwide and is associated with a wide spectrum of benign and malignant neoplasias (17). HPV is the second infectious agent implicated in cancer development, after *Helicobacter pylori* (17). It is estimated that 5.2% of all types of cancer can be attributed to HPV infection; HPV has been associated with 90-93% of anal cancer cases, 12-63% of oropharyngeal cancer cases, 36-40% of penile cancer cases, 40-51% of vulvar cancer cases, 40-64% of vaginal cancer cases and 99.7% of cervical cancer cases (17,18). Approximately 100 HPV subtypes with genetic variations and different oncogenic potentials have been identified and classified into three groups: high-risk (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82); probable high-risk (types 26, 53 and 66) and low-risk (types 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81 and CP6108) (4,19).

3. HPV and cervical cancer

Epidemiological and molecular studies have documented the causal link between HR-HPV infection and cervical cancer (3,18,20). HR-HPV subtypes HPV 16, HPV 18, and HPV 31 have been detected in 99.7% of squamous cell cervical carcinomas and in 94-100% of adenocarcinomas and adenosquamous carcinomas (12). It is estimated that ~11.4% of women worldwide and 9.4% of Mexican women are at risk of contracting an HPV infection at some point during their lives (21). Furthermore, it is estimated that >80% of sexually active women become infected with HPV, and >50% of young women are infected after their first sexual intercourse. Almost 90% of infections are spontaneously eliminated during a 3-year period, with only 10% becoming persistent infections. Among the latter, only 1% develop into cervical cancer (10,21).

Persistent HPV infection is required for normal cells to transform into cancerous cells (21). An important step in malignant progression is the integration of HPV into the host's genome (9,10,21). It appears that the integration event does not happen randomly. Although almost all chromosomes are susceptible, certain regions of the human genome are favored for viral DNA insertion, such as fragile sites, rupture and translocation points, and transcriptionally active regions (10). HPV integration into the cellular genome has several implications: i) it allows permanent expression of the E6 and E7 oncoproteins, which promote cell transformation and immortalization by inactivating the p53 and Rb tumor-suppressor genes, respectively, as well as other proteins that participate in cell adhesion, apoptosis, cell cycle, DNA repair, cellular metabolism, and signal transduction regulating transcription and translation; ii) viral integration near or within a gene can eventually lead to cell growth and proliferation alterations and iii) viral integration can induce epigenetic modification of viral and cellular genes, which may affect their expression. A series of epigenetic alterations in the cellular and viral genomes can occur during each stage of cervical cancer (7,10,21).

4. DNA methylation in cervical cancer

Regulation of gene expression is a vital process that determines the profile of proteins required to ensure the proper occurrence of processes including development, cellular differentiation, organogenesis, cellular stress response and programmed cell death (22). In normal tissues, epigenetic events such as DNA methylation, histone acetylation and expression of miRNAs, and other small RNAs regulate the expression of genes participating in the activation of differentiation processes as well as cellular functions that contribute to cellular homeostasis (23,24). Twenty-five years ago, it was discovered that epigenetic modifications participate in cancer development, leading to uncontrolled cell proliferation (23). One of the most widely studied epigenetic mechanisms is DNA methylation, a reversible reaction catalyzed by DNA methyltransferase (DNMT) enzymes. DNMT1 is a maintenance methyltransferase that preserves the methylation pattern during each cellular division. DNMT3a and DNMT3b are *de novo* methyltransferases (25,26). DNMTs add a methyl group onto carbon 5 of cytosine residues adjacent to guanine residues (5'-CpG-3'), which mainly occurs in CpG islands. CpG islands are generally found in the promoter regions of protein-coding genes, and expression is silenced upon their methylation. Non-coding genes, such as miRNAs, are also susceptible to regulation by methylation (25,26).

Global DNA hypomethylation in repetitive regions and hypermethylation in CpG island regions of tumor-suppressor gene promoters are DNA modifications that are commonly found early during cancer development (3,27). Alterations to the DNA methylation pattern, which have also been described in cervical cancer, contribute to genomic instability, chromosomal rearrangements, and silencing of coding and non-coding genes, such as miRNAs (2,20,28-30). Silencing of tumor-suppressor genes through DNA hypermethylation has been linked to the development of different types of cancers, including cervical cancer, and is frequently associated with poor clinical results (Table I). However, silencing of tumor suppressor miRNAs through hypermethylation of CpG islands in their promoter regions has also been implicated in carcinogenesis (30,31).

Table I. Hypermethylated genes associated with cancer development and the biological processes altered during carcinogenesis.

Hypermethylated genes in cancer	Biological process
hMLH1, *WRN*, *BRCA1*, ***MGMT***	DNA repair
CRBP1, ***RAR-β2***	Vitamin response
NOREIA, ***RASSF1A***	Ras signaling
p15INK4b, *Rb*, ***P16INK4a***, ***CCNA1***, ***FHIT***	Cell cycle
P14ARF, ***p73***, ***HIC-1***	p53 pathway
E-cadherin, *H-cadherin*, *FAT*, *EXT-1*, *SLIT2*, *EMP3*, ***CADM1***	Cell adherence and invasion
TMS1, *WIF-1*, *SFRP1*, ***hTERT***, ***DcR1***, ***DcR2***, ***DAPK1***	Apoptosis
DKK-1, *IGFBP-3*, ***APC***	Wnt signaling pathway
SOCS.1, *SOCS-3*, ***SYK***	Tyrosine kinase signaling cascade
GATA-4, *GATA-5*, *ID4*	Transcription factors
GSTP1, *LKB1/STK11*, *THBS-14*, *COX-2*, *SRBC*, *RIZ1*, *SLC5 A8*, *TPEF/HPP1*, *Laminin*, ***PTEN***, ***CDH1***, ***TSLC1***	Other pathways

Bold font, promoters reported to be hypermethylated in cervical cancer.

5. HPV and DNA methylation

It is thought that HR-HPV can induce changes in DNA methylation and histone acetylation and also cause aberrant miRNA expression (6). Little is known concerning the role of oncogenic viruses in the modification of cellular DNA methylation patterns (35). The hepatitis B, hepatitis C, Kaposi's sarcoma-associated and Epstein-Barr viruses interact with DNMTs, modulating their expression. As a result, viral and cellular genes are trans-activated and trans-repressed, respectively (6,35,36). Although the relationship between HPV and aberrant methylation in cervical cancer is not well understood, some authors have suggested that HPV interferes with the cellular DNA methylation machinery, either to conceal itself or as part of its viral cycle (6,35,37). Some investigators have described that upon HPV 16 infection, cellular DNA undergoes epigenetic alterations induced by the E6 and E7 oncoproteins (35,38,39). It has been proposed that methylation has arisen as a defense mechanism by the host cell to silence viral DNA (6,40,41).

The E6 and E7 oncoproteins of HR-HPV increase the expression and activity of DNMT1 (39). E6 does so by degrading p53 (35,39) (Fig. 1A). In the cervical cancer cell lines SiHa and CaSki, knockdown of E6 is associated with an increase in p53 and a decrease in DNMT1 expression (35,39). In contrast, Lin *et al* (42) showed that p53 negatively regulates DNMT1 expression both in cell lines and in lung cancer patients. p53 binds to the specificity protein 1 (Sp1) and chromatin-remodeling proteins, and this complex then binds to the promoter region of DNMT1. The formation of the complex inhibits Sp1 from activating the transcription of DNMT1 (42). Normally, Sp1 induces degradation of p53 by MDM2-mediated ubiquitination (Sp1/p53/MDM2 complex) and induces overexpression of DNMT1 (42). Non-small cell lung cancer patients with mutations in p53 and patients with alterations in p53 and/or Sp1 showed hypermethylated promoter regions of tumor-suppressor genes (p=0.003-0.016), most likely due to DNMT1 overexpression (42).

Modulation of DNMT1 expression by E7 (Fig. 1B) can occur in two different ways: i) indirectly, through E7 binding to pRb, which releases the transcription factor E2F; given that conserved E2F-binding sequences exist at the transcription start site for DNMT1, the release of E2F results in the regulation of the DNMT1 promoter activity and ii) directly, by binding of E7 to DNMT1 (38). It has been proposed that the E7/DNMT1 complex induces a conformational change in DNMT1, exposing its active site, promoting DNMT1/DNA binding, and binding to S-adenosyl-L-methionine (AdoMet) (38). Once the E7/DNMT1/DNA complex forms, E7 dissociates from the complex, and DNMT1 closes on the DNA, maintaining a stable DNMT1/DNA interaction (35,40). The increase in DNMT1 activity causes aberrant methylation of the cellular genome, resulting in the silencing of tumor-suppressor genes and favoring cellular transformation (38,40).

The role of DNMT1 in cervical carcinogenesis has been reported by Jin-Tao *et al* (43), who used *in vitro* and *in vivo* studies and found that low levels of serum folate and high expression of DNMT1 protein or mRNA were significantly associated with cervical carcinogenesis (p=0.001). Integration of HR-HPV DNA into the host's genome is an essential step in cervical carcinogenesis, and changes to viral DNA methylation are associated with the oncogenic capacity of HPV (28,29). After its integration into the human genome, the DNA of HPV 16 and HPV 18 is methylated (28,45). However, there are still controversial results regarding the participation of HPV in the aberrant DNA methylation that has been observed in cervical cancer.

Henken *et al* (28) used primary human foreskin keratinocytes (PHFKs) transfected with HPV 16 and HPV 18 to decipher the most important events in HR-HPV-mediated transformation. The authors used a longitudinal *in vitro* system utilizing serial passages and found that the transfected keratinocytes gradually developed dysplastic characteristics that were similar to pre-malignant cervical lesions. After immortalization, only the keratinocytes with HPV DNA integrated into their genome accumulated changes in the methylation

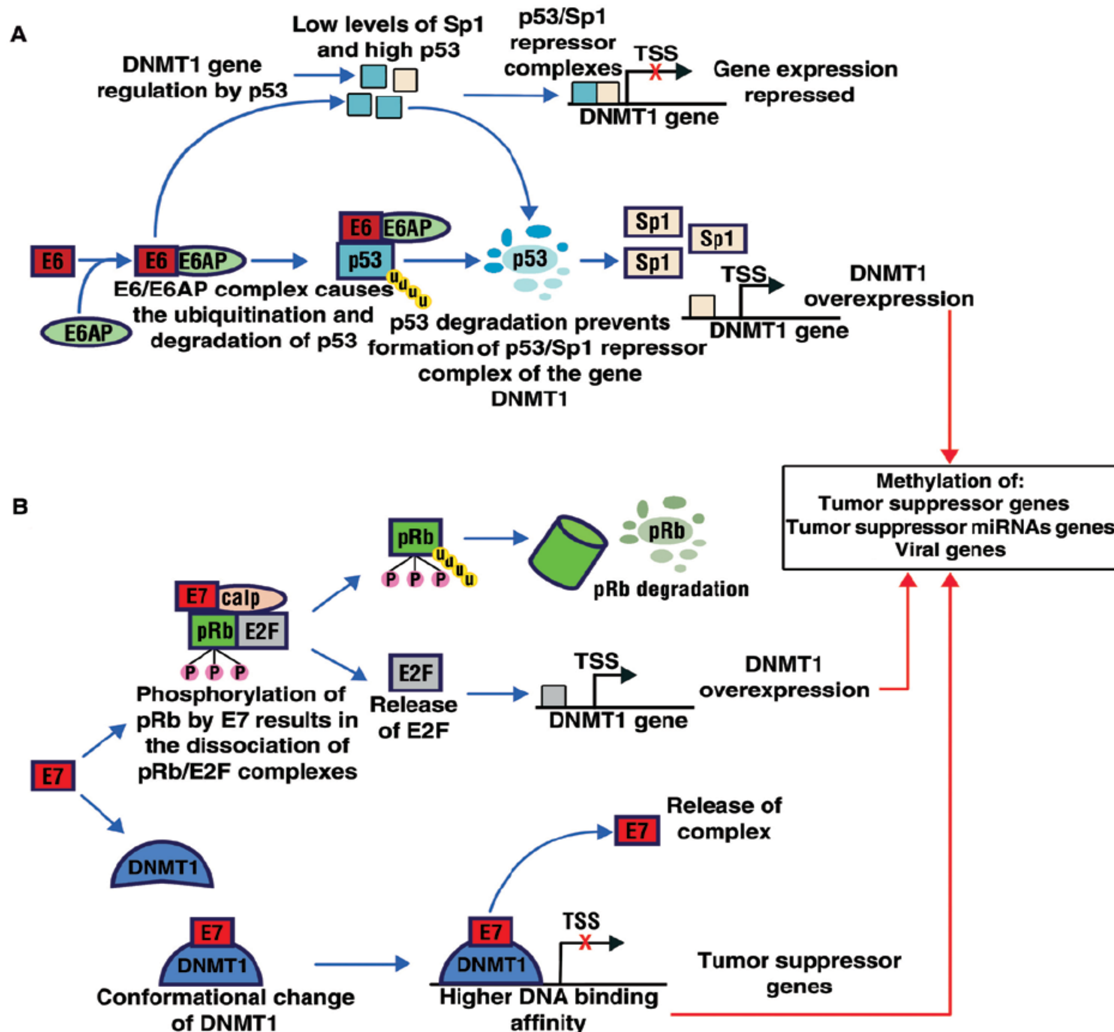

Figure 1. Regulation of viral and cellular gene methylation by the E6 and E7 oncoproteins of HR-HPV. Integration of viral DNA into the cellular genome causes genetic and epigenetic alterations. The E6 and E7 oncoproteins of HR-HPV increase the expression and activity of DNA methyltransferases, particularly DNMT1. (A) Binding of p53 to Sp1 (p53/Sp1) forms a repressor complex for DNMT1 transcription. Degradation of p53 by E6 avoids the formation of this repressor complex and Sp1 induces the expression and activity of DNMT1. (B) E2F positively regulates the promoter activity of DNMT1. Binding of E7 to pRb (E7/pRb) causes the release of E2F, favoring the expression of DNMT1. Binding of E7 to DNMT1 (E7/DNMT1) induces a conformational change in DNMT1, exposing its DNA binding site and promoting DNA binding; once the E7/DNMT1 complex binds DNA, DNMT1 closes on the DNA and maintains a stable DNMT1/DNA interaction, and E7 dissociates from the complex. Overexpression of DNMT1 results in hypermethylation of tumor-suppressor gene promoters, which leads to cellular transformation and tumorigenesis. HR-HPV, high-risk HPV; DNMT, DNA methyltransferase.

patterns in the promoter regions of different genes. After transfecting PHFKs with episomal forms of both HPV 16 and HPV 18, Leonard *et al* (35) and Henken *et al* (28) observed overexpression of DNMT1 and DNMT3B. They also found changes in the methylation status of different cellular genes that were previously reported to be methylated in cervical cancer, most likely due to an early epigenetic reprogramming induced by HR-HPV. Furthermore, Leonard *et al* (35) observed differences in the expression topography of DNMTs in relation to the viral genotype. Following transfection with HPV 16, nuclear expression of DNMT1 was restricted to cells in the basal and early differentiation layers and was decreased in more differentiated cells. However, in cells transfected with HPV 18, nuclear DNMT1 expression was observed in the basal and suprabasal layers as well as in the stratum granulosum, with some cells displaying intense DNMT1 staining. Changes in the expression topography of DNMT3B were similar to those observed for DNMT1. However, the global staining intensity was weaker. Moreover, Leonard *et al* (35) analyzed the whole-genome methylation profile after transfection with the episomal forms of HPV 16 and HPV 18 and found a significant increase in the methylation status of 5,607 and 2,387 genes, respectively. They also found a decrease in the methylation status of 3,568 and 4,160 genes, respectively.

Non-overlapping increases and decreases in methylation were found for 2,295 and 1,023 genes, respectively. It is possible that the altered miRNA expression observed in cervical cancer is related to the aberrant methylation of miRNA promoters. Thus, HR-HPV could indirectly induce aberrant miRNA methylation (2,20,46).

6. miRNAs and their deregulation in cancer

miRNAs are small, non-coding RNA molecules ~22-25 nucleotides (nt) in size. They are usually phylogenetically conserved with a tissue- and time-specific expression pattern (47,48). miRNAs have been recognized as epigenetic regulators, controlling gene expression without altering the DNA sequence (49). The expression profile of miRNAs in cell lines and cervical cancer tissues suggests that aberrant miRNA expression contributes to the development of cervical cancer and HR-HPV-induced precursor lesions (1,7,50). Defects in miRNA expression have been associated with: i) genetic alterations, such as deletions, amplifications and point mutations and ii) epigenetic alterations, such as histone modifications and aberrant DNA methylation (25,46,48,52).

Regulation of miRNA expression is important to maintain cellular homeostasis. However, the molecular mechanisms regulating miRNA gene transcription are not well understood to date (31,54). Currently, it is thought that miRNA biogenesis is regulated at several levels: i) at a transcriptional level, which consists of pri-miRNA transcription by RNA polymerase II and III; ii) at a post-transcriptional level, consisting of miRNA maturation, which involves the processing of pri-miRNA to pre-miRNA, export into the cytoplasm, and incorporation into the RISC complex and iii) at a level of miRNA localization within the genome (23,46).

Regulation of miRNA expression at the transcriptional level is one of the most important steps in their biogenesis, and genome localization influences their transcription (23,54,55). miRNA genes are encoded within the genome as a unit or in groups of 2 to 19 miRNAs and can reside within introns or exons of coding genes or in intergenic regions (1,23,46,55,56). The miRNA genes localized to intergenic regions have their own promoter, which allows them to be independently transcribed. miRNAs localized to intragenic regions (in introns or exons) can be transcribed independently from the gene in which they reside, as long as they have their own promoter, or they can be transcribed together with the host gene (1,23,46,55,56).

Identification of transcriptional start sites and regulatory regions is critical to understand the mechanisms and transcription factors that mediate miRNA expression (56). In general, miRNA expression can be regulated by: i) DNA-binding factors, such as c-myc and p53; ii) specific transcription factors, such as myocyte enhancer factor-2 (MEF2), PU.1 and REST and iii) growth factors, such as platelet-derived growth factor (PDGF) and transforming growth factor β (TGF-β), among others (46). Given that miRNA genes are expressed in a tissue- and time-specific manner and their promoters contain characteristics such as CpG islands, TATA boxes, TFIIB recognition elements, and initiators that are similar to the promoters of protein-coding genes, miRNA expression can also be regulated by epigenetic mechanisms, such as nucleosome remodeling and DNA methylation (23,31-33,46,54,57).

Regulation of miRNA expression at a post-transcriptional level is essential for the specificity and function of certain miRNAs in a tissue- and time-specific context. miRNAs that are clustered in groups are individually expressed independently from the other miRNAs in the group (58). This suggests that miRNAs are individually regulated at a post-transcriptional level (58). Their localization within the genome appears to be an important factor for the regulation or deregulation of certain miRNAs, given that several miRNAs have been mapped to or fragile sites, minimal regions of loss of heterozygosity, amplification, common breakpoint regions and transcriptionally active regions that have been linked to cancer in humans (2,23,46,50,54). In cancer tissues, miRNA expression profiling has revealed that their expression is either increased or decreased compared with healthy tissue. They are differentially expressed in different types of tumor, cell lineages and tumor stages (32,59). miRNAs play an important role in cervical carcinogenesis, from HPV infection to cancer progression (Table II) (50).

7. HPV and miRNA promoter methylation in cervical cancer

It is possible that the aberrant methylation of miRNA promoters is responsible for the altered expression of some miRNA genes with tumor-suppressor or oncogenic functions in cancer (Table III). The role of HR-HPV in altering the cellular DNA methylation status is still controversial. However, it is possible that HR-HPV plays a role in the deregulation of miRNA gene methylation in cervical cancer (2,69,70). Although few studies exist regarding DNA methylation in the deregulation of miRNA expression in cancer, it has been proposed that alterations to the methylation status of miRNA genes could explain the deregulation of miRNA expression in cervical cancer (2,69,70).

In patients and cervical cancer cell lines, it has been observed that silencing of tumor-suppressor miRNAs through aberrant promoter methylation favors cervical carcinogenesis (2,20,69,70). It has been proposed that HR-HPV can lead to modifications in the methylation pattern of miRNA promoters (20,69). Leonard *et al* (35) reported that changes to cellular DNA methylation associated with HPV 16 and HPV 18 are not randomly distributed but rather cluster in specific chromosomal regions, such as the HR-HPV integration regions and regions of chromosomal loss and gain. After cervical cancer cell lines (HeLa, SiHa, CaSki and C33A) were subjected to treatment with hypomethylating agents, decreased methylation levels were found for certain miRNAs, which resulted in their increased expression and concomitant decreased expression of their target genes (2,69,70). It is possible that the HR-HPV genotypes are involved in the methylation processes of miRNAs in cervical cancer (20). However, *in vitro* findings suggest that the methylation events take place after cellular immortalization and are not directly related to the presence of HR-HPV (2). It is likely that identifying the methylation status of miRNAs could be useful for the prognosis of precursor lesions and cervical cancer (2).

Analysis of the methylation status of the three loci encoding the mature hsa-miR-124 (hsa-miR-124-1/-2/-3) in cervical cancer cell lines by Wilting *et al* (2) showed that the

Table II. Expression of miRNAs in cervical and uterine cancer.

Study groups	miRNA expression	Significance in cervical cancer	Refs.
Cervical tissue and serum from patients with SCCC with LNM and without LNM and samples from healthy patients	Upregulated: miR-1246, miR-20a, miR-2392, miR-3147, miR-3162-5p and miR-4484	Overexpression of miRNAs in serum can predict gangliar metastasis in patients with early-stage SCCC.	(60)
Samples from patients with primary CAC and SCCC	Upregulated: miR-21, miR-27a, miR-34a, miR-155, miR-196a, miR-203 and miR-221	Differential expression of miRNAs correlates with the histopathological diagnosis of primary CAC and SCCC, independently of clinical stage and HPV infection.	(61)
Samples from patients with HSIL and CAC and samples from healthy patients	Upregulated: hsa-miR-9, hsa-miR-15b and hsa-miR-28-5p Downregulated: hsa-miR-100 and hsa-miR-125b	Altered expression of these five miRNAs, is associated with chromosomal alterations in cervical cancer.	(62)
Samples from patients with SCCC (FIGO IB2-IV) and patients with early-stage SCCC (FIGO IB1)	Downregulated: hsa-let-7c, hsa-miR-10b, hsa-miR-100, hsa-miR-125b, hsa-miR-143, hsa-miR-145 and hsa-miR-199a-5p	Decreased expression of let-7c, miR-10b, miR-100, miR-125b, miR-143, miR-145 and miR-199a-5p is associated with advanced-stage SCCC. Decreased expression of let-7c, miR-100, miR-125b, miR-143, miR-145 and miR-199a-5p is associated with LNM and decreased patient survival. Decreased expression of miR-10b and miR-100 is associated with a poor prognosis for SCCC.	(63)
Samples from patients with SCCC (CIN2, CIN3) and samples from healthy patients	Upregulated: miR-518a, miR-34b, miR-34c, miR-20b, miR-338, miR-9, miR-512-5p, miR-424, miR-345 and miR-10a Downregulated: miR-193b and miR-203	Differential miRNA expression was found in tissues from patients with SCCC and samples from healthy patients. Predictive target analysis revealed that the miRNAs with decreased expression control signaling pathways regulating cell cycle and apoptosis.	(64)
Samples from patients with cervical cancer (IB-IIB) and patients with benign gynecological diseases	Upregulated: hsa-miR-15a, hsa-miR-19a, hsa-miR-20b, hsa-miR-21, hsa-miR-141, hsa-miR-106b and miR-hsa-224 Downregulated: hsa-let-7c, hsa-miR-143, hsa-miR-199a-5p, hsa-miR-203 and miR-145	hsa-miR-15a, hsa-miR-106b, and hsa-miR-20b regulate a large number of target genes and have strong regulatory effects on the differential expression of genes in cervical cancer.	(65)
Samples from patients with cervical cancer, LSIL, HSIL and healthy patients	Upregulated: miR-522*, miR-512-3p, miR-148a, miR-302b, miR-10a, miR-196a and miR-132 Downregulated: miR-26a, miR-143, miR-145, miR-99a, miR-203, miR-513, miR-29a, miR-199a, miR-106a, miR-205, miR-197, miR-16, miR-27a and miR-142-5p	Different miRNA expression was found between normal cervix, precursor lesions, and cancer tissues. This suggests that deregulated miRNAs play a role in malignant transformation of cervical cells.	(66)
Samples from patients with cervical cancer	Upregulated: miR-21, miR-200a and miR-9 Downregulated: miR-203 and miR-218	miR-200a affects the metastatic potential of cervical cancer cells by suppressing the expression of genes that are important for cell motility	(67)
Samples from patients with cervical cancer and healthy patients	Upregulated: miR-15b, miR-16, miR-146a, miR-155, miR-223, miR-21, miR-205 and let-7f Downregulated: miR-126, miR-424, miR-143, miR-145	Functional studies showed that miR-143 and miR-145 suppress cell growth, whereas miR-146a promotes cell proliferation in cervical cancer.	(1)

Table II. Continued.

Study groups	miRNA expression	Significance in cervical cancer	Refs.
Cervical cancer cell lines CaSki, SiHa, HeLa and C33A. Samples from patients with cervical cancer and healthy patients	Upregulated: miR-182, miR-183 and miR-210 Downregulated: miR-143, miR-145, miR-126, miR-195, miR-218, miR-368 and miR-497	miR-218 expression was decreased in HPV-positive cell lines and cervical cancer tissue compared with C33A cells and normal cervix tissue. Expression of the HPV 16 (high-risk) E6 oncoprotein decreases the expression of miR-218 compared with HPV 6 (low-risk). This suggests that some miRNAs are regulated by HPV.	(11)
Samples from patients with invasive SCCC and healthy patients	Upregulated: miR-199a, miR-199s, miR-9, miR-199a, miR-199b, miR-145, miR-133a, miR-133b, miR-214 and miR-127 Downregulated: miR-149 and miR-203	Overexpression of miR127 is associated with LNM. *In vitro*, transfection with anti-miR-199a in cervical cancer cell lines inhibits cell growth.	(68)
Cervical cancer cell lines SW756, C4I, C33A, CaSki, SiHa and ME-180 as well as samples from patients with benign gynecological pathologies	Upregulated: miR-21 Downregulated: let-7b, let-7c, miR-23b, miR-196b and miR-143	Decreased expression of miR-143 and overexpression of miR-21 in cervical cancer samples is reproducible, which highlights the potential value of miRNAs as tumor markers.	(5)

SCCC, squamous cervical cell carcinoma; CAC, cervical adenocarcinoma; HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion; LNM, lymph node metastasis; FIGO, classification criteria of the International Federation of Gynecology and Obstetrics.

three promoter regions of hsa-miR-124 were methylated in SiHa and CaSki cells. In contrast, the methylation levels of the three hsa-miR-124 regions, in particular hsa-miR-124-1, were extremely low in HeLa cells compared with those observed in SiHa and CaSki cells. A decrease in methylation levels and overexpression of hsa-miR-124 were observed after SiHa cells were treated with the hypomethylating agent 5-aza-2'-deoxycytidine (5-Aza) (2). Ectopic expression of hsa-miR-124 in SiHa and CaSki cells decreased the proliferation rate and migratory capacity of the cells (2). Yao *et al* (69) proposed that hypermethylation of miR-432, miR-1286, miR-641, miR-1290, miR-1287 and miR-95 may be related to HR-HPV infection. Following treatment with 5-Aza, miRNAs were only overexpressed in cervical cancer cell lines infected with HR-HPV (CaSki, HeLa and SiHa) and not in a cell line without HPV (C33A). Wilting *et al* (70) reported that transcriptional repression of hsa-miR-149, hsa-miR-203, hsa-miR-375 and hsa-miR-638 was associated with an increase in methylation levels of these miRNAs. Treatment of the SiHa cell line with the hypomethylating agent 5-Aza induced an increase in the expression of hsa-miR-149, hsa-miR-203 and hsa-miR-375. However, no increased expression was observed for hsa-miR-638. In accordance with these findings, a decrease in the methylation levels of hsa-miR-149, hsa-miR-203 and hsa-miR-375 but not hsa-miR-638 was observed in cells treated with 5-Aza (70). It is likely that high concentrations of the hypomethylating agent are needed to decrease methylation of the hsa-miR-638 promoter region or that more complex epigenetic mechanisms are regulating this locus (70). Ectopic expression of hsa-miR-203 in cervical cancer cell lines decreased their rates of proliferation and anchorage-independent growth (70). The results described by Wilting *et al* (70) indicate that decreased expression of hsa-miR-149, hsa-miR-203 and hsa-miR-375 in cervical cancer cell lines is associated with the methylation status of their promoter regions.

To investigate the stage in the HR-HPV-mediated transformation process at which hsa-miR-124 becomes methylated, Wilting *et al* (2) analyzed the three loci encoding the mature form of hsa-miR-124 (hsa-miR-124-1/-2/-3) using a longitudinal panel of human foreskin keratinocytes immortalized with HPV 16 and HPV 18. This panel represented the morphological, genetic and epigenetic aspects of the different transformation stages that are observed in high-grade lesions. These authors found that in the late passages of keratinocytes immortalized with HR-HPV, there was an increase in the methylation levels of the loci encoding hsa-miR-124. Furthermore, this increase was correlated with a decrease in miRNA expression and high levels of IGFBP7, which is considered a potential target gene of hsa-miR-124. Wilting *et al* (70) reported that methylation of the promoter regions of hsa-miR-149, hsa-miR-203, hsa-miR-375 and hsa-miR-638 was increased in keratinocytes immortalized with HPV 16 and HPV 18 compared with primary keratinocytes. Moreover, these authors found that the increase in hsa-miR-149, hsa-miR-203 and hsa-miR-375 methylation correlated with malignant progression and that expression of these miRNAs can be restored by treatment with 5-Aza.

The findings in samples from patients with precursor lesions and cervical cancer support the *in vitro* findings. Wilting *et al* (2) reported that no methylation of hsa-miR-124-1 and hsa-miR-124-2 was found in normal cervical tissue.

Table III. miRNA genes regulated by methylation in certain types of cancer.

Type of cancer	Methylation status	Refs.
Colon cancer	Hypermethylated: miR-126, miR-34a, miR-34b/c, miR-1-1, miR-133a-2 and miR-149	(60,71-73)
Gastric cancer	Hypermethylated: miR-433, miR-127, miR-148a, miR-34b, miR-129, miR-9, miR-10b, miR-195 and miR-378	(74-79)
Leukemia	Hypermethylated: miR-663	(80)
Bladder cancer	Hypermethylated: miR-200b, miR-152 and miR-10a	(81)
Hepatocellular carcinoma	Hypermethylated: miR-129-2, miR-10a, miR-122 and miR-1-1	(19,50,82,83)
	Hypomethylated: miR-191	(84)
Breast cancer	Hypermethylated: miR-31, miR-130a, let-7a-3/let-7b, miR-155, miR-137, miR-34b/miR-34c, miR-125b and miR-34a	(72,85-87)
Prostate cancer	Hypermethylated: miR-205, miR-132 and miR-193b	(88-90)
Non-small cell lung cancer	Hypermethylated: miR-9-3, miR-122, miR-124-2, miR-124-3 and miR-34b/c	(91-94)
Multiple myeloma	Hypermethylated: miR-203	(95)
Pancreatic cancer	Hypermethylated: miR-132	(85)
	Hypomethylated: miR-200a and miR-200b	(14)
Ovarian cancer	Hypermethylated: miR-34a and miR-34b/c	(72)

However, in cervical cancer, 90% of samples showed methylation of these genes. Furthermore, these authors observed that the increase in methylation levels of hsa-miR-124-1 and hsa-miR-124-2 was correlated with reduced expression of hsa-miR-124 (2). However, the authors also found that the methylation status of hsa-miR-124-1 and hsa-miR-124-2 was predictive of high-grade lesions in 43 cervical shave biopsy samples from women who tested positive for HR-HPV. They concluded that silencing of hsa-miR-124 by DNA methylation is functionally implicated in cervical carcinogenesis and can be used as a valuable indicator to improve the timely detection of cervical cancer and high-grade precursor lesions. Botezatu *et al* (20) assessed the methylation status of CpG islands surrounding the hsa-miR-124a, hsa-miR-34b and hsa-miR-203 genes in cervical cancer and precursor lesions. They further evaluated the relationship between methylation status and the presence of HPV DNA and the viral genotype. They found significant differences in the methylation levels of the miRNA promoter regions in cervical tumor samples compared with control samples. Hypermethylation of *miR-124a* and *miR-203* was also observed in precursor lesion samples compared with control samples. Botezatu *et al* (20) further observed significant differences in the methylation levels of the miR-124a and miR-203 CpG islands between the HR-HPV group and the low-risk HPV (LR-HPV) group and found a strong association between the methylation process and HR-HPV genotypes. In cervical tumor and precursor lesion samples with LR-HPV genotypes, methylation levels were similar to the ones found in normal samples. This finding strengthens the possibility that the HR-HPV genotypes are involved in miRNA methylation processes (20). Yao *et al* (69) reported that in primary cervical tumors compared with normal tissue, the expression levels of hsa-miR-432, hsa-miR-1286, hsa-miR-641, hsa-miR-1290, hsa-miR-128 and hsa-miR-95 were inversely correlated with the methylation status found in cervical cancer cell lines treated with 5-Aza. Wilting *et al* (2) reported significantly increased methylation levels for hsa-miR-149, hsa-miR-203 and hsa-miR-375 in patients with cervical carcinomas. In high-grade lesions, methylation levels were only significantly increased for hsa-miR-203 and hsa-miR-375. Moreover, Wilting *et al* (70) found an increase in the methylation levels of hsa-miR-203 in cervical samples from women with high-grade lesions who were infected with HPV compared with control samples.

8. Conclusion

The finding that alterations in miRNA expression and methylation of key genes involved in cell cycle regulation are frequent events in the process of carcinogenesis represents a challenge and an incentive for this field of research. Extensive study has been devoted to identifying variations in miRNA expression and the expression of miRNA targets in cervical cancer and its precursor lesions. However, knowledge concerning the role of miRNAs in the carcinogenic process is still in its early stages. Evidence suggests that in cervical cancer, hypermethylation of miRNA promoters contributes to the decreased expression of miRNAs with tumor-suppressor gene functions and favors overexpression of miRNAs with oncogenic functions. Methylation is an important mechanism in the HPV viral cycle. Alterations to the methylation status of cellular DNA are influenced by HPV infection, the viral genotype, the physical state of the viral DNA, and oncogenic risk. The E6 and E7 oncoproteins of HPV 16 induce the overexpression of DNA methyltransferase enzymes, which can catalyze the aberrant methylation of protein-coding and miRNA genes that are susceptible to regulation by methylation. Furthermore, HPV

deregulates the expression of miRNAs with loci located at fragile sites through the E6 and E7 oncoproteins. Targets of these proteins include transcription factors of miRNAs, such as p53.

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Capitulo II

Methylation and expression of miRNAs in precancerous lesion and cervical cancer with HPV16 infection

Methylation and expression of miRNAs in precancerous lesion and cervical cancer with HPV16 infection

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Abstract

Abnormal expression and promoter methylation of microRNAs are common events during cervical carcinogenesis. Worldwide, infection by types 18 and 16 of Human Papillomaviruses (HPVs) is considered the major risk factor for cervical cancer development. It has been reported that the miRNAs expression can be deregulated by specific HPV genotypes. In this work we analyzed the promoter methylation of 22 miRNAs and the expression of two miRNAs in 10 non-SIL samples without HPV16 infection, and 7 non-SIL, 16 LSIL and 15 cervical cancer samples, all with HPV16 infection. The methylation levels of miR-124-2, miR-218-1, miR-218-2 and miR-34b/c promoters were significantly higher in cervical cancer than in LSIL sample. The methylation levels of miR-193b promoter were significantly lower in cervical cancer than in LSIL samples. The expression of miR-124 was significantly lower in cervical cancer than in LSIL and Non-SIL samples. The expression of miR-193b was significantly higher in cervical cancer than in LSIL. In conclusion, our results suggest that the abnormal promoter methylation and expression of miR-124 and miR-193b are common events during cervical carcinogenesis.

Keywords

miRNAs, methylation, expression, cervical cancer, HPV16

Introduction

Cervical cancer is the second most common neoplasia in women worldwide [1]. Persistent infection with high-risk Human Papillomavirus (HR-HPV) is associated with origin and development of this cancer [2-3]. Cervical cancer is characterized by the progression of squamous intraepithelial lesions (SIL) of the cervix. Based on their cytopathological characteristics, these lesions are divided into low grade SIL (LSIL), high grade SIL (HSIL), and invasive cervical cancer [4]. HPV16 is the most prevalent HPV genotype found in invasive cervical cancer worldwide [5], and in México, it is the most frequent genotype found in cervical cancer [6], LSIL and HSIL [7].

miRNAs are non-coding RNAs of ~23 nucleotides long that regulate expression of protein-coding genes via base pairing to the 3' untranslated region and subsequent induction of degradation, destabilization or translation inhibition of their target mRNAs [8]. It has been found that miRNAs regulate the expression of several oncogenes and tumor suppressor genes [9]. miRNAs are differentially expressed in different human cell types, and its aberrant expression has been implicated in the development of several human cancers, including cervical cancer [9-10]. Epigenetic alterations such as aberrant DNA methylation and histone modifications, appear to be a major mechanism by which the normal patterns of miRNAs expression are disrupted in cancer [11]. Abnormal promoter methylation of miR-124 family genes has been reported in colorectal [12], liver [13] renal cancer [14] and non-Hodgkin´s lymphoma [15], Similarly, promoter hypermethylation of miR-34 family genes has been found in lung [16], gastric [17], breast [18] and esophageal cancer [19].

Few studies have analyzed the methylation of miRNAs in cervical cancer. The methylation of miR-124 family genes has been detected in cervical cancer cell lines SiHa, CaSki and HeLa, as well as in HPV16 and HPV18 immortalized keratinocytes [20]. The methylation levels of miR-149, miR-203, miR-375 [21], miR-124a, miR-34b and miR-203 [22] were found to be significantly higher in cervical tumor than in normal tissue. On the other hand, it was recently found that miRNAs can be regulated specifically by HPV16 or HPV18 in an *in vitro* model systems. Elevated expression of

miR-16, miR-25, miR-92a, and miR-378, and the decreased expression of miR-22, miR-27a, miR-29a, and miR-100 could be attributed to viral oncoproteins E6 or E7 of HPV16 and HPV18 [23]. In summary, expression of miRNAs can be regulated specifically by HPV16 or HPV18, and abnormal promoter methylation and expression of miRNAs are common events during cervical carcinogenesis. Therefore in this work we analyzed the promoter methylation of 22 miRNAs and the expression of two miRNAs in 10 non-SIL samples without HPV infection, and 7 non-SIL, 16 LSIL and 15 cervical cancer samples, all positive to HPV16 infection.

Results

We analyzed the promoter methylation of 22 miRNAs in 48 cervical samples with or without HPV16 infection. The mean age was 32.2 years old (range, 24-42 years) for Non-SIL without HPV16 infection, 32.0 years old (range, 22-57 years) for Non-SIL, 29.9 years old (range, 19-43 years) for LSIL and 52.8 years old (range, 38-86 years) for cervical cancer. To determine the differences in the methylation of 22 miRNA promoters in all cervical samples, we analyzed the promoter methylation using a Human Cancer miRNA EpiTect Methyl II Signature PCR Array, as described under materials and methods. The heat map diagram shows the hierarchical clustering of miRNAs genes in all samples. The methylation levels are showed for 22 miRNAs in the four groups (Figure 1). The frequencies of miRNAs promoter methylation in cervical samples are shown in Table 1. Only the methylation of miR-124-2, miR-193b, miR-218-1, miR-218-2 and miR-34b/c showed a statistically significant difference between the analyzed groups. Based upon this results we analyzed the methylation levels of 5 miRNAs in the four groups (Figure 2). We found that the methylation levels of miR-124-2, miR-218-1, miR-218-2 and miR-34b/c were significantly higher in cervical cancer that in LSIL samples (p=0.001, p<0.001, p=0.070 and p=0.047, respectively). The methylation level of miR-193b was significantly lower in cervical cancer than in LSIL (p=0.012) and Non-SIL without HPV16 infection samples (p=0.006). Finally we evaluated the expression of miR-124 and miR-193b in the four samples groups. As shown in figure 3, the expression of miR-124 was significantly lower in cervical cancer than in LSIL samples (p=0.015)

and Non-SIL (*p*=0.026). The expression of miR-193b was significantly higher in cervical cancer than in LSIL (*p*=0.023).

Discussion

In this study, we analyzed the promoter methylation of 22 miRNAs and the expression of two miRNAs in cervical samples without and with HPV16 infection. In this study we found abnormal methylation of miR-124-2 in cervical cancer with HPV16 infection. The methylation of this miRNA has been reported previously in cervical cancer cell lines SiHa, CaSki, HeLa, and in cervical adenocarcinoma and cell squamous carcinoma [20], as well as in others types of human cancer [12]. The methylation promoter of miR-124-1 and miR-124-2 in cervical tissue and cervical cancer cell lines has been correlated with lower miR-124 expression levels [20]. In agreement with these data, we also found that the expression of miR-124 was significantly lower in cervical cancer than in Non-SIL and LSIL samples. The fact that methylation and low or null expression of miR-124 is frequently found in samples of cervical cancer and cell lines, suggests that this miRNA may possess tumour suppressive traits in cervical cancer. Indeed, ectopic expression of miR-124 significantly decreased the proliferation rate in SiHa and CaSki cells, and decreased the migratory potential in SiHa cells [20]. These data suggest that miR-124-2 may act as a tumor suppressor in cervical cancer. The IGFBP7 mRNA has been identified as a potential target of miR-124 in cervical cancer [24]. IGFBP7 is part of the insulin-like growth factor (IGF) axis, which was shown to influence the persistence of HR-HPV infection [25]. In summary, the tumor suppressor function of miR-124 in cervical cancer could be, at least in part, explained by its effect on the regulation of IGFBP7 expression.

Few studies have evaluated the miR-193b expression level in cervical precancerous lesions and cervical cancer. We found that the methylation level of miR-193b was significantly lower in cervical cancer than in LSIL and Non-SIL samples, while its expression was significantly higher in cervical cancer than in LSIL. However, the expression of miR-193b was significantly lower in LSIL that in Non-SIL without HPV16 infection. In agreement with these observations, Cheung et al, reported that miR-193b

was down regulated in high grade cervical intraepithelial neoplasia (similar to HSIL) when compared to normal cervical epithelium [26]. The over-expression of miR-193b has been reported in head and neck squamous cell carcinomas [27], glioma tissue [28] and multiple myeloma [29]. Moreover, it has been suggested that in lung [30] and breast cancer [31] miR-193b act as a tumour suppressor. We found that, miR-193b is overexpressed in cervical cancer, suggesting that it may have oncogenic functions. The opposing effects of the same miRNA in different cancer types have been previously reported [32-33].

In conclusion, our results indicate that the methylation of miR-124-2, miR-218-1, miR-218-2 and miR-34b/c is higher in cervical cancer than in LSIL and the methylation of miR-193b is lower in cervical cancer than in LSIL and Non-SIL. The results of the methylation and expression of miR-124-2 and miR-193b suggest an inverse correlation. However, our study included only small number of samples therefore, it is necessary to increase the number of samples to obtain conclusive results about the methylation and expression of miRNAs in cervical cancer and its precursor lesions.

Materials and Methods

Sample collection

The samples were collected at the Guerrero State Cancer Institute, located in southern Mexico. The population consisted of 48 cervical samples; 10 non-SIL without HPV16 infection, and 7 non-SIL, 16 LSIL and 15 cervical cancer all with HPV16 infection. Exo-endocervical exfoliated cell samples were collected by sampling the ectocervix with an Ayre spatula and endocervix with a cytobrush. Smears were used for cytomorphological examination through conventional Papanicolaou staining. Samples were analyzed by a local, experienced cytopathologist and were classified according to the Bethesda System [34]. Women whose cytological diagnosis was Non-SIL or LSIL, a biopsy was no taken. For women diagnosed with HSIL or cervical cancer, a biopsy was obtained for histopatological confirmation of the diagnostic. Two different pathologists performed the diagnosis using the International Federation of Gynecology and Obstetrics (FIGO) classification system [4]. All patients signed an informed consent and filled out a

questionnaire containing demographic and gynecological risk factors data. The study was approved by the Bioethics and Research Committee of the Guerrero State Cancer Institute, and was performed in accordance to ethical guidelines of the 2008 Helsinki Declaration.

HPV detection and genotyping

Genomic DNA was extracted from cervical scrapes or biopsies by the phenol chloroform method [35]. DNA samples were stored at -20°C until used. The concentration and purity of the DNA were evaluated using a NanoDrop 2000c UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, USA). HPV genotyping was done using the INNO-LiPA HPV Genotyping Extra assay (Innogenetics, Gent Belgium), following the manufacturer´s protocol. Briefly, the DNA samples were subjected to PCR amplification (40 cycles) using the Inno-LiPA HPV Genotyping Extra Amp. The PCR products were then denatured, and 10 µl of each sample were hybridized onto nitrocellulose strips where the HPV type-specific oligonucleotides were already bound. After 60 min incubation at 49°C, the PCR product bound to a specific probe was detected by an alkaline phosphatase-streptavidin conjugate. The INNO-LiPA test allows identification of 13 established HR-HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 66), 5 putative HR-HPV types (26, 53, 68, 73, and 82), 7 low-risk HPV types (6, 11, 40, 43, 44, 54, and 70), and 2 types with undefined risk (74 and 69/71).

DNA methylation analysis

DNA methylation levels were evaluated using the Human Cancer miRNA EpiTect Methyl II Signature PCR Array (QIAGEN, Maryland, USA) following the manufacturer´s protocol. This assay is based on the digestion of unmethylated and methylated DNA, using methylation-sensitive and methylation-dependent restriction enzymes. The remaining DNA after digestion is added to the array and ABI 7500 Real Time-PCR (Applied Biosystems, Foster City, CA) instrument was used to read the plates. The relative amount of methylated and unmethylated DNA was calculated using a standard ΔCt method, normalizing the amount of DNA in each digestion against the total amount

of input DNA in the mock digest, using the manufacturer supplied Excel macro spreadsheet.

Total RNA isolation and expression levels of miR-124 and miR-193b

Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer´s instructions. The concentration and purity of RNA were evaluated using a NanoDrop spectrophotometer (Thermo Scientific). The reverse transcription was carried out using 10 ng of total RNA. Expression of miR-124 (001182) and miR-193b (002367) was measured using TaqMan microRNA assays following the manufacturer´s instructions (Applied Biosystems) in an Applied Biosystems 7500 Real-Time PCR system. Expression of miR-92a (000431) was used as an internal reference for miR-124 and miR-193b expression. The relative expression of both miRNAs was analyzed by comparative Ct method [36].

Statistical analysis

Statistical analysis was performed using the STATA 10.0 software package (Stat corporation, College Station, TX, USA). Comparisons of groups defined were performed using the Fisher exact test for categorical variables and Mann-Whitney test for continuous variables. A p value of <0.05 was considered statistically significant.

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Author Contributions

G.F.T. and O.P.Z. Conceived and designed the experiments and coordinated the study and wrote the draft; H.J.W. Performed the experiments and contributed in preparation of paper; D.N.M.C. did the data analysis; L.delC.A.R. and M.A.J.L. Luz did the cytological

and histopathological diagnosis; J.G.M.C. takes the samples; D.H.S. Wrote the paper. V.H.G.B. Contributed with reagents/materials. B.I.A. Contributed with molecular diagnosis of HPV and reagents/materials. All authors have been reading and approved the final manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

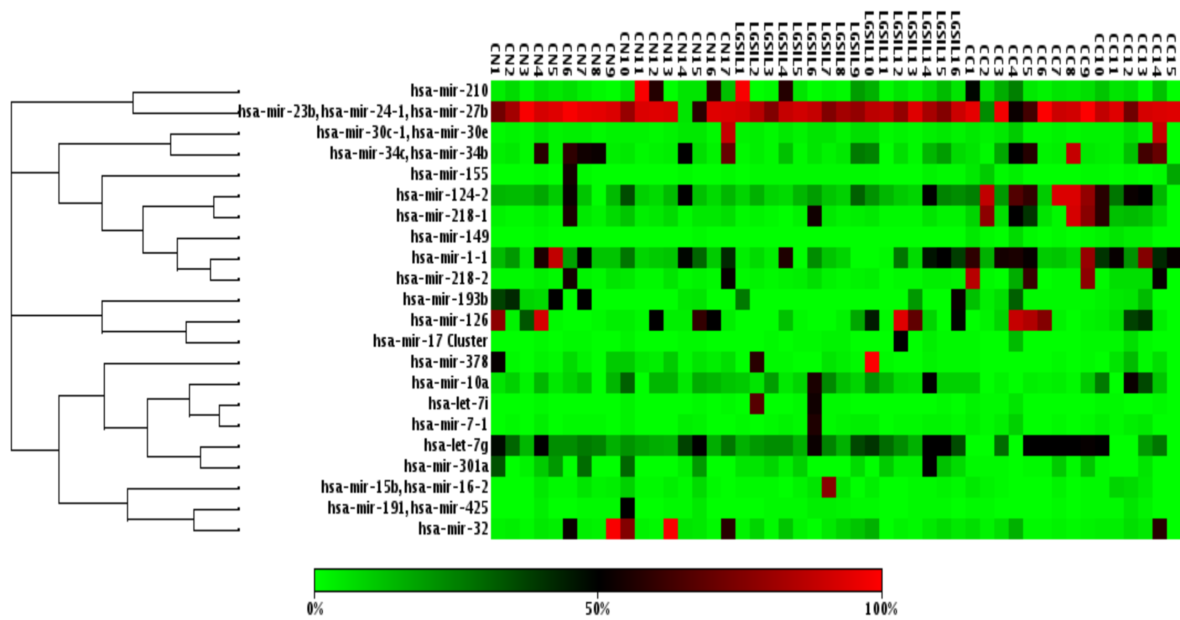

Figure 1. Heat map diagram of the promoter methylation analysis of 22 miRNAs in cervical samples. The diagram shows the unsupervised hierarchical clustering of the 22 miRNAs according to their promoter methylation status. Each row represented a miRNA and each column represents a sample. The color scale shown at the bottom illustrates the relative methylation level of a miRNA: red color represents a high methylation level and green color represents a low methylation level. CN, non-squamous intraepithelial lesion; LGSIL, low grade squamous intraepithelial lesion; CC, cervical cancer.

Table 1. Frequency of miRNAs promoter methylation in cervical samples

	Non-SIL n = 10 %	Non-SIL HPV-16 n = 7 %	LSIL HPV-16 n = 16 %	CC HPV-16 n = 15 %	*P*
let-7g	22.2	28.6	37.5	70	0.176
miR-10a	11.1	0	12.5	18.2	0.916
miR-1-1	30	42.9	30.8	66.7	0.191
miR-124-2	22.2	14.3	12.5	62.5	**0.014**
miR-126	16.7	42.9	40	46.7	0.692
miR-193b	57.1	0	33.3	7.1	**0.047**
miR-210	0	50	14.3	14.3	0.080
miR-218-1	10	0	6.2	43.8	**0.021**
miR-218-2	20	14.3	0	35.7	**0.049**
miR-23b, miR-24-1, miR-27b	100	85.7	100	93.3	0.306
miR-301a	50	0	8.3	8.3	0.118
miR-32	20	28.	0	6.7	0.124
miR-34c, miR-34b	40	28.6	0	43.8	**0.009**
Let-7i	0	0	12.5	0	0.518
miR-155	11.1	0	0	6.2	0.773
miR-15b, miR-16-2	0	0	6.2	0	1.000
miR-378	0	0	13.3	0	0.652
miR-7-1	0	0	6.2	0	1.000
miR-30c-1, miR-30e	0	1	0	1	0.187
Cluster miR-17	0	0	6.2	6.2	1.000
miR-149	0	0	0	0	-
Cluster miR-191, miR-425	10	0	0	0	0.354

SIL squamous intraepithelial lesion, LSIL low grade squamous intraepithelial lesion, CC cervical cancer, HPV human papillomavirus.
P value was calculated with Fisher exact test.
Statistically significant differences are indicated in bold.

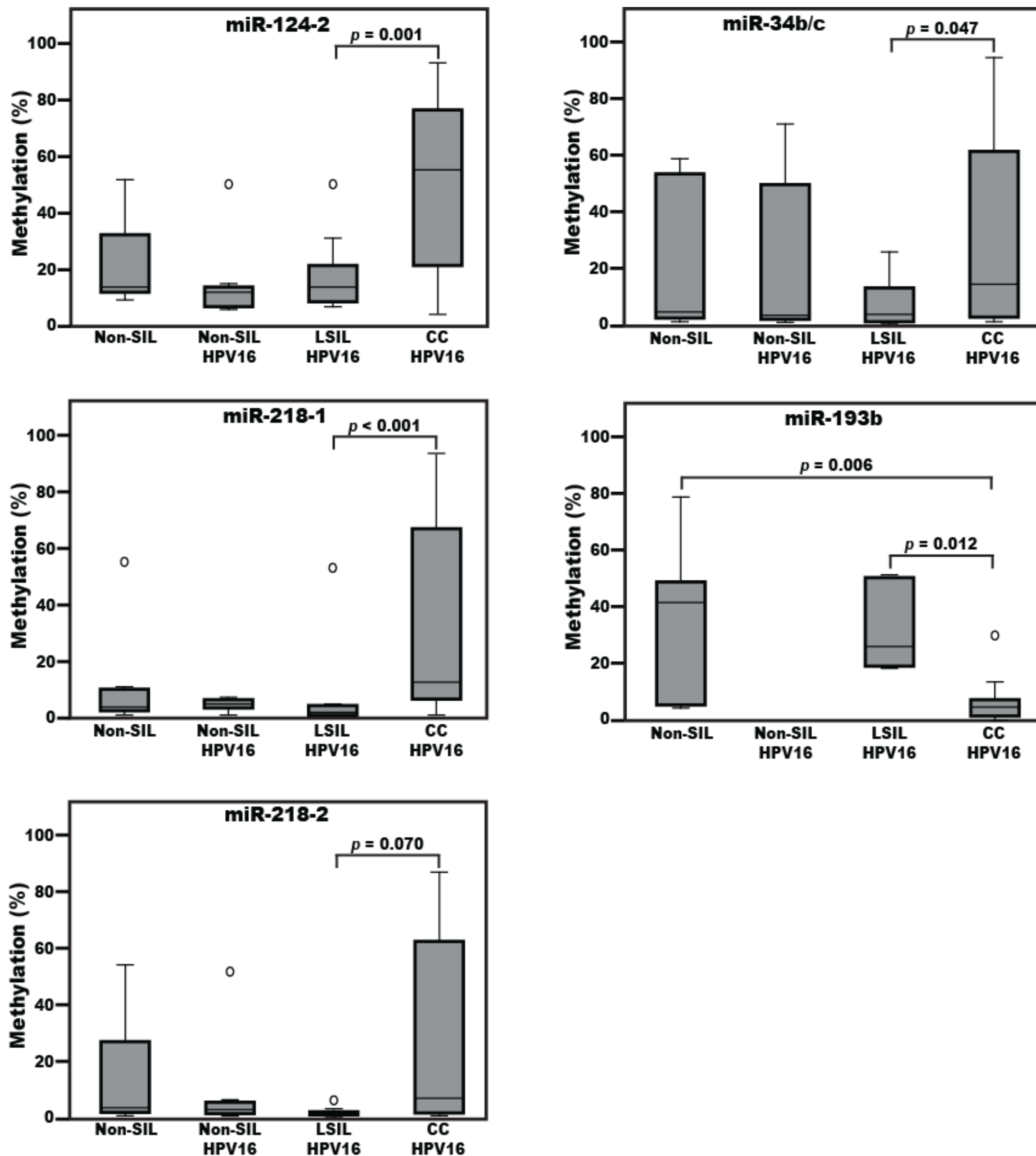

Figure 2. Methylation levels of five miRNAs in cervical samples. The methylation levels were determined in non-squamous intraepithelial lesions (Non-SIL, n = 10), Non-SIL with HPV16 infection (n = 7), low grade squamous intraepithelial lesions with HPV16 infection (LSIL, n = 16), and cervical cancer with HPV16 infection (CC, n = 15). Average methylation level for each group is indicated as a horizontal line. *p* value is indicated when the differences are statistically significant.

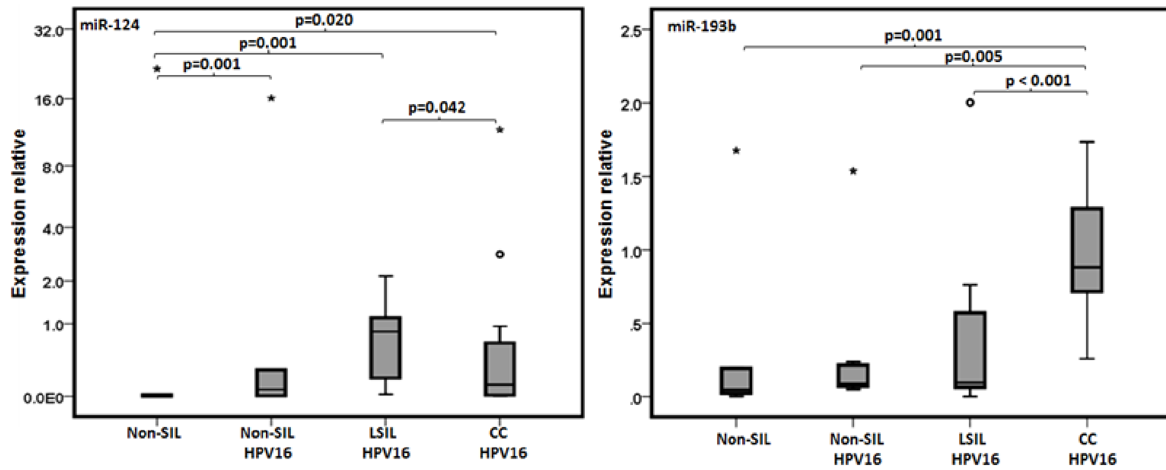

Figure 3. Expression of miR-124 and miR-193b in cervical samples. The expression levels were determined in non-squamous intraepithelial lesions (Non-SIL), Non-SIL with HPV16 infection, low grade squamous intraepithelial lesions with HPV16 infection (LSIL), and cervical cancer with HPV16 infection (CC). Average expression level by samples group is indicated as a horizontal line. *p* value is indicated when the differences are significant.

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Capítulo III

miR-23b as a potential tumor suppressor and its regulation by methylation of DNA in Cervical Cancer

miR-23b as a potential tumor suppressor and its regulation by DNA methylation in Cervical Cancer

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Abstract

Background: The aberrant expression of miR-23b is involved in the development and progression of cancer. In Cervical Cancer (CC), miR-23b is expressed at low levels; however, the mechanisms regulating its expression are not fully understood. The aim of this study was to evaluate the potential role of methylation in the silencing of miR-23b in CC cell lines and determine its expression in stages of malignant progression and in CC tissues containing HPV16 DNA.

Methods: The methylation status of the miR-23b promoter was determined in HeLa, SiHa, CaSki and C33A cells using Human Cancer miRNA EpiTectMethyl II Signature PCR Array®. The cells were treated with 5-Aza-2'-deoxycytidine (5'-Aza-CdR), and the expression of miR-23b was determined before and after hypomethylating treatment by qRT-PCR using individual TaqMan assays. miR-92a was used for data normalization. The expression pattern of miR-23b was determined in cervical scrapes and biopsies of women without squamous intraepithelial lesion (without SIL), with precursor lesions and with CC, all with infection by HPV16.

Results: In C33A, HeLa and CaSki cells, methylation levels were associated with decreased expression of miR-23b; after treatment with 5'-Aza-CdR, the expression of miR-23b increased. The expression levels of miR-23b decreased in relation the increase in the severity of the lesion, and were significantly lower in CC ($p<0.05$). In women with premalignant lesions and infection by HPV16, the decrease of miR-23b increased the risk of CC (OR=26, 95% CI=6.7-192.6, $p <0.05$).

Conclusions: The results of this research suggest that, in CC, the expression of miR-23b is regulated by methylation of its promoter region and that this microRNA plays a functionally important role in cervical carcinogenesis. In women with premalignant lesions and CC infected by HPV16, the expression of miR-23b corresponded to a tumor suppressor microRNA.

Keywords: Expression, miR-23b, premalignant lesions, DNA methylation, Cervical Cancer, HPV16.

Introduction

Persistent infection with High-Risk Human Papillomavirus (HR-HPV) is the main etiological factor of Cervical Cancer (CC) [1,2,3]. In addition, other molecular events such as genetic and epigenetic abnormalities also contribute to the transformation and immortalization of epithelial cells infected with HR-HPV [4]. There are reports of the involvement of DNA methylation, histone covalent modifications and expression of microRNAs (miRNAs) in the development and progression of CC [5].

It has been estimated, using bioinformatic analysis, that miRNAs can regulate 60 to 90% of the protein-encoding genes and that a single miRNA can regulate, directly or indirectly, the expression of hundreds of target mRNAs [6,7,8,9]. The aberrant expression of miRNAs has been associated with the maintenance of the undifferentiated state of cancer cells [10].

MiRNA biogenesis is highly regulated by multiple processes. About 40% of human miRNAs are organized in conserved clusters, with distances of at least 5000 bp between them [11], and are co-transcribed as discrete polycistronic pri-miRNAs [8,12]. Although the miR-23b gene is encoded in the human chromosome 9q22.32, in a cluster that includes miR-24-1 and miR-27b, the mature sequences of each miRNA are differentially expressed [13,14].

The altered expression of miR-23b has been found to be associated with many types of cancer. In breast cancer, the overexpression of miR-23b is correlated with cell proliferation and metastasis. In addition, the overexpression of miR-23b has been linked with increased Her2, EGF and TNF-α signaling, and is thus recognized as an oncogene [15]. In contradiction with this, there are reports that the expression of miR-23b in breast cancer increases the formation of focal adhesions and cell-cell junctions, and modulates the formation of lamellipodia and cell spreading, indicating a metastatic suppressor role

for this microRNA [16]. Furthermore, the expression of miR-23b has been found to decrease in castration-resistant prostate cancer tissue, while its overexpression suppresses migration and invasion; thus, miR-23b is also recognized as a metastatic suppressor in this type of cancer [8,9,11].

The expression of miR-23b is decreased in tissue and cell lines of HR-HPV Cervical Cancer [17], and is associated with the overexpression of the urokinase-type plasminogen activator (uPA), the target gene of this miRNA [18]. In particular, uPA has been directly associated with migration and invasion in CC [19]. Although it is proposed that the E6 oncoprotein of HR-HPV regulates the expression of miR-23b in CC, the mechanism by which the expression of this miRNA is regulated has not been fully elucidated.

Recently, it was shown that a significant number of miRNAs are embedded in CpG islands and that they are targets of epigenetic regulation [20,10,19, 21]. In human neoplasia, hypermethylation of CpG sites is associated with transcriptional silencing of tumor suppressor genes, including genes that encode miRNAs [22]. There is evidence that miR-23b is subjected to epigenetic silencing in glioblastoma and prostate cancer [10,8]; for the latter, there are reports of the methylation of two CpG islands located 1.0 kb upstream of the sequence of the gene encoding miR-23b [9]. Interestingly, it has been suggested that, in Cervical Cancer, HPV16 E6 increases the levels of DNA methyltransferase 1 (DNMT1) by degradation of p53, causing hypermethylation of miRNA genes, among others [23,24].

The mechanisms that regulate the expression of miR-23b in CC are still unknown; it is proposed that inhibition of p53 expression by HR-HPV E6 contributes to the decreased expression of this miRNA. The presence of CpG sites in the promoter sequence of miR-23b allows the regulation of this miRNA by methylation. It is known that epigenetic silencing of miRNAs is associated with processes of invasion and metastasis. Contributing to the elucidation of the mechanism by which the expression of miR-23b is

regulated in CC will provide useful information to increase the understanding of this pathology and to create therapies targeting specific epigenetic modifications.

The objective of this research was to evaluate the potential role of methylation in the silencing of miR-23b in CC cell lines and to determine the pattern of expression of this microRNA in cervical tissues of patients without squamous intraepithelial lesion (without SIL), with precursor lesions and HPV16 CC. In this study, we found decreased expression of miR-23b in cervical tissue with premalignant lesions in CC and in CC cell lines. Treatment with 5'Aza-2-deoxycytidine restored the expression of miR-23b in C33A, HeLa and CaSki cells.

Material and methods

Culture of Cervical Cancer cell lines

The following cell lines were cultures: HeLa (50 copies of integrated HPV 18), SiHa (1-2 copies of integrated HPV16), CaSki (450 to 600 copies of integrated HPV16) and C33A (HPV negative). The cells were cultured in Dulbecco's modified Eagle medium (DMEM), supplemented with fetal bovine serum (10%) and penicillin/streptomycin (1%) (Invitrogen, Carlsbad, CA, USA). The cultures were incubated at 37 °C in humidified atmosphere with 5% CO_2 [17,25,26].

Treatment of cell lines with 5'-Aza-2-deoxycytidine (5-aza-CdR)

Cells were seeded in six-well plates (25 x 10^3/well) and cultured for 72 h before treatment. The cells were treated with 10 μM of 5-aza-CdR dissolved in DMSO and added to fresh culture medium. The cultures were incubated at 37 °C to 5% CO_2 for 24 h; after this time the treatment was repeated and the incubation was continued for 24 h under the same conditions [27,28]. The assay was performed in triplicate for each cell line. Untreated cells were included as a control [26].

Patients and samples of cells or cervical tissue

We studied 54 biopsies of women with cytopathological and histopathological diagnosis of squamous intraepithelial lesion (SIL) or CC with infection by HPV16, of which 19 biopsies were of low-grade squamous intraepithelial lesion (LSIL), 7 of high-grade squamous intraepithelial lesion (HSIL) and 28 of CC. The samples were obtained during routine screening for detection of premalignant lesions or CC at the State Cancer Institute "Dr. Arturo Beltran Ortega" in Acapulco, Guerrero. We included eighteen cervical scrapings of women of cervical cytologies without squamous intraepithelial lesions (Non-SIL), infected with and without HPV16, who attended the Immunohistochemistry and Cytopathology Laboratory of the Autonomous University of Guerrero (Chilpancingo, Guerrero, Mexico) for timely detection of CC. Before sampling, all women signed an informed consent to participate in the study.

Extraction and Purification of Nucleic Acids

Extraction of total RNA and DNA from the cell lines before and after treatment with 5-aza-CdR, as well as from cervical samples, was done with TRIzol Reagent according to the manufacturer's instructions. The integrity of both nucleic acids was determined by electrophoretic shift in agarose gel 1% [29]. The DNA was stored at -20 °C, and the RNA at -70 °C.

Detection and typing of HPV 16

Detection and typing of HPV was performed using INNO-LiPA genotyping extra kit (Innogenetics, Barcelona, Spain) according to the manufacturer's instructions.

Methylation analysis of miR-23b by RT-PCR

The methylation status of the promoter region of miR-23b was determined using Human cancer miRNA EpiTect Methyl II Signature PCR Array® (QIAGEN Sciences, Maryland, USA) following the manufacturer's instructions. Briefly, this assay was based on the digestion of methylated and unmethylated DNA using restriction enzymes sensitive to methylation and methylation-dependent. The DNA that remained after digestion was added to the matrix. An ABI 7500 equipment for Real-Time PCR was used to read the

plates. The relative amount of methylated and unmethylated DNA was calculated using the standard ∆Ct method, normalizing the amount of DNA in each digestion against the total amount of input DNA in null digestion using an Excel spreadsheet provided by the manufacturer.

Quantitative analysis of miR-23b expression by RT-PCR

The expression of miR-23b was determined using the ABI 7500 system for Real-Time PCR (Applied Biosystems, Foster City, CA). Reverse transcription was performed using 10 ng of total RNA. The expression of miR-23b (000400, AUCACAUUGCCAGGGAUUACC) was measured using TaqMan microRNA assays following the manufacturer's instructions (Applied Biosystems). The expression of miR-92a (000431, UAUUGCAUUGUCCCGGCCUGU) was used as internal reference for the expression of miR-23b. The relative expression of both miRNAs was analyzed by the comparative Ct method.

Data Analysis

Using the statistical package STATA, version 9.2, we determined the frequency of methylation of the promoter region of miR-23b between cells treated with 5'-Aza-CdR and untreated cells; Fisher's exact test was used to compare the differences between the two conditions. The median and interquartile range of the expression of miR-23b was determined and the difference between groups was calculated by the Mann-Whitney test. The association between the expression of miR-23b and the presence of Cervical Cancer was determined by logistic regression, with a confidence level of 95%. A value of $p < 0.05$ was considered statistically significant.

Results

The expression level of miR-23b is decreased in Cervical Cancer cell lines due to the methylation of its promoter region.

Bioinformatics analysis and published data allowed us to identify miR-23b as a candidate for regulation by methylation of its promoter region in Cervical Cancer. The methylation status of the promoter region of miR-23b in HeLa, SiHa, CaSki and C33A

cell lines was evaluated by qRT-PCR. Methylation of the miR-23b promoter is close to 100% in the four cell lines; figure 1a.

To check whether the methylation status corresponds to the expression level of miR-23b, we determined the relative expression of miR-23b in HeLa, SiHa, CaSki and C33A cells. In C33A, HeLa and CaSki cells, the low expression of miR-23b was negatively related with methylation of the promoter of this miRNA; in contrast, SiHa cells showed a higher level of miR-23b expression than C33A, HeLa and CaSki cells; figure 1b.

Furthermore, to confirm that methylation is a mechanism that affects the expression of miR-23b in HeLa, SiHa, CaSki and C33A cell lines, the cells were exposed to 5'-Aza-CdR, a compound that inhibits DNA methylation. The expression level of miR-23b was higher in all cell lines after treatment with 5'-Aza-CdR compared with untreated cells. The increase in the expression of miR-23b was statistically significant in C33A, HeLa, SiHa and CaSki ($p<0.05$) cells; figure 1c.

Expression of miR-23b in scrapes Non-SIL, with premalignant lesions and CC.

To investigate whether the expression level of miR-23b was similar in cell lines and in samples from the cervix, we analyzed cervical scrapes Non-SIL (n = 18) and tissues of patients diagnosed with LSIL (n=19), HSIL (n=7) or CC (n=28), all with HPV16.

The expression of miR-23b was significantly lower in samples Non-SIL ($p <0.05$) and in Cervical Cancer tissue ($p <0.05$) than in tissue with LSIL and HSIL; figure 2a. Risk analysis for the expression of miR-23b indicated that the decrease in the expression levels of miR-23b in precursor lesions with HPV16 increased the risk of developing CC (OR=36; 95% CI =6.7-192.6; $p<0.05$); figure 2b.

Discussion

Although Cervical Cancer is one of the most widely studied tumor models, the role played by epigenetic factors such as the expression of miRNAs and DNA methylation in tumorigenesis is not yet fully understood. The miRNAs regulate cellular processes such

as proliferation, growth, differentiation, cell metabolism, apoptosis and cell signaling through the regulation of the expression of multiple target genes. The deregulation of the expression of miRNAs can lead to abnormal expression of genes associated with human diseases such as cancer.

Because the presence of CpG islands upstream of the start site of transcription of miR-23b makes it susceptible to epigenetic silencing, this study investigated whether methylation of CpG dinucleotides influences the level of expression of this miRNA in CC cell lines.

The most significant findings of this study were: 1) the miR-23b promoter is methylated in Cervical Cancer cell lines; 2) the expression of miR-23b is low in CC cell lines; 3) the expression of miR-23b increases significantly in SiHa, CaSki and C33A cells after treatment with 5'-Aza-CdR; 4) the expression of miR-23b is higher in LSIL than in HSIL and CC, that is, it decreases as the grade of the lesion increases; 5) in biopsies positive for HPV16 CC, the expression level of miR-23b was similar to that found in HeLa, SiHa, CaSki and C33A cell lines.

Our results on the methylation of miR-23b in Cervical Cancer cell lines are consistent with those found by Majid *et al.,* in cell lines and tumor tissue of prostate cancer. The miR-23b promoter contains two high density CpG islands in a 1-kilobase region located upstream of the start site of transcription; in prostate cancer, it was found that the expression of miR-23b is regulated by methylation of these CpG islands [9]. It is likely that in CC the expression of miR-23b is also regulated by dinucleotides methylation in the CpG islands of its promoter.

Our findings show that, with respect to the methylation level of the miR-23b promoter, which is close to 100%, the expression of miR-23b is low in Cervical Cancer cell lines, suggesting a possible regulation mechanism of the expression of miR-23b in these cells. Interestingly, we found a higher level of expression of miR-23b in SiHa cells containing one to two copies of HPV16 DNA integrated into the cellular genome, than in CaSki cells

containing 450 to 600 copies of the HPV16 genome and that in HeLa cells with 50 copies of HPV18 in their genome. There is evidence that the expression of several miRNAs is differentiated in the presence of HPV16 or HPV18 [30]. The lowest expression of miR-23b was detected in C33A cells without HPV. These results are consistent with those found by Lui *et al.*, in 2007, who reported a reduced expression of miR-23b in CC cell lines. It is likely that differences in the expression level of miR-23b between CC cell lines are related to the tissue of origin of the cells (miRNAs are tissue-time-specific), the type and number of copies of HPV integrated into the cell genome and the methylation of miR-23b.

miR-23b was found deregulated in Cervical Cancer associated to HR-HPV, and this event is related to the ability of the E6 oncoprotein of HPV16 and HPV18 to bind to E6-AP. The formation of this complex induces rapid degradation of P53 dependent on ubiquitination [1]. The absence of p53 leads to a decreased expression of miRNAs exhibiting consensus response elements, and which are regulated by this transcription factor, including miR-23b [31]. Furthermore, there are reports that in C33A cells, P53 is expressed at normal levels but is non-functional, because it has a point mutation at codon 273, in an evolutionarily conserved domain. This mutation results in the substitution of an arginine by a cysteine [32]. It is likely that mutated P53 is unable to transactivate miR-23b, which would explain the low levels of this miRNA in CC cells negative to HPV.

In 2013, Bisio *et al.,* found that although miR-23b can be moderately transactivated by P53, its promoter sequence has no high affinity binding domains for this transcription factor; they concluded that cis-regulation by P53 seems to modulate in part the expression of miR-23b [33]. It was observed that the silencing of E6 in SiHa and CaSki cells increased the level of P53 and downregulated the expression of DNMT1 [23]. Conversely, the absence of P53 due to the action of HR-HPV E6 induced increased levels of DNMT1 and increased the probability of miRNAs silencing by hypermethylation of their promoters.

We found that the expression of miR-23b is higher in LSIL than in HSIL and CC, that is, the expression of this miRNA decreased as the grade of the lesion increased. It was also observed that the decreased expression of miR-23b in biopsies from patients with cervical cancer positive for HPV16 was similar to that found in HeLa, SiHa, CaSki and C33A cells. In cancer, miRNAs are classified as oncogenic miRNAs (oncomiRs) or tumor suppressor miRNAs, based on the increase or decrease of their expression and on their function on their corresponding target genes [2]. The expression of miR-23b varies substantially between normal cervix and cervical carcinoma cell lines [17]; thus, the decreased expression of miR-23b found by this study in cell lines and tissue of CC, as well as its overexpression in LSIL, suggested that the behavior of the expression of miR-23b in cervical carcinogenesis corresponds to that of a tumor suppressor. This finding is consistent with the findings of Au Yeung *et al.,* in 2011, who described a decreased expression of miR-23b in SiHa and CaSki cells. These authors also demonstrated that overexpression of miR-23b in SiHa cells induced lower levels of mRNA and of the uPA protein, a serine protease, with oncogenic properties [31]. When uPA binds to its receptor, it contributes to the activation of the proteolytic cascade and to the turnover of the extracellular matrix, migration, invasion, metastasis and proliferation of different tumor cell types [34].

Interestingly, the expression of miR-23b has been reported to have dual functions in breast cancer. In 2013, Jin *et al.*, reported that miR-23b and miR-27b were overexpressed in breast cancer and that, together, they have oncogenic properties *in vitro.* Controversially, in the same year Pellegrino *et al.*, suggested that miR-23b acts as a metastatic-suppressor in breast cancer.

We found significantly decreased expression of miR-23b in cervical scrapes Non-SIL, as well as in CC. This similarity may be because the area of metaplastic transformation, from where cervical scrapings are obtained, contains highly undifferentiated normal cells; metaplastic cells generally suffer a reprogramming process and lose cell-cell junctions due to the decreased expression of E-cadherin (CDH1), a phenotypic condition also exhibited by cancer cells [35]. The aberrant activation of epithelial-mesenchymal

transition (EMT) is the primary mechanism of tumor spread, because during tumorigenesis the cells manage to separate, enhance their mobility and are subjected to metaplasia [35,36]. In hepatocellular carcinoma cell lines, decreased levels of miR-23b are correlated with the loss of expression of CDH1. Furthermore, it was observed that overexpression of miR-23b *in vitro* induces overexpression of CDH1 in these cells [18]. Conversely, Pellegrino *et al.,* found overexpression of miR-23b in MCF-7 cells, associated with a more stable epithelial phenotype, without change in the expression levels of CDH1 [16]. The decreased levels of miR-23b in CC and in normal cervical scrapings found in this study are probably related to the metaplastic phenotype present in both conditions; however, there is no data to support this hypothesis.

There are reports that miR-23b is involved in the inhibition of mesenchymal markers in endometrial carcinosarcoma [37], and this miRNA has been linked to the regulation of transcription factors Snai1, ZEB2 and ZEB1, negative regulators of the expression of CDH1 and, therefore, key inducers of EMT [37, 35,36]. In highly malignant Cervical Cancer tissue, besides the expression of uPA and the loss of expression of CDH1, there is nuclear expression of SNAI1 and ZEB1, which is strongly associated with advanced stages of Cervical Cancer and lymph node metastasis [36,38].

In bladder cancer, ZEB1 is a direct target of miR-23b, and the suppression of ZEB1 by this miRNA results in inhibition of cell proliferation and invasion, in cell cycle arrest and apoptosis [39]; however, none of these events has been described in CC.

After determining the baseline expression of miR-23b, HeLa, SiHa, CaSki and C33A cells were subjected to hypomethylating treatment with 5'-Aza-CdR [10 μM], a cytosine analog that is incorporated into DNA and prevents its methylation during the following replications. It was found that the expression levels of miR-23b increased in the four cell lines. Methylation inhibition increased the expression of miR-23b; therefore, it is likely that miR-23b is silenced by methylation. These results are in agreement with those reported in prostate cancer, where miR-23b is silenced by methylation of its promoter region and is considered a tumor supperssor [9].

Conclusions

The results of this research suggest that, in CC, the expression of miR-23b is regulated by methylation of its promoter region. In women with premalignant lesions and infected by HPV16, the decreased expression of miR-23b significantly increases the risk of Cervical Cancer. It is likely that miR-23b has tumor suppressor functions in CC.

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Author Contributions

G.F.T. and H.J.W. Conceived and designed the experiments and coordinated the study and wrote the draft; G.E.C.V. Performed the experiments and wrote the paper; G.T.A. and D.S.F. contributed to performed the experiments; L.delC.A.R. and M.A.J.L. Did the cytological and histopathological diagnosis; D.H.S. and O.P.Z. Made the donation of cell lines. and contributed with reagents/materials. B.I.A. Contributed with molecular diagnosis of HPV and reagents/materials. All authors have been reading and approved the final manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

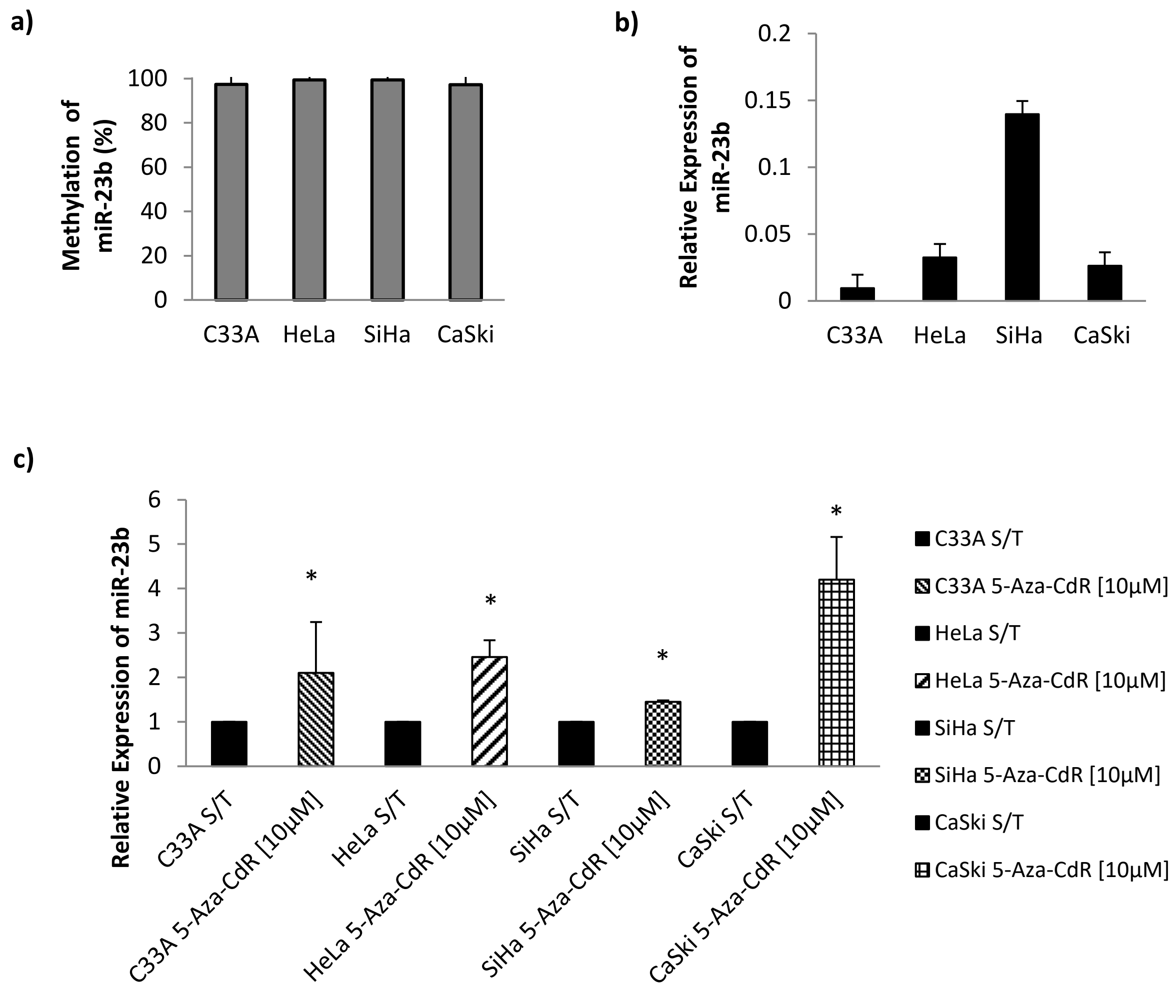
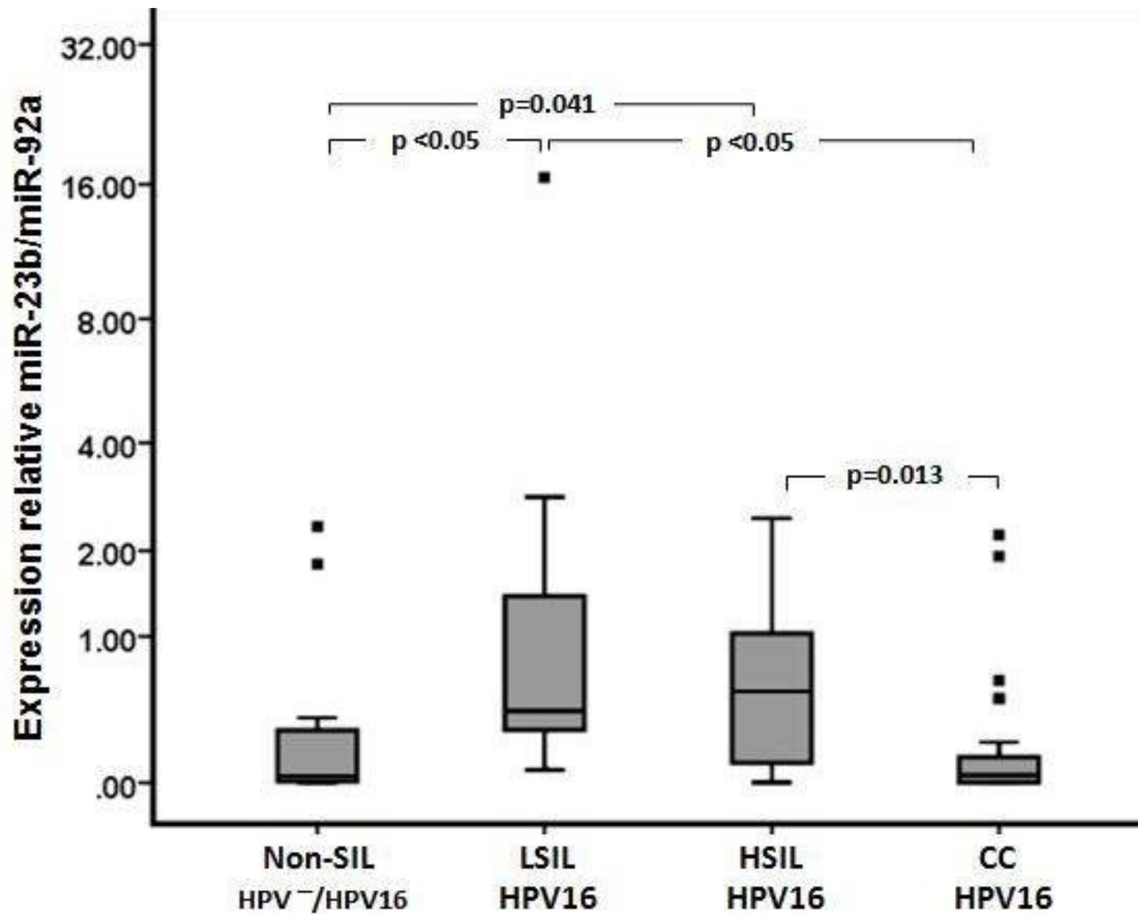


Figure 1. miR-23b is deregulated by methylation of its promoter region in CC cell lines. a) Analysis of the methylation status of the promoter region of miR-23b showed that it was methylated in HeLa, SiHa, CaSki and C33A cell lines. **b)** The relative expression levels of miR-23b were found decreased in C33A, HeLa and CaSki cells compared to the levels observed in SiHa cells. **c)** Hypomethylating treatment with [10 µM] 5'-Aza-CdR increased the expression of miR-23b in the four cell lines tested. After treatment, there was a significant increase in the expression of miR-23b in C33A, HeLa, SiHa and CaSki ($p < 0.05$) cells.

a)



b)

Expression of miR-23b	Clinical diagnosis		OR (95% CI)	Value of *p**
	SILs (n = 26)	**CC** (n=28)		
Low level	2 (7.7 %)	**21 (75%)**	**36 (6.7-192.6)**	<0.05
High level	**24 (92.3%)**	7 (25%)	1.0*	

Figure 2. Expression of miR-23b in cervical scrapes Non-SIL, and in tissues with LSIL, HSIL and CC. a) The expression of miR-23b was significantly lower in CC tissue (*p<0.05) compared with LSIL and HSIL tissue. miR-92a was used for normalization of the data. **b)** Women with precursor lesions (SILs) and low expression levels of miR-23b have 36 fold the risk of developing CC. SILs: LSIL+HSIL; OR: Odds ratio; 95% CI, 95% confidence interval.

Capítulo IV

Expresión de miR-24 y miR-27b en la carcinogénesis cervical.

Disminución de la expresión de miR-24 y miR-27b en cáncer cervical con VPH 16

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Abstrac

Los microRNAs (miRNAs) son una clase de RNAs pequeños no codificantes involucrados en diversos procesos biológicos (proliferación, diferenciación y apoptosis) y su desregulación se ha ligado al desarrollo del cáncer. Estudios sugieren que miR-24 y miR-27b están involucrados en el desarrollo del cáncer cervical por tal motivo evaluamos la expresión de miR-24 y miR-27b en la carcinogénesis cervical. Se incluyeron 19 mujeres con LEIBG, 7 con LEIAG y 28 con cáncer cervical todas con VPH 16 y 18 mujeres sin LEI con y sin VPH 16. La expresión relativa de miR-24 y miR-27b se realizó por qPCR. Las líneas celulares SiHa, HeLa y CaSki sobreexpresaron miR-24 y bajos niveles fueron observados en C33A, mientras que miR-27b se encontró disminuido en las 4 líneas celulares. Tanto miR-24 y miR-27b se encontraron sobreexpresados en lesiones precursoras y disminuidos en mujeres sin LEI y cáncer cervical. La disminución de la expresión de miR-24 y miR-27 en cáncer cervical fue estadísticamente significativa en comparación con LEIAG (p=0.031, p=0.019 respectivamente). La sobreexpresión de miR-24 y miR-27b en mujeres con LEIBG y su disminución en mujeres con cáncer cervical sugieren que miR-24 y miR-27b tiene una función supresora de tumor en la carcinogénesis cervical.

Palabras clave

sin LEI, LEIBG, LEIAG, cáncer cervical, VPH 16, miR-27b, miR-24.

Introducción

El cáncer cervical, es uno de los tumores malignos más frecuentes entre las mujeres de todo el mundo, con una incidencia de aproximadamente 528,000 nuevos casos y alrededor de 266,000 muertes por año (Wang *et al.*, 2008; GLOBOCAN, 2012). Las lesiones escamosas intraepiteliales de bajo grado (LEIBG) y alto grado (LEIAG) anteceden al carcinoma cervical de células escamosas, que representa cerca del 80% de los casos de cáncer cervical (Apgar *et al.*, 2003; Wilting *et al.*, 2010). La infección persistente por virus del papiloma humano de alto riesgo (VPH-AR) es el principal factor de riesgo para el desarrollo del cáncer cervical, siendo el VPH 16 el más frecuente (Zheng & Wang, 2011). El efecto de la infección por el VPH-AR a través, de sus oncoproteínas E6 y E7 se refleja en la desregulación del metabolismo y del ciclo celular (Kalantari *et al.*, 2004; Lechner *et al.*, 2013; Lui *et al.*, 2007; Saavedra *et al.*, 2012; Whiteside *et al.*, 2008; Wilson *et al.*, 2013). Por otra parte, el VPH-AR induce inestabilidad genómica y alteraciones epigenéticas como la metilación del DNA, modificaciones de histonas y alteraciones en la expresión de RNAs pequeños como los miRNAs, eventos que se ven favorecidos por la integración del VPH al genoma celular (Balderas-Loaeza *et al.*, 2007; Botezatu, 2010; Kalantari *et al.*, 2004; Lechner *et al.*, 2013; Lui *et al.*, 2007; Saavedra *et al.*, 2012; Wilson *et al.*, 2013). Actualmente se sabe que alteraciones en la expresión de miRNAs están involucrados en el desarrollo del cáncer cervical y que las oncoproteínas E6 y E7 del VPH-AR pueden modular la expresión de ciertos miRNAs que favorecen la progresión de las lesiones precursoras a cáncer cervical invasor (Au Yeung *et al.*, 2010; Li *et al.*, 2010; Martinez *et al.*, 2008; McKenna & McMenamin, 2014; Wang *et al.*, 2008).

En los últimos años, los microRNAs (miRNA) han emergido como nuevos protagonistas moleculares que regulan a nivel transcripcional y postranscripcional más del 30% de todos los genes humanos y su desregulación ha sido implicado en el desarrollo de diversas enfermedades incluyendo cáncer cervical (Bandres *et al.*, 2009; Deiters, 2009; Jost *et al.*, 2011; Siomi & Siomi, 2010). Los miRNAs son moléculas pequeñas de RNA no codificantes de aproximadamente 22-25 nucleótidos (nt), a menudo conservados filogenéticamente y que presentan un patrón de expresión tejido-tiempo-específico (Jost

et al., 2011; Shivdasani, 2006). A nivel postranscripcional la regulación por miRNAs se lleva a cabo cuando el miRNA a través de una secuencia de 2-8 nt ubicada en su extremo 5´ se une a sitios afines en la región 3´ no traducida (3´UTR) del RNAm blanco. La complementariedad perfecta miRNA/RNAm induce la degradación del mRNA blanco y la complementariedad parcial de 1 o más bases inhibe la traducción del mRNA (Chu & Rana, 2007; Heneghan *et al.*, 2009; Jost *et al.*, 2011; Siomi & Siomi, 2010). Estudios previos han reportado que miR-24 y miR-27b están involucrados en el desarrollo de diversas patologías como el cáncer actuando como supresores de tumor o como oncogenes al tener como blanco genes que regulan negativamente o positivamente los procesos de proliferación, migración e invasión celular (Bang *et al.*, 2012; Ishteiwy *et al.*, 2012; Salvi *et al.*, 2009). Los miRNAs; miR-24 y miR-27 se encuentran en clúster con miR-23, codificados en 2 loci genómicos diferentes (Chan *et al.*, 2010; Zaman *et al.*, 2012).

El clúster miR-23b/miR-24-1/miR-27b, es un gén intrónico localizado en el cromosoma humano 9q22.32 mientras que el gen intrónico del clúster miR-23a/24-2/27a se localiza en el loci 19q13.13 (Chan *et al.*, 2010; Zaman *et al.*, 2012). La secuencia madura de miR-24-1 y miR-24-2 son idénticas. En contraste, con miR-23a/b y miR-27a/b que tienen secuencias diferentes (Guo *et al.*, 2014) . El miR-24 ha sido descrito como supresor de tumor por regulación de c-myc y E2F2 causando inhibición de la proliferación celular (Lal *et al.*, 2009; Salvi *et al.*, 2009). El miR-24 también ha sido descrito como un oncomir al tener como blanco mRNAs que inhiben el ciclo celular o que favorecen la apoptosis como p16 (p16INK4A) y el factor 1 asociado a Fas (FAF1) (Lal *et al.*, 2009; Qin *et al.*, 2010). Por su parte, miR-27b también ha sido descrito como supresor de tumor por regulación de la proteína de especificidad 1 (Sp1) y LIM quinasa de dominio 1 (LIMK1) y como oncogén al tener como blanco al factor de transcripción pro-apoptótico FOXO1 y al inhibidor de la angiogénesis semaporina 6A (SEMA6A) (Jiang *et al.*, 2009; Mozos *et al.*, 2014; Urbich *et al.*, 2012; Walter *et al.*, 2013; Wan *et al.*, 2014). Estudios en líneas celulares de cáncer cervical sugieren que miR-24 y miR-27b pueden estar involucrados en el desarrollo del cáncer cervical (Cheng *et al.*, 2005; Lui *et al.*, 2007), por tal motivo en este estudio evaluamos la expresión relativa de miR-

24 y miR-27b en las líneas celulares de cáncer cervical y muestras de pacientes desde el espectro de sin LEI hasta cáncer cervical. En este estudio la disminución significativa de la expresión de miR-24 y miR-27 en cáncer cervical en relación con LEIAG sugiere que miR-24 y miR-27b tiene una función supresora en esta neoplasia.

Metodología

Cultivo de líneas celulares de cáncer cervical

Se cultivaron las líneas celulares de cáncer cervical: HeLa (50 copias de VPH18 integradas), SiHa (de 1-2 copias de VPH 16 integradas), CaSki (de 450 a 600 copias de VPH 16 integradas) y la línea celular C33A (VPH-). Las células fueron cultivadas en medio Eagle modificado por Dulbecco (DMEM), suplementado con 10% de Suero Fetal Bovino y 1% de penicilina/estreptomicina (INVITROGEN, Carlsbad, CA, USA). Los cultivos se incubaron a 37ºC, en atmósfera húmeda con 5% de CO_2 (He *et al.*, 2011; Lui *et al.*, 2007; Wang *et al.*, 2008).

Pacientes

Se realizó un estudio observacional comparativo en el que se incluyeron 72 mujeres originarias del Estado de Guerrero, México. De acuerdo al estudio molecular para la detección y tipificación del VPH 16 y al estudio citológico y/o histopatológico, se incluyeron en el estudio 18 mujeres sin Lesión Escamosa Intraepitelial (sin LEI) con y sin infección por VPH 16, 19 mujeres con diagnóstico de Lesión Escamosa Intraepitelial de Bajo Grado (LEIBG), 7 mujeres con Lesión Escamosa Intraepitelial de Alto Grado (LEIAG) y 28 mujeres con diagnóstico de cáncer cervical, todas con infección por VPH 16. Las muestras de mujeres sin LEI se captaron en el Laboratorio de Citopatología e Inmunohistoquímica de la Universidad Autónoma de Guerrero (UAGro), Chilpancingo, Guerrero. México. Las muestras de mujeres con LEIBG, LEIAG y cáncer cervical en el Instituto Estatal de Cancerología "Dr. Arturo Beltrán Ortega", Acapulco, Guerrero. México. El proyecto se aprobó por el comité de bioética de la UAGro y del Instituto participante. Solo se incluyeron en el estudio las pacientes que aceptaron participar y firmaron su consentimiento informado.

Obtención de Ácidos nucleícos

La extracción del DNA y RNA se realizó por el método de Fenol cloroformo y TriZol Reagent (Invitrogen, Carlsbad CA, USA) respectivamente de acuerdo a los protocolos de manufactura. El DNA fue re-suspendido en 35 µl de agua desionizada estéril, libre de DNasas y se conservó a -20 °C hasta su procesamiento. Para verificar la integridad del DNA se amplificó por PCR un fragmento del gen constitutivo de β-Globina (Vossler *et al*., 1995). Los productos de PCR se corrieron en geles de agarosa al 1.5%. El RNA fue re-suspendido en 35 µl de agua desionizada estéril tratada con DPEC y libre de en RNasas. Para verificar la integridad del RNA se corrió 1 µg de RNA total en geles de agarosa al 1.0%. La concentración y pureza de los ácidos nucleícos se determinaron en un NanoDrop 2000c UV-Vis Spectrophotometer (Thermo Scientific) en una relación 260/280 y una pureza de 1.85.

Detección y tipificación de VPH 16

La detección y tipificación del VPH se realizó con el kit INNO LiPA genotyping Extra kit (Innogenetics, Barcelona, España), de acuerdo a las indicaciones del fabricante.

Transcripción reversa y cuantificación por PCR en tiempo real de miRNAs

La expresión de miR-24 y miR-27b se determinó en un equipo AB 7500 Real Time PCR System (Applied Biosystems) utilizando ensayos TaqMan para miRNAs de acuerdo a las indicaciones del fabricante (000402 y 000409; Applied Biosystems). Para la RT se utilizaron 10 ng de RNA total, 3 µl de primer de RT en stem-loop (1X), 1.5 µl de buffer RT (1X), 0.15 µl de dNTPs (1 mM), 1 µl de transcriptasa reversa MultiScribe (1U/µl) y 0.19 µl de Inhibidor de RNasa (0.25 U/µl), en un volumen final de 15 µl. Las reacciones se incubaron en un termociclador eppendorf durante 30 minutos a 16ºC, 30 minutos a 42ºC, 5 minutos a 85ºC y posteriormente mantenidas a 4ºC hasta su posterior uso. Para la PCR se utilizó 1.33 µl del producto RT, 10 µl de Master mix PCR TaqMan Universal (1X), 1µL de la mezcla de cebadores (1X) y 10 µl de sonda TaqMan Universal sin UNG (1X), en un volumen final de 10 µL según el protocolo de Ensayos TaqMan MicroRNA (Applied Biosystems). Las reacciones se incubaron a 95ºC durante 10 minutos, seguido de 45 ciclos de 95ºC durante 15 segundos y 60 grados durante 30 segundos. La

expresión relativa se determinó por el método ΔCT. La expresión de los miRNAs se normalizo usando la expresión del gen hsa-miR-92 como control endógeno en cada muestra (000431, Applied Biosystems).

Análisis estadístico

Se utilizó el programa estadístico STATA versión 9.0. Los valores *P* para las variables dicotómicas se calcularon por la prueba exacta de Fisher. Los valores *P* de variables no paramétricas se calcularon por las pruebas de Mann-Whitney. Un valor de $p<0.005$ fue considerado significativo.

Resultados

Expresión de miR-24 y miR-27b en líneas celulares de cáncer cervical

En la última década se ha demostrado que cambios en los niveles de expresión de miRNAs están involucrados en el desarrollo del cáncer. Estudios recientes reportan que miR-24 y miR-27b pueden jugar un papel dual en cáncer. Debido a que miR-24 y miR-27b tienen como blanco genes que participan en la proliferación, diferenciación y metástasis, en este estudio evaluamos la expresión relativa de miR-24 y miR-27b en líneas celulares de cáncer cervical SiHa y CaSki positivas a VPH 16, HeLa positiva a VPH 18 y C33A negativa a VPH. Los niveles de expresión de miR-24 se encontraron sobreexpresados en las líneas celulares SiHa, HeLa y CaSki y disminuido en la línea celular C33A, Figura 1A. Mientras que miR-27b se encontró disminuido en las 4 líneas celulares de cáncer cervical, Figura 1B. La línea celular SiHa expresó 45.1 veces más miR-24 que la línea celular C33A, mientras que HeLa expresó 37.7 veces más miR-24 y CaSki 3.8 veces más miR-24 que la línea celular C33A, Figura 2A. A pesar de que miR-27b se encontró disminuido en las cuatro líneas celulares de cáncer cervical, C33A expreso los niveles más bajos. Sin embargo, se observó que las líneas celulares VPH positivas expresan en promedio 3.7 veces más miR-27b en comparación a C33A, Figura 2B.

Expresión de miR-24 y miR-27b en la carcinogénesis cervical asociada a infección por VPH 16

Para valorar el papel de miR-24 y miR-27b en la carcinogénesis cervical y para conocer en qué etapa la expresión de estos miRNAs se ve alterada, se determinaron los niveles de expresión de miR-24 y miR-27b en 72 muestras cervicales de mujeres originarias del Estado de Guerrero, México. La edad media fue de 33.2 años (DE=11.6) para mujeres sin LEI, 30.8 años (DE=6.7) para LEIBG, 42.4 años (DE=14.3) para LEIAG y de 53.0 años (DE=17.6) para cáncer cervical. Con ayuda de ensayos TaqMan específicos para cada miRNA, se determinaron los niveles de expresión de la forma madura de miR-24 y miR-27b por qPCR. A través de la comparación de los valores de Ct se evaluó el nivel de expresión de miR-24 y miR-27b en los diferentes grupos de estudio. Los niveles de expresión de miR-24 se encontraron sobreexpresados en mujeres con lesiones precursoras (LEIBG M=2.73, DE=151.23 y LEIAG M=5.53, DE=10.43) y disminuidos en mujeres sin LEI (M=0.70, DE=2.32) y cáncer cervical con VPH 16 (M=0.94, DE=42.92). La disminución de la expresión de miR-24 en mujeres con cáncer cervical fue estadísticamente significativas en comparación con los niveles de expresión en mujeres con LEIAG ($p=0.031$), Figura 3.

Los niveles de expresión de miR-27b se encontraron sobreexpresados en mujeres con lesiones precursoras (LEIBG M=0.59, DE=39.48 y LEIAG M=0.60, DE=0.69) y disminuidos en mujeres sin LEI (M=0.19, DE=0.67) y en mujeres con cáncer cervical (M=0.04, DE=1.58). La disminución de la expresión de miR-27b en mujeres con cáncer cervical fue estadísticamente significativas en comparación con los niveles de expresión en mujeres sin LEI ($p=0.019$), mujeres con LEIBG ($p=0.001$) y mujeres con LEIAG ($p=0.006$), Figura 4. Los niveles de expresión de miR-24 y miR-27b se categorizaron en dos grupos: bajos niveles de expresión (miR-24 ≤1.92; miR-27b ≤0.15) y altos niveles de expresión (miR-24 >1.92; miR-27b >0.15) de acuerdo al percentil 50 de los niveles de expresión relativa de cada miRNA. El 68.4% (13/19) de las mujeres con LEIBG y el 85.7% (6/7) de las mujeres con LEIAG presentaron altos niveles de expresión de miR-24, mientras que sólo el 42.9% (12/28) de las mujeres con cáncer cervical presentaron altos niveles de miR-24 ($p=0.037$), Tabla 1. El 73.7% (14/19) de las mujeres con LEIBG

y el 85.7% (6/7) presentaron altos niveles de expresión de miR-27b y sólo el 28.6% (8/20) de las mujeres con cáncer cervical presentaron altos niveles de miR-27b (p=0.004), Tabla 1. El análisis de asociación arrojó 3.6 veces el riesgo de desarrollar cáncer cervical si expresan bajos niveles de miR-24 y 8.3 veces el riesgo de desarrollar cáncer cervical si expresan bajos niveles de miR-27b, Tabla 2.

Discusión

Con el fin de conocer los niveles de expresión de miR-24 y miR-27b en la carcinogénesis cervical asociado a la infección por VPH 16, en este estudio determinamos la expresión de miR-24 y miR-27b en líneas celulares derivadas de cáncer cervical y en muestras de raspados cervicales de mujeres sin LEI y con LEIBG así como, en biopsias de mujeres con LEIGB y cáncer cervical. En este estudio encontramos que las líneas celulares de cáncer cervical VPH positivas (SiHa, HeLa y Caski) sobreexpresan miR-24 en diferentes niveles así como la disminución de la expresión de miR-24 en la línea celular VPH negativa (C33A). Los resultados obtenidos en este estudio son consistentes con otros estudios en los cuales reportan la expresión de miR-24 en las líneas celulares HeLa, SiHa y CasKi (Cheng *et al.*, 2005; Lal *et al.*, 2009; Lui *et al.*, 2007; Merkerova *et al.*, 2008; Wang *et al.*, 2008), así como la disminución de la expresión de miR-24 en la línea celular C33A (Lui *et al.*, 2007). A pesar de que se ha encontrado que las líneas celulares de cáncer cervical VPH positivas expresan miR-24,Cheng *et al.*, 2005, mencionan que en cáncer cervical miR-24 desempeña un papel supresor de tumor ya que en las células HeLa la inhibición de miR-24 aumentó el crecimiento celular, lo que sugiere que miR-24 es un importante regulador del crecimiento celular.

Al realizar la comparación de los niveles de expresión entre las líneas celulares VPH positivas en comparación con la línea celular negativa (C33A), encontramos que las células SiHa y HeLa son las que presentaron mayor expresión de miR-24. Por otra parte, también se ha reportado por To *et al.*, 2012, que la línea celular HeLa expresa bajos niveles de miR-24 y altos niveles del Factor ADP ribosilante (ARF), un gen blanco de miR-24. Los niveles de expresión de miR-27b se encontraron disminuidos en las

cuatro líneas celulares, de las cuales C33A presentó los niveles de expresión más bajos de este miRNA. Resultados similares fueron obtenidos por Lui *et al*., 2007 quienes reportan que miR-27 se encuentra disminuido en las líneas celulares SiHa, CasKi y C33A y Martinez *et al*., 2008, mencionan que las líneas celulares positivas a VPH 16 sobreexpresan 4.9 veces más miR-27b en comparación con la línea celular C33A negativa a VPH.

Los niveles de expresión de miR-24 se encontraron disminuidos en raspados cervicales de mujeres sin LEI y en biopsias de mujeres con cáncer cervical y sobreexpresados en biopsias de mujeres con LEIBG y LEIAG. Además de observar que las mujeres con bajos niveles de expresión de miR-24 tienen un mayor riesgo de desarrollar cáncer. Los bajos niveles de expresión de miR-24 en mujeres sin LEI probablemente se debe al contenido de células indiferenciadas presentes en el raspado cervical, ya que se ha reportado que las células indiferenciadas expresan bajos niveles de miR-24 y en células diferenciadas los niveles de expresión de miR-24 se encuentran sobreexpresados inhibiendo la progresión del ciclo celular (Lal *et al*., 2009; To *et al*., 2012). La sobreexpresión de miR-24 en lesiones precursoras y su disminución en cáncer cervical hace especular que miR-24 es un supresor de tumor en cáncer cervical. Existen estudios que soportan el papel supresor de tumor de miR-24 en cáncer y aunque la función de miR-24 es controversial, se ha demostrado que miR-24 regula genes que controlan la progresión del ciclo celular y genes de reparación del DNA. En cáncer colorectal miR-24 actúa como supresor de tumor a través de su blanco dihidrofolato reductasa (DHFR), (Mishra *et al*., 2007), en carcinoma de laringe de células escamosas a través de su blanco la calgranulina A (S100A8) (Guo *et al*., 2014) y en células hematopoyéticas diferenciadas miR-24 inhibe la proliferación celular por supresión de la expresión de E2F2, MYC y otros genes que regulan el ciclo celular (Lal *et al*., 2009).

Algunos de los blancos reportados para miR-24 han sido estudiados en la carcinogénesis cervical y presentan un patrón de expresión inverso a los niveles de expresión de miR-24 encontrados en este estudio. Un estudio de casos y controles en cáncer cervical realizado por Arvanitis and Spandidos, 2008, reportan que ciertos genes

se activan en etapas precancerosas y otros genes durante la transformación maligna. Uno de los genes que se activan en LEIBG es la DHFR, que convierte el dihidrofolato en tretafolato que transfiere el grupo metilo requerido para la síntesis de *novo* de purinas necesarias para la replicación del DNA (Arvanitis & Spandidos, 2008). Entre los genes de respuesta tardía están E2F2 y MYC que se han encontraron sobreexpresados solamente en tumores cervicales (en un 80 y 74% de los casos de cáncer cervical) y no en lesiones precancerosas (Arvanitis & Spandidos, 2008). El factor de transcripción E2F2 activa la transcripción de muchos genes esenciales para la replicación del DNA, la progresión del ciclo celular y la reparación del DNA por lo que la disminución de miR-24 en cáncer cervical permitiría la sobreexpresión de estos genes (Lal *et al*., 2009). Koskimaa *et al*., 2010, reportan que S100A8 y S100A9 se encuentran expresados en bajos niveles en epitelio normal, pero sus niveles incrementan en queratinocitos activados durante su proliferación y diferenciación. La desregulación de la expresión de DHFR, S100A8 E2F2 y MYC (blancos de miR-24) en cáncer cervical se relacionó con la infección por VPH-AR. Además McKenna *et al.,* 2014, reportan que la expresión de miR-24 y miR-205 se ve afectada por las oncoproteínas de E6 y E7 del VPH-AR.

La disminución de la expresión de miR-24 en mujeres con cáncer cervical observado en este estudio, y considerando la información que soportan que las oncoproteínas E6 y E7 del VPH-AR, afectan la expresión de miR-24 y la sobreexpresión de genes blancos de miR-24 (DHFR, S100A8 E2F2 y MYC) en cáncer cervical sugieren un posible mecanismo por el cual el VPH-AR puede interrumpir el balance entre la proliferación y diferenciación de los queratinocitos.

Resultados similares fueron obtenidos para miR-27b, observándose una disminución de la expresión de miR-27b en raspados cervicales sin LEI y en biopsias de cáncer cervical, mientras que en pacientes con LEIBG y LEIAG miR-27b se encontró sobreexpresado. Por otra parte se encontró que las mujeres con bajos niveles de expresión de miR-27b tienen un mayor riesgo de desarrollar cáncer. La función biológica de miR-27b en cáncer cervical aún no ha sido reportada. Sin embargo, el papel supresor de tumor de miR-27b ha sido soportado en otros tipos de cáncer. En

neuroblastoma miR-27b tiene como blanco el receptor gamma activado *por el factor* proliferador *de* peroxisomas (PPARγ) y observaron que tanto *in vivo* como *in vitro* la sobreexpresión de miR-27b o el bloqueo de su blanco PPARγ inhibe el crecimiento del neuroblastoma (Lee *et al.,* 2012). En neuroblastoma el papel supresor de miR-27b se asoció con la disminución de la expresión del intercambiador Na+/H+ (NHE1), un gen diana de PPARγ, así como a una respuesta inflamatoria reducida (Lee *et al.*, 2012). Por otra parte, miR-27b a través de PPARγ regula la actividad de NF-κB y la transcripción de genes inflamatorios diana (Lee *et al.*, 2012). En cáncer colorectal reportan que miR-27b tiene un papel antitumoral debido a que la expresión de miR-27b se encontró disminuida en la mayoría de los tejido de cáncer colorectal y observaron que tanto *in vivo* como *in vitro* la sobreexpresión de miR-27b reprime la formación de colonias, la proliferación celular y disminuye el crecimiento tumoral, a través de su blanco el factor de crecimiento endotelial vascular (VEGFC), inhibiendo así la progresión del tumor y la angiogénesis (Ye *et al.*, 2013).

Conclusiones

La sobreexpresión de miR-24 y miR-27b en mujeres con LEIBG y la disminución de su expresión en mujeres con cáncer cervical sugieren que miR-24 y miR-27b podría tener una función supresora de tumor en cáncer cervical. Además es posible que la sobreexpresión de miR-24 y miR-27b en lesiones precursoras de cáncer cervical puede ser de mejor pronóstico para la reversión de las lesiones precursoras y la disminución de la expresión de estos miRNAs puede ser un riesgo para desarrollar cáncer cervical. Por otra parte, es posible que la desregulación de la expresión de algunos genes por las oncoproteínas E6 y E7 del VPH-AR pueda ser vía desregulación de miRNAs como miR-24 y miR-27b.

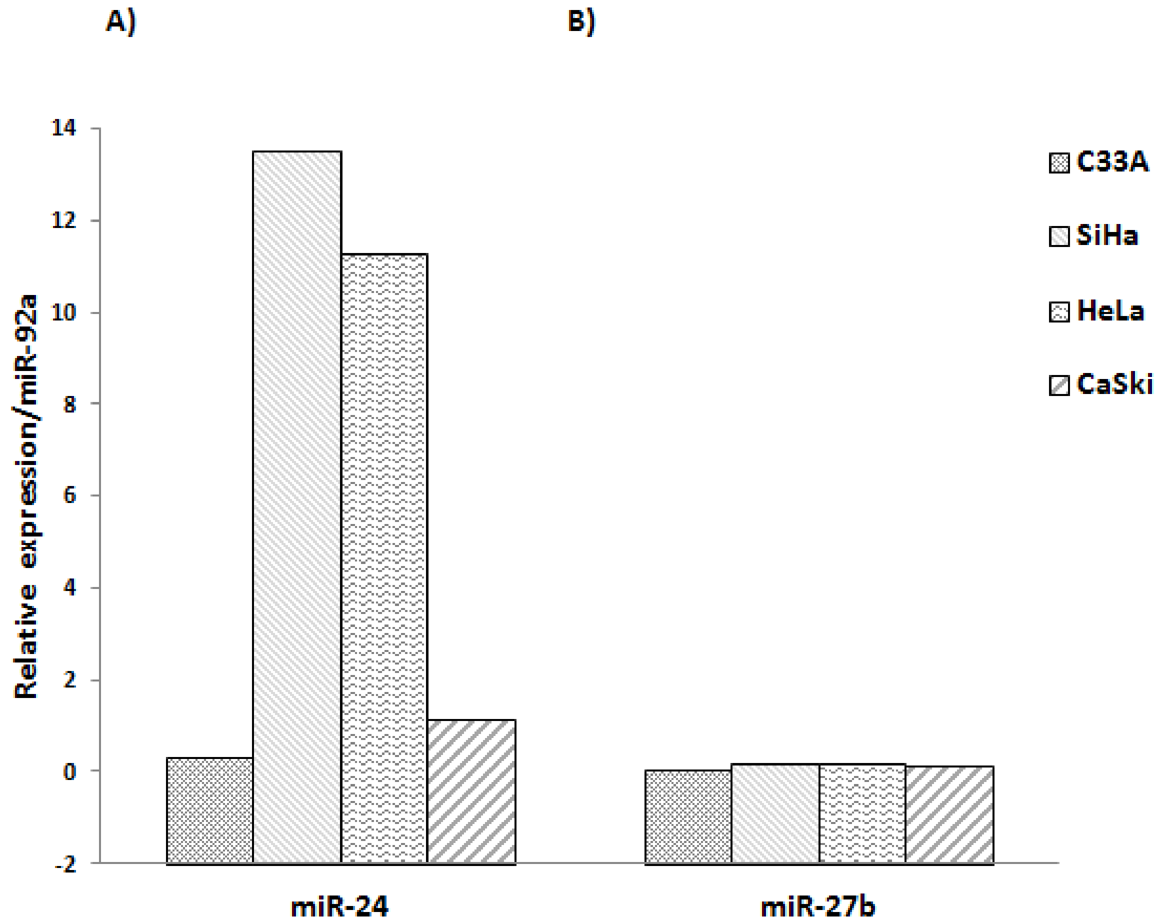

Figura 1. Expresión relativa de miR-24 y miR-27b en líneas celulares de cáncer cervical. A) Expresión relativa de miR-24 en línea celular C33A negativa a VPH, HeLa VPH 18, SiHa y CasKi VPH 16. B) Expresión relativa de miR-27b en línea celular C33A negativa a VPH, HeLa VPH 18, SiHa y CasKi VPH 16. Los datos fueron medidos por qPCR y calculado por el método de delta CT. La expresión relativa de miR-24 y miR-27b fueron normalizados por los niveles de miR-92a. Los valores son expresados como la media de dos cultivos independientes.

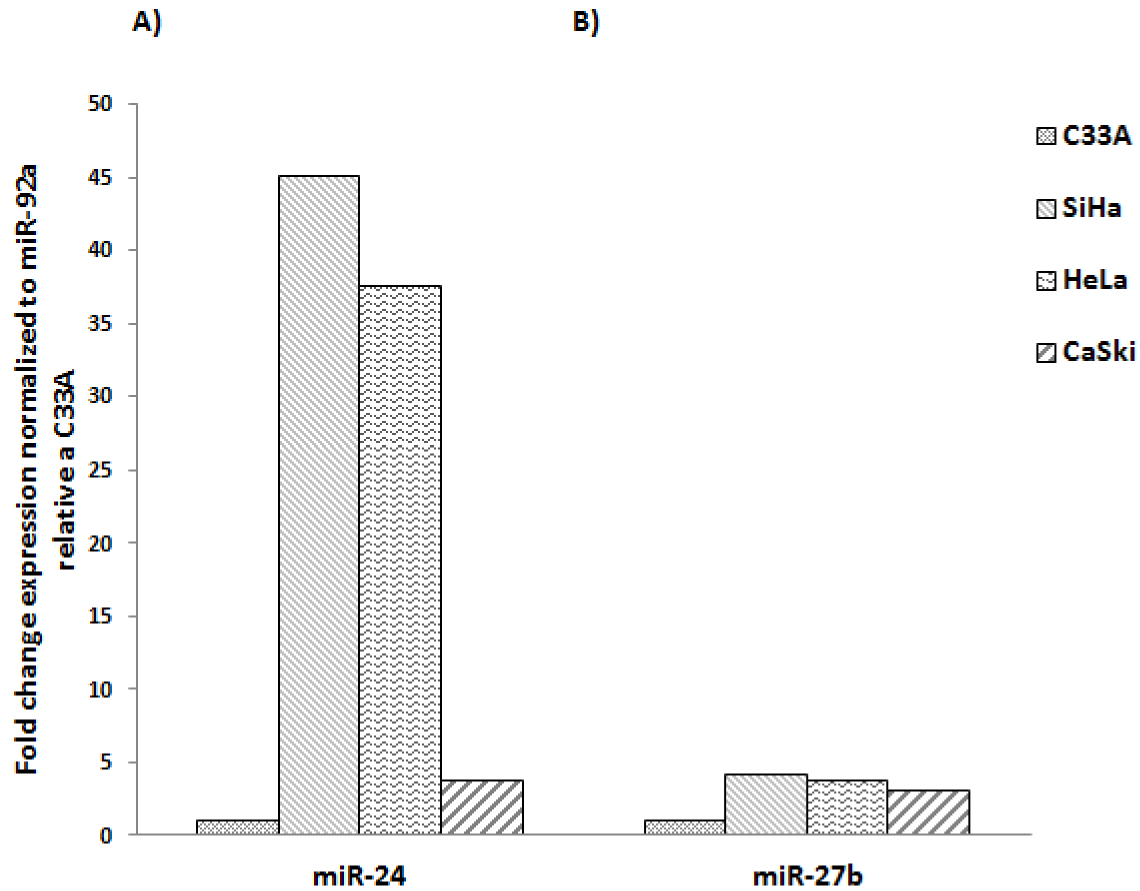

Figura 2. Diferencias en los nivel de expresión de miR-24 y miR-27b en las líneas celulares de cáncer cervical con VPH en comparación con la línea celular negativa a VPH. A) Comparación de los niveles de expresión de miR-24 las líneas celulares VPH positivas (SiHa, CasKi y HeLa) en comparación a la línea celular VPH negativa (C33A). Comparación de los niveles de expresión de miR-27b las líneas celulares VPH positivas (SiHa, CasKi y HeLa) en comparación a la línea celular VPH negativa (C33A). Los datos fueron medidos por qPCR y calculado por el método de delta CT. La expresión relativa de miR-24 fueron normalizados por los niveles de miR-92a. Los valores son expresados como la media de dos cultivos independientes.

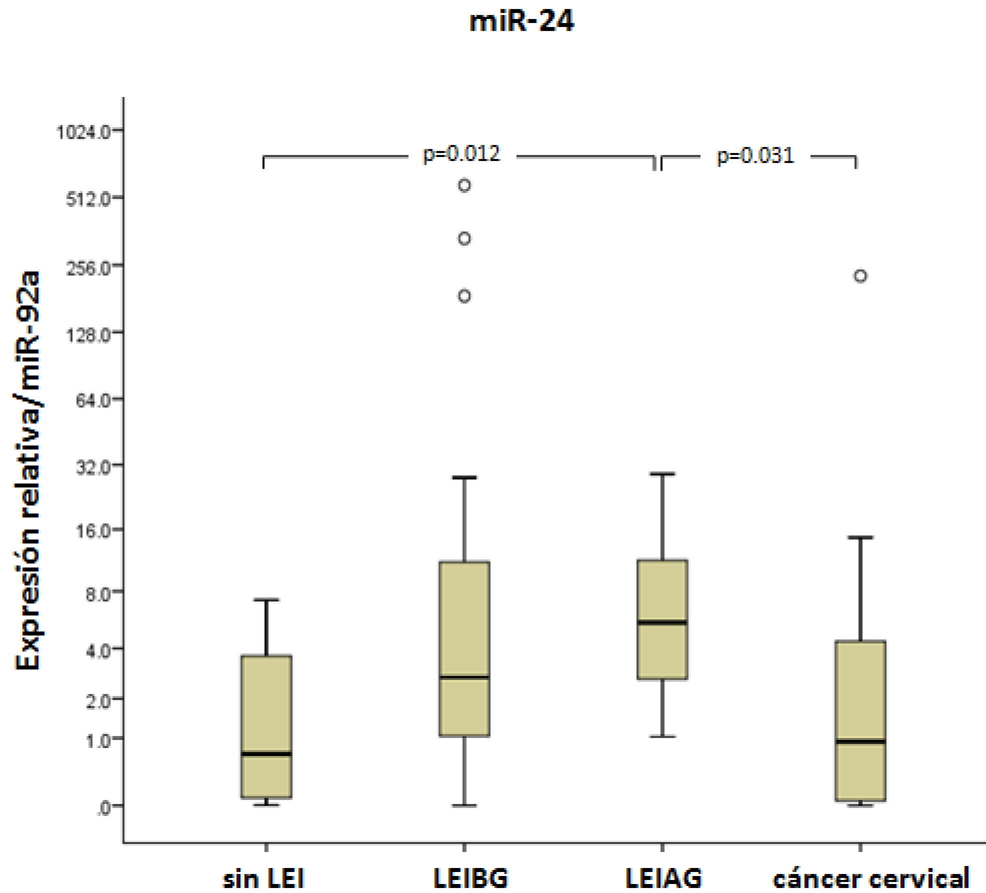

Figura 3. Expresión relativa de miR-24 en muestras de pacientes con cáncer cervical. Los datos fueron medidos por qPCR y calculado por el método de delta CT. La expresión relativa de miR-24 fueron normalizados por los niveles de miR-92a y mostrados en una escala log 2. Los valores son expresados como la media de dos corridas independientes.

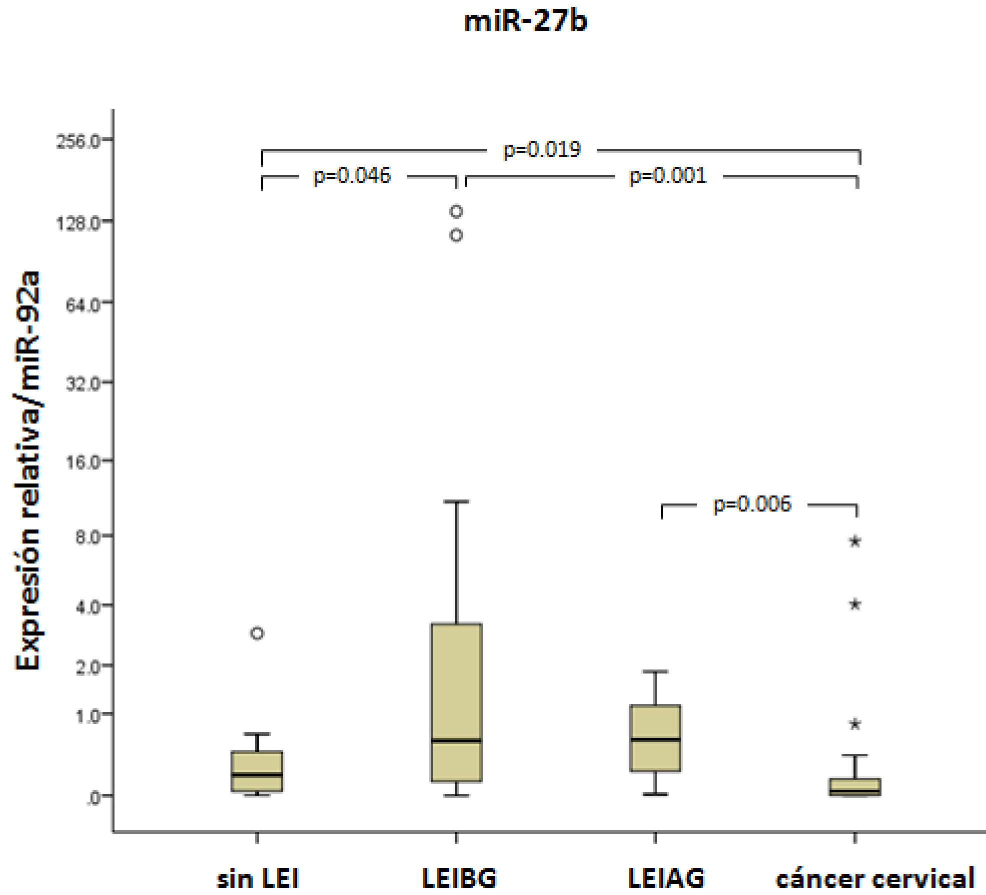

Figura 4. Expresión relativa de miR-27b en muestras de pacientes con cáncer cervical. Los datos fueron medidos por qPCR y calculado por el método de delta CT. La expresión relativa de miR-24 fueron normalizados por los niveles de miR-92a y mostrados en una escala log 2. Los valores son expresados como la media de dos corridas independientes.

Tabla 1. Niveles de expresión de miR-24 y miR-27b en muestras cervicales.

	Sin LEI VPH(-) (n=18)	LEIBG VPH 16 (n=19)	LEIAG VPH 16 (n=7)	CaCU VPH 16 (n=28)	Valor p
Expresión de miR-24 n (%)					
Baja	12 (66.7%)	6 (31.6%)	1 (14.3%)	16 (57.1%)	0.037
Alta	6 (33.3%)	13 (68.4%)	6 (85.7%)	12 (42.9%)	
Expresión de miR-27b n (%)					
Baja	9 (50.0%)	5 (26.3%)	1 (14.3%)	20 (71.4%)	0.004
Alta	9 (50.0%)	14 (73.7%)	6 (85.7%)	8 (28.6%)	

Prueba exacta de Fisher

Tabla 2. Riesgo de cáncer cervical asociado a la disminución de la expresión miR-24 y miR-27b.

	LEIBG+LEIAG VPH 16 (n=26)	CaCU VPH 16 (n=28)	OR (IC95%)	p
Expresión de miR-24 n (%)				
Baja	7 (26.9%)	16 (57.1%)	3.6 (1.2-11.4)	0.028
Alta	19 (73.1%)	12 (42.9%)		
Expresión de miR-27b n (%)				
Baja	6 (23.1%)	20 (71.4%)	8.3 (2.4-28.4)	0.001
Alta	20 (76.9%)	8 (28.6%)		

OR: Odds ratio IC: Intervalo de confianza al 95%

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Capítulo V

Metilación de miR-218-1 en lesiones escamosas intraepiteliales de bajo grado y cáncer cervical con VPH 16

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Metilación de miR-218-1 en lesiones escamosas intraepiteliales de bajo grado y cáncer cervical con VPH 16

Methylation of miR-218-1 in squamous intraepithelial lesions of low grade and cervical cancer with HPV 16

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RESUMEN

Introducción: alteraciones en la expresión de algunos microRNAs (miRNAs) en la carcinogénesis cervical parecen estar relacionados con la metilación aberrante de sus promotores. Objetivo: evaluar la frecuencia de metilación del promotor del microRNA-218-1 (miR-218-1) en lesiones escamosas intraepiteliales de bajo grado (LEIBG) y cáncer cervical (CC) con virus del papiloma humano (VPH 16). Metodología: se incluyeron 16 muestras de LEIBG y 16 de CC con VPH 16. La metilación del promotor de miR-218-1 se determinó con EpiTect® Methyl II PCR Array (QIAGEN). Resultados: el promotor de miR-218-1 se encontró metilado en un 43.75% (7/16) en CC y en un 6.25% (1/16) en LEIBG ($p = 0.0007$). Conclusiones: el promotor de miR-218-1 se encontró metilado en un mayor porcentaje en muestras de CC que en LEIBG. Es posible que la metilación del promotor de miR-218-1 sea un mecanismo epigenético implicado en su expresión aberrante en CC.

ABSTRACT

The alterations in the expression of some microRNAs (miRNAs) in cervical carcinogenesis seems to be related to the aberrant methylation of their promoters. This study evaluates the methylation frequencies of microRNA-218-1 (miR-218-1)'s promoter in low grade squamous intraepithelial lesions (LGSIL) and cervical cancer (CC) with Human Papillomavirus type 16 (HPV-16). In addition,16 samples with LGSIL and 16 with CC were included. The methylation status was performed with qPCR Epitect Methyl II Arrays. The promoter of miR-218-1 was found methylated in 43.75% (7/16) in CC and 6.25% (1/16) in LGSIL ($p = 0.037$). The promoter of miR-218-1 was found methylated in a higher percentage in CC samples than in LGSIL. It is possible that promoter methylation of miR-218-1 to be an epigenetic mechanism involved in their aberrant expression in CC.

Recibido:
Aceptado:

Palabras clave:
LEIBG, cáncer cervical, miR-218-1, metilación.

Keywords:
LGSIL; cervical cancer; miR-218-1; methylation.

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INTRODUCCIÓN

El cáncer cervical (CC) es una enfermedad compleja que involucra la expresión anormal de oncogenes y genes supresores de tumor (Peng *et al.*, 2012). La infección persistente por virus del papiloma humano de alto riesgo (VPH-AR) como el VPH 16 se considera el factor causal más importante para desarrollar CC (Zheng & Wang, 2011). La actividad transformante del VPH-AR se explica principalmente por la actividad de sus oncoproteínas E6 y E7. Éstas regulan importantes procesos biológicos celulares, como la apoptosis, proliferación celular, estabilidad cromosómica, diferenciación celular, la respuesta inmunológica y mecanismos epigenéticos como la metilación de genes y expresión de microRNAs (miRNAs), entre otros (Lizano-Soberón, 2009).

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Los *miRNAs* son pequeñas moléculas de Ácido Ribonucleico (ARN) monocatenario de longitud de 20-23 nucleótidos (nt) que se localizan, tanto en regiones intragénicas como intergénicas (Gil, 2012; Palmero *et al.*, 2011). Los genes de miRNAs constituyen el 1%-2% de los genes conocidos en los eucariotas (John *et al.*, 2004). Se considera que los miRNAs son reguladores de importantes procesos biológicos, tales como el crecimiento celular, la apoptosis, la eliminación de la infección viral y el desarrollo del cáncer (Hu *et al.*, 2010).

Las alteraciones genéticas y epigenéticas son algunos de los mecanismos responsables de la desregulación de los miRNAs en cáncer (Li, Cao, Zhang, Yin & Xu, 2009). En el cáncer, los cambios epigenéticos de los miRNAs, tales como la metilación del Ácido Desoxirribonucleico (ADN) y las modificaciones que sufren las histonas, son importantes en la regulación de su expresión (Li *et al.*, 2009). La disminución en la expresión de genes supresores de tumor en cáncer está vinculado con la hipermetilación de regiones ricas en citosinas adyacentes a guaninas (islas CpG) en sus promotores (Esteller, 2007). El microRNA-218 (miR-218) se encuentra frecuentemente desregulado en CC (Yamamoto *et al.*, 2013). El silenciamiento de miR-218 en CC se ha relacionado con la infección por virus del papiloma humano (VPH), sin embargo la expresión disminuida de miR-218 se ha observado tanto en líneas celulares VPH positivas como en líneas celulares negativas a VPH (Yamamoto *et al.*, 2013). En cáncer oral, la disminución de la expresión de miR-218 se ha relacionado con la hipermetilación del ADN (Yamamoto *et al.*, 2013). Debido a estos hallazgos es posible que la metilación aberrante de promotores de miRNAs sea responsable de la expresión alterada de algunos genes de miRNAs con funciones supresoras de tumor, por lo que el objetivo de este estudio fue evaluar el estado de metilación del promotor de miR-218-1 en muestras de lesiones escamosas intraepiteliales de bajo grado (LEIBG) y CC con infección por VPH 16.

MATERIALES Y MÉTODOS

Muestras de pacientes

Se realizó la captación de biopsias de pacientes con diagnóstico de CC que acudieron al Instituto Estatal de Cancerología Dr. Arturo Beltrán Ortega, en Acapulco, Guerrero, durante el periodo de mayo a noviembre de 2013; durante el mismo periodo se captaron muestras de pacientes con diagnóstico de LEIBG en el Laboratorio de Citopatología e Inmunohistoquímica de la Unidad Académica de Ciencias Químico-Biológicas de la Universidad Autónoma de Guerrero (UACQB-UAG), Chilpancingo, Guerrero. Sólo se incluyeron en el estudio las pacientes con diagnóstico de LEIBG y CC con infección por VPH 16, y que aceptaron participar en el estudio y firmar su consentimiento informado. El ADN obtenido de las muestras se almacenó a -20 °C hasta su procesamiento.

Restricción del ADN de muestras de pacientes con LEIBG y CC

Previo al análisis de metilación, el ADN total de cada muestra fue digerido utilizando EpiTect II *DNA Methylation Enzyme Kit* (QIAGEN). El kit contiene dos enzimas, una enzima sensible a metilación (Enzima A) que digiere ADN desmetilado y una enzima dependiente metilación (Enzima B) que digiere ADN metilado. Se preparó una reacción que consistió de 1 µg de ADN total, 26 µl de *buffer* de digestión [5X] y se llevó a un volumen final de 120 µl utilizando agua libre de DNasas y RNasas. Posteriormente, se repartieron 28 µl en cuatro tubos eppendorf para obtener cuatro reacciones de restricción; una reacción en la cual no se le agregaron enzimas de restricción (Mo) (que representó la cantidad total de ADN), una reacción con 1 µl de enzima sensible a metilación "A" (Ms), una reacción con 1 µl de enzima dependiente metilación "B" (Md), y una reacción de doble digestión en la cual se agregaron 1 µl de enzima A y 1 µl de enzima B (Msd) y se llevaron a un volumen final de 30 µl. Las cuatro reacciones se incubaron a 37 °C durante toda la noche. Posteriormente las enzimas fueron inactivadas a 65 °C por 20 min y almacenadas a -20 °C hasta su utilización.

Reacción en cadena de la polimerasa en tiempo real (qPCR) para determinar el estado de metilación del promotor de miR-218-1

El estado de metilación del promotor de miR-218-1 se determinó utilizando *Human Cancer* miRNA EpiTect Methyl II *Signature* PCR *Array* (QIAGEN, Maryland USA). No se requiere conversión del ADN con bisulfito de sodio. Después de la digestión, el ADN residual se cuantificó mediante Applied Biosystems 7500 Real-Time PCR Systems (qPCR), utilizando cebadores que flanquean la región promotora del gen de miR-218-1 (EpiTect Methyl II PCR *Primer Assay for Human* hsa-mir-218-1 CpG *Island* 190453-QIAGEN). Se realizaron por muestra cuatro reacciones una para cada producto de digestión (Mo, Ms, Md y Msd) agregando 30 µl del producto de digestión, 330 µl de master mix para qPCR (SYBR® Green

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ROX qPCR Mastermix-QIAGEN) y 300 µl de agua libre de DNasas y RNasas (volumen final 660 µl). Se dispensaron 25 µl en una placa de qPCR que contenía los cebadores para el promotor de miR-218-1. El programa de amplificación que se utilizó fue 95°C × 10 mn para activar la DNA polimerasa, 90°C × 30 seg y 72°C × 1 min (tres ciclos), 97°C × 15 s y 72°C × 1 min (40 ciclos).

Análisis de metilación del ADN

Los niveles de metilación del ADN fueron determinados de acuerdo con las instrucciones del *software* EpiTect Methyl DNA *Methylation* PCR *Data Analysis* (QIAGEN) (http://www.sabiosciences.com/dna_methylation_data_analysis.php).

Detección y tipificación de VPH 16

La detección del VPH 16 se llevó a cabo utilizando el kit INNO LiPA genotyping Extra (Innogenetics, Barcelona, España), siguiendo las indicaciones de la casa comercial. Amplificando un segmento de 65 pb de la región L1 del genoma viral, que permite detectar por lo menos 54 tipos distintos de VPH, entre ellos el VPH 16.

RESULTADOS

Se incluyeron en el estudio 30 muestras de mujeres, divididas en dos estratos; 16 muestras de LEIBG con infección por VPH 16 y 16 muestras de CaCU con infección por VPH 16. La edad media para el grupo de LEIBG fue de 33 años y para CC de 52.71 años. Al analizar la frecuencia de metilación del promotor de miR-218-1 fue mayor en CC (VPH 16+), encontrándose en un 43.75% (7/16) de las muestras en comparación con las muestras de pacientes con LEIBG (VPH 16+) que se encontró en un 6.25% (1/16).

Por otra parte, la comparación del porcentaje de secuencias de ADN metiladas en ambos grupos de estudio a través de la prueba no paramétrica de Mann-Whitney arrojó diferencias estadísticamente significativas ($p = 0.0007$) entre CC y LEIBG, encontrándose un porcentaje mayor de secuencias metiladas en CC que en LEIBG (figura 1).

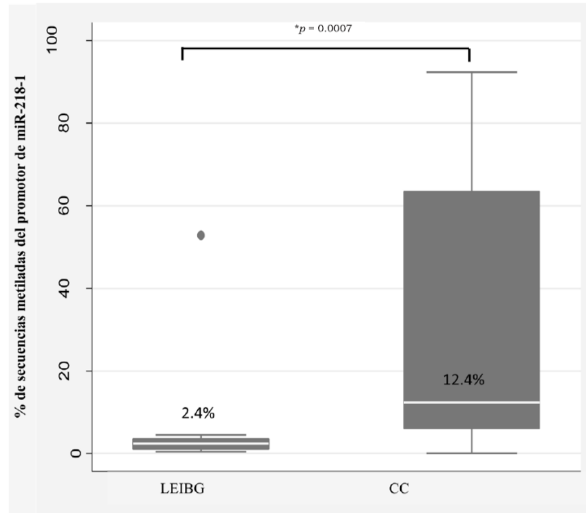

Figura 1. Comparación de secuencias metiladas del promotor de miR-218-1 en muestras de pacientes con LEIGB y CC con infección por VPH 16 (*Prueba de Mann-Whitney).
Fuente: Elaboración propia.

DISCUSIÓN

Los miRNAs desempeñan un papel importante en la carcinogénesis cervical, desde la infección por el VPH hasta la progresión a cáncer. Alteraciones en el perfil de expresión de miRNAs han sido observadas en cáncer cervical y en lesiones precursoras inducidas por VPH en comparación con tejido normal sugiriendo su participación en la carcinogénesis cervical (Zheng & Wang, 2011).

Defectos en la expresión de miRNAs han sido asociados con fallas en su regulación pos-transcripcional y/o represión transcripcional relacionados principalmente a: 1) alteraciones genéticas como deleciones, amplificaciones y mutaciones puntuales, y 2) a alteraciones epigenéticas como modificación de histonas y metilación aberrante del ADN, mecanismos que pueden verse favorecidos por los VPH-AR, como el VPH 16 (Lujambio *et al.*, 2007).

En este estudio realizado se encontró que en pacientes con CC es más frecuente encontrar metilado el promotor de miR-218-1 en comparación con pacientes con LEIBG, además de que estos pacientes presentaron un porcentaje mayor de secuencias de

ADN metiladas. En CC no existen reportes del estado de metilación del promotor de miR-218, sin embargo, la regulación epigenética por metilación de miR-218 ha sido reportada. En cáncer oral la disminución de la expresión de miR-218 por hipermetilación del ADN ha sido reportada (Yamamoto *et al.*, 2013). Por otra parte, se ha reportado que miR-218-1 es un supresor de tumor y se encuentra disminuido en cáncer. Estudios en CC reportan que la expresión de miR-218 se encuentra desregulada (Martínez *et al.*, 2008) y que su sobreexpresión inhibe la invasión y migración celular (Tie *et al.*, 2010). Los resultados obtenidos en este estudio pueden sugerir que posiblemente la expresión de miR-218-1 esté silenciada por metilación de su promotor en CC y que la metilación aberrante de su promotor esté involucrada en la patogénesis del CC.

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Discusión

La infección persistente por VPH-AR es la principal causa para desarrollar cáncer cervical sin embargo, alteraciones genéticas y epigenéticas son necesarias para la progresión de las lesiones precancerosas a cáncer invasor. En cáncer cervical se ha observado un incremento en la expresión de miRNAs oncogénicos y una disminución de miRNAs supresores de tumor (Zheng *et al.,* 2011). En cáncer cervical la infección por VPH-AR y la expresión de sus oncoproteínas E6 y E7 juegan un papel importante en la desregulación de la expresión de los miRNAs. Además, se ha descrito que las modificaciones epigenéticas posiblemente inducidas por el VPH-AR pueden estar implicadas en la desregulación de la expresión de los miRNAs (Zheng *et al.,* 2011). Evidencia acumulada sugiere que los miRNAs juegan un papel importante en la patogénesis del cáncer cervical y estudios previos han demostraron su silenciamiento epigenético en esta enfermedad.

Con la finalidad de identificar genes de miRNAs silenciados por metilación de su región promotora en cáncer cervical, se evaluó el estado de metilación de 22 promotores de genes de miRNAs que contienen dinucleótidos o islas CpG, lo cual los hace susceptibles de ser regulados por metilación. En este estudio se identificaron cuatro genes de miRNAs (miR-124-2, miR-218-1, miR-218-2, el clúster miR-34c/b y el miR-23b/miR-24-1/miR-27b) que se encontraron frecuentemente metilados tanto en las líneas celulares como en tejidos de mujeres con cáncer cervical, quienes presentaron altos niveles de metilación (>15%) en comparación con las mujeres sin LEI y con LEIBG. El análisis de la expresión de miR-124, miR-218, miR-193b reveló una correlación negativa con sus niveles de metilación. El tratamiento con 5'-Aza-CdR un agente hipometilante permitió significativamente la sobreexpresión de miR-124 en las líneas celulares C33A y HeLa y la sobreexpresión de miR-218 y miR-23b en las células SiHa, HeLa, CaSki y C33A. El tratamiento con 5'-Aza-CdR y el resultado en población sugiere que la metilación contribuye al silenciamiento de la expresión de estos miARNs en cáncer cervical. Por otra parte, el promotor de miR-193b se encontró frecuentemente desmetilado y sobreexpresado mujeres con cáncer cervical en comparación con las mujeres sin LEI y con LEIBG. En las líneas celulares SiHa, HeLa, CaSki y C33A el

promotor de miR-193b se encontró desmetilado, sin embargo sólo se observó sobreexpresión de miR-193b en la línea celular SiHa.
Nuestros resultados en líneas celulares y población coinciden con reportes previos en los que han descrito que miR-124-2, miR-218-1, miR-218-2, miR-23b y miR-34b/c se encuentran frecuentemente metilados y que presentan una correlación negativa con sus niveles de expresión (silenciados o disminuidos) en cáncer. Otros estudios soportan que miR-124-2, miR-218-1, miR-218-2 y miR-34b/c son regulados por metilación ya que el tratamiento con agentes hipometilantes permite la sobreexpresión de estos miRNAs. (Rauhala *et al.,* 2010; Suzuki et al., 2011; Baer *et al.*, 2012; Formosa et al., 2013). Además, miR-124, miR-218, miR-34b/c miR-23b/miR-24-1/miR-27b y miR-193b son miRNAs supresores de tumor ya que la sobreexpresión ectópica de estos miRNAs disminuye o silencia la expresión de genes blanco que favorecen la proliferación y metástasis (Rauhala et al., 2010; Suzuki et al., 2011; Baer *et al.*, 2012; Formosa et al., 2013).

La metilación de miR-124-2, ha sido identificada en cáncer colon (Lujambio et al., 2007; Suzuki et al., 2011), cáncer gástrico (Ando et al., 2009), malignancias hematológicas (Agirre et al., 2009; Baer *et al.,* 2012) y carcinoma hepatocelular (Shimizu et al., 2013), cáncer de pulmón (Kitano *et al.,* 2011). En este estudio la frecuencia de metilación del promotor de miR-124-2 en mujeres con cáncer cervical fue mayor (62.5%) que los reportados en cáncer colon (50.6%) y cáncer de pulmón de células pequeñas (50.0%), mientras que Suzuki et al., 2011 reportan una frecuencia de hasta el 96.6% al realizar un análisis combinado de metilación del promotor de miR-124-1 y miR-124-2. Wilting *et al.,* 2010, reportan que el silenciamiento de miR-124 por metilación parece ocurrir durante la carcinogénesis inducida por el VPH pero que parece que no está directamente relacionada con la presencia de VPH-AR ya que ocurre en la etapa de post-inmortalización.

La frecuencia de metilación del promotor de miR-34b/c encontrada en este estudio (43.8%), están dentro de los porcentajes reportados por otros autores en cáncer de colón 35%, pulmón 54%, mama 69%, cuello y cabeza 8% y en melanoma de 25%

(Lujambio *et al.,* 2008). En líneas celulares de cáncer de colon se encontró metilado en todas las líneas celulares estudiadas (9 de 9, 100%) y en el 90% de tumores primarios de colon (Toyota *et al.,* 2008). El tratamiento con 5'-Aza-CdR en líneas celulares de cáncer metastasico de melanoma (IGR37), y de cuello y cabeza (SIHN-011B) y colon (SW620) establecidas de nódulos linfáticos permitió la sobreexpresión miR-34b/c sólo en la línea celular SIHN-011B (Lujambio et al., 2008).

En general, se ha demostrado que miR-218 esta disminuido en varios tipos de cáncer y asociado con fenotipos malignos. El miR-218 se ha caracterizado como un miRNA supresor de tumor que inhibe la invasión, migración y proliferación celular en células de cáncer de tiroides y cáncer cervical (Yamamoto et al., 2010; Guan et al., 2013). El tratamiento con 5'-Aza-CdR en líneas celulares de cáncer metastasico de melanoma (IGR37), y de cuello y cabeza (SIHN-011B) establecidas de nódulos linfáticos permitió la sobreexpresión de miR-218 (>2 veces más que las células no tratadas), (Lujambio et al., 2008). Martinez et al., 2008, reportan que el VPH 16 reduce la expresión de miR-218 en células de cáncer cervical a través de su oncoproteína E6 al observar que la expresión de miR-218 sólo se encontró disminuida en líneas celulares positivas a VPH y en lesiones cervicales y cáncer que contienen DNA del VPH 16 y determinaron que LAMB3 es un blanco de miR-218 en cáncer cervical. Otros estudios soportan la participación del VPH-AR en la disminución de la expresión de miR-218 (Li et al., 2010) Interesantemente observamos que el promotor de miR-218-1 solo se encontró metilado en las líneas celulares positivas a VPH (HeLa, SiHa y CaSki), lo cual podría ser un puente entre la metilación de genes de miRNAs inducida por VPH-AR.

El promotor de miR-193b se encontró frecuentemente desmetilado (92.9%) y sobreexpresado en cáncer cervical, y metilado en mientras que en mujeres sin LEI sin infección por VPH y mujeres con LEIBG. El miR-193b ha sido identificado como un supresor de tumor silenciado por metilación de su promotor en cáncer de próstata y otros tipos de cáncer (Lujambio *et al.,* 2007; Rauhala *et al.,* 2010). El promotor de miR-193b también se ha encontrado desmetilado en líneas celulares de cáncer. Además, la sobreexpresión de miR-93b ha sido reportada en células y muestras de gliomas

asociándose con un pronóstico pobre. En gliomas miR-193b promueve la proliferación celular a través de la vía del factor de crecimiento transformante β (TGF-β) por medio de su blanco Smad3 (Zhong *et al.,* 2014). El miR-193b ha sido sugerido que puede ser regulado epigenéticamente en líneas celulares de ganglios linfáticos procedentes de cáncer de colon, piel y cuello y cabeza (Lujambio *et al.,* 2007). Lujambio *et al.,* encontraron que en líneas celulares miR-193b responde al tratamiento con 5'-Aza-CdR, y demostraron que miR-193b se metila en un área que abarca 10 dinucleótidos CpG alrededor del sitio de inicio de la transcripción (pre-miR-193b~365-1) en líneas celulares de cáncer. Sin embargo, similar hipermetilación se encontró en tejidos normales. En líneas celulares de cáncer de próstata encontraron que 6 líneas celulares (22Rv1, VCAP, LNCaP, PC-3, EP156T y las células PrEC) presentaron metilación en miR-193b mientras que no se observó metilación en las líneas celulares (DU145 y LAPC-4) (Rauhala et al., 2010). En cáncer cervical miR-193b se encontró sobreexpresado en líneas celulares VPH 16 positivas comparado con la línea celular VPH negativa y con cérvix normal (Martinez et al., 2008). El tratamiento con 5'-Aza-CdR en las líneas celulares IGR37, SIHN-011B y SW620 permitió la sobreexpresión miR-193b (>2 veces más que las células no tratadas) (Lujambio et al., 2008).

Entre los genes analizados en las líneas celulares de CaCU el promotor de miR-23b se encontró metilado y su expresión disminuida así como el aumento significativo de su expresión después del tratamiento con 5'-Aza-CdR. Mientras que la expresión de miR-23b, miR-27b y miR-24 disminuyó conforme aumentó el grado de lesión. Nuestros resultados sobre miR-23b coinciden con reportados por Majid *et al.,* 2012, en líneas celulares y tejido tumoral de cáncer de próstata en el cual concluyen que la expresión de miR-23b es regulada por metilación de estas islas CpG. Por lo que es probable que en cáncer cervical la expresión de miR-23b también este regulada por metilación de dinucleótidos en las islas CpG de su promotor. La sobreexpresión de miR-24 y miR-27b en mujeres con LEIBG y su disminución en mujeres con cáncer cervical sugieren que miR-24 y miR-27b tiene una función supresora de tumor en la carcinogénesis cervical. Estudios en líneas celulares de cáncer cervical sugieren que miR-23b, miR-24 y miR-

27b pueden estar involucrados en el desarrollo del cáncer cervical (Cheng *et al.,* 2005; Lui *et al.,* 2007).

Los resultados obtenidos en este estudio sobre la metilación y expresión de miR-124-2, miR-218-1, miR-218-2, miR-23b/miR-24-1/miR-27b y miR-34b/c en líneas celulares de cáncer cervical y en muestras de pacientes con LEIBG y cáncer cervical, así como la sobreexpresión de estos miRNAs en líneas celulares de cáncer cervical después del tratamiento con el agente desmetilante 5'-Aza-CdR sugiriere que la metilación de dinucleótidos o islas CpG contribuyen al silencio en la expresión de estos miRNAs.

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Anexos

Anexo 1. PCR Array Catalog # EAHS-591ZA (QUIAGEN, Duesseldorf, Germany).

Anexo 1. miRNAs candidatos a silenciamiento por metilación.	
Oncogénicos	**Papel en Cáncer**
Grupo miR-23b	Inhibe la expresión de POX (Prolina oxidasa), proteína supresora de tumor que inhibe la proliferación celular e induce apoptosis a través de especies reactivas del oxígeno (ROS) (Lui *et al.,* 2010).
miR-301a	Sobre-expresado en cáncer pancreático. Favorece la activación de NF-kB (Factor Nuclear kB), regulando negativamente la expresión del gen NKRF (Factor de represión de NF-kB) (Lu *et al.,* 2011).
miR-32	Sobre-expresado en cáncer de riñón y mieloma múltiple. Reduce la expresión del factor pro-apoptótico Bim (Pichiorri *et al*., 2008; Petillo *et al*., 2009; Gocek *et al.,* 2011).
miR-378	En la línea celular de cáncer de mama MT-1 miR-378 promueve la supervivencia celular, el crecimiento del tumor y la angiogénesis a través de sus blanco SuFu y Fus-1 (Lee *et al.,* 2007).
miR-10a-MDM4	Mediador de la metástasis en células tumorales del páncreas (Weiss *et al*., 2009).
miR-155	Sobre-expresado en carcinoma hepatocelular, su gen blanco es SOX6 un factor de transcripción de p21waf1/cip1 (Xie *et al.,* 2011).
miR-191 **miR-425**	La inhibición de miR-191 disminuye la proliferación celular e induce apoptosis *in vitro*. Reducen la masa tumoral *in vivo* en modelos murinos de carcinoma hepatocelular (Elyakim *et al.,* 2010).
miR-210	Favorece la diferenciación, proliferación, migración e invasión celular en líneas celulares de cáncer de mama (Rothe *et al.,* 2011).
Grupo miR-17-92	Promueve la proliferación, evita la activación de la muerte celular inducida, Tienen un papel clave en la respuesta Th1, favorece la inducción de IFN-γ y suprime la diferenciación de células Treg. La pérdida de miR-17-92 en células T CD4 resulta en la evasión del tumor (Jiang *et al.,* 2011; Sasaki *et al*., 2010).
Supresores de Tumor	**Papel en Cáncer**
miR-218-1	Disminuido en cáncer gástrico, de pulmón, de cuello y cabeza, de vejiga y próstata. Disminuido en líneas celulares VPH 16^+, lesiones cervicales y cáncer en comparación con líneas celulares VPH^- y tejido cervical normal. Favorece la apoptosis e Inhibe la proliferación celular, la migración y la invasión al regular la expresión de los genes ROBO1, SLIT2, VOPP1 y el gen TMX1 (Tatarano *et al.,* 2011).
mir-30c-1 mir-30e	Regula negativamente al factor de crecimiento de tejido conectivo (CTGF), desempeñando un papel importante en el control de cambios estructurales en la matrix extracelular (Duisters *et al*., 2009).
miR-34a	Tiene como blanco al gen E2F3 y CDK6. Regula la progresión del ciclo celular, la proliferación, la apoptosis, daño al DNA y la angiogénesis. Se ha encontrado hipermetilado en cáncer de mama, ovario, colón, pulmón y tumores malignos hematopoyéticos. La disminución de miR-34a en CaCU se ha asociado con la infección por VPH-AR (Li *et al*., 2010; Nalls *et al.,* 2011).
miR-34c	Disminuye la proliferación celular, la migración, la invasión e incrementa la apoptosis. Regula negativamente a E2F3 y BCL2, los cuales estimulan la proliferación y suprimen la apoptosis en células de cáncer de próstata respectivamente (Hagman *et al.,* 2010).
miR-34b	Silenciado en varios tipos de cáncer: melanoma, colón y cáncer de cuello y cabeza. La sobreexpresión de miR-34b reduce la motilidad celular y la invasión (Mazar *et al.,* 2011).
miR-7-1	Inhibe la proliferación celular por desregulación del gen BCL-2 miR-7 (Xiong *et al*., 2011).
Let-7g	Regula la proliferación celular de manera negativa y positiva al tener como blanco a c-Myc y p16 respectivamente (Lan *et al*., 2011).
Let-7i	Regula negativamente la entrada a la fase S del ciclo celular. Además, disminuye la expresión de reguladores críticos de ciclo celular tales como las ciclinas y CDKs (Bueno *et al.,* 2010).
miR-1-1	Inhibe la proliferación celular al tener como blanco al gen CCND2 que codifica para la ciclina D2, que favorece la transición de G1/S. Inhibe la invasión y la metástasis a través de Factor derivado de células estromales SDF-1α y el receptor de quimiocinas CXC 4 (CXCR4) (Leone *et al.,* 2011).
miR-124-2	Inhibe la invasión y/o la metástasis por sus genes blanco ROCK2 y EZH2 (Zheng *et al*., 2011).
miR-15b -miR-16-2	Inhiben la expresión de ciclina E, la cual es necesaria para la transición de G1/S (Ofir *et al*., 2011).
miR-193b	Reprime la proliferación celular y regula la ciclina D necesaria para la transición de G1/S del ciclo celular (Chen *et al.,* 2011).
miR-196a-1	Se ha observado que promueve el crecimiento celular (Chen *et al.,* 2011).
miR-126	Inhibe la proliferación celular en cáncer de pulmón de células no pequeñas a través de su blanco EGFL7 (Sun *et al.,* 2010).

Anexo 2. PCR Array Catalog # EAHS-591ZA (QUIAGEN, Maryland, USA).

Precursor Accession #	Precursor miRNA ID	Chr	CpG Start	CpG End	Precursor Position
MI0000433	hsa-let-7g	3	52312069	52313057	chr3: 52302294-52302377
MI0000434	hsa-let-7i	12	62996159	62997632	chr12: 62997466-62997549
MI0000266	hsa-mir-10a	17	46658948	46660003	chr17: 46657200-46657309
MI0000651	hsa-mir-1-1	20	61148081	61148990	chr20: 61151513-61151583
MI0000444	hsa-mir-124-2	8	65290108	65290946	chr8: 65291706-65291814
MI0000471	hsa-mir-126	9	139559804	139561005	chr9: 139565054-139565138
MI0000478	hsa-mir-149	2	241374410	241376320	chr2: 241395418-241395506
MI0000681	hsa-mir-155	21	26934424	26934805	chr21: 26946292-26946356
MI0000438, MI0000115	hsa-mir-15b, hsa-mir-16-2	3	160117012	160118878	chr3: 160122376-160122473
MI0000071, MI0000072, MI0000073, MI0000074, MI0000076, MI0000093	hsa-mir-17 Cluster	13	91999541	92001441	chr13: 92002859-92002942
MI0000465, MI0001448	hsa-mir-191, hsa-mir-425	3	49058325	49059986	chr3: 49058051-49058142
MI0003137	hsa-mir-193b	16	14395604	14397075	chr16: 14397824-14397906
MI0000286	hsa-mir-210	11	567938	569461	chr11: 568089-568198
MI0000294	hsa-mir-218-1	4	20253276	20256868	chr4: 20529898-20530007
MI0000295	hsa-mir-218-2	5	168727429	168728275	chr5: 168195151-168195260
MI0000439, MI0000080, MI0000440	hsa-mir-23b, hsa-mir-24-1, hsa-mir-27b	9	97848140	97848525	chr9: 97848303-97848370
MI0000745	hsa-mir-301a	17	57231855	57232655	chr17: 57228497-57228582
MI0000736, MI0000749	hsa-mir-30c-1, hsa-mir-30e	1	41157162	41157975	chr1: 41220027-41220118
MI0000090	hsa-mir-32	9	111881482	111882278	chr9: 111808509-111808578
MI0000742, MI0000743	hsa-mir-34c, hsa-mir-34b	11	111383168	111383892	chr11: 111383663-111383746
MI0000786	hsa-mir-378	5	149109570	149111750	chr5: 149112388-149112453
MI0000263	hsa-mir-7-1	9	86594326	86596142	chr9: 86584663-86584772

Anexo3. Expresión relativa de let-7g, let-7i, miR-218 y miR.218*

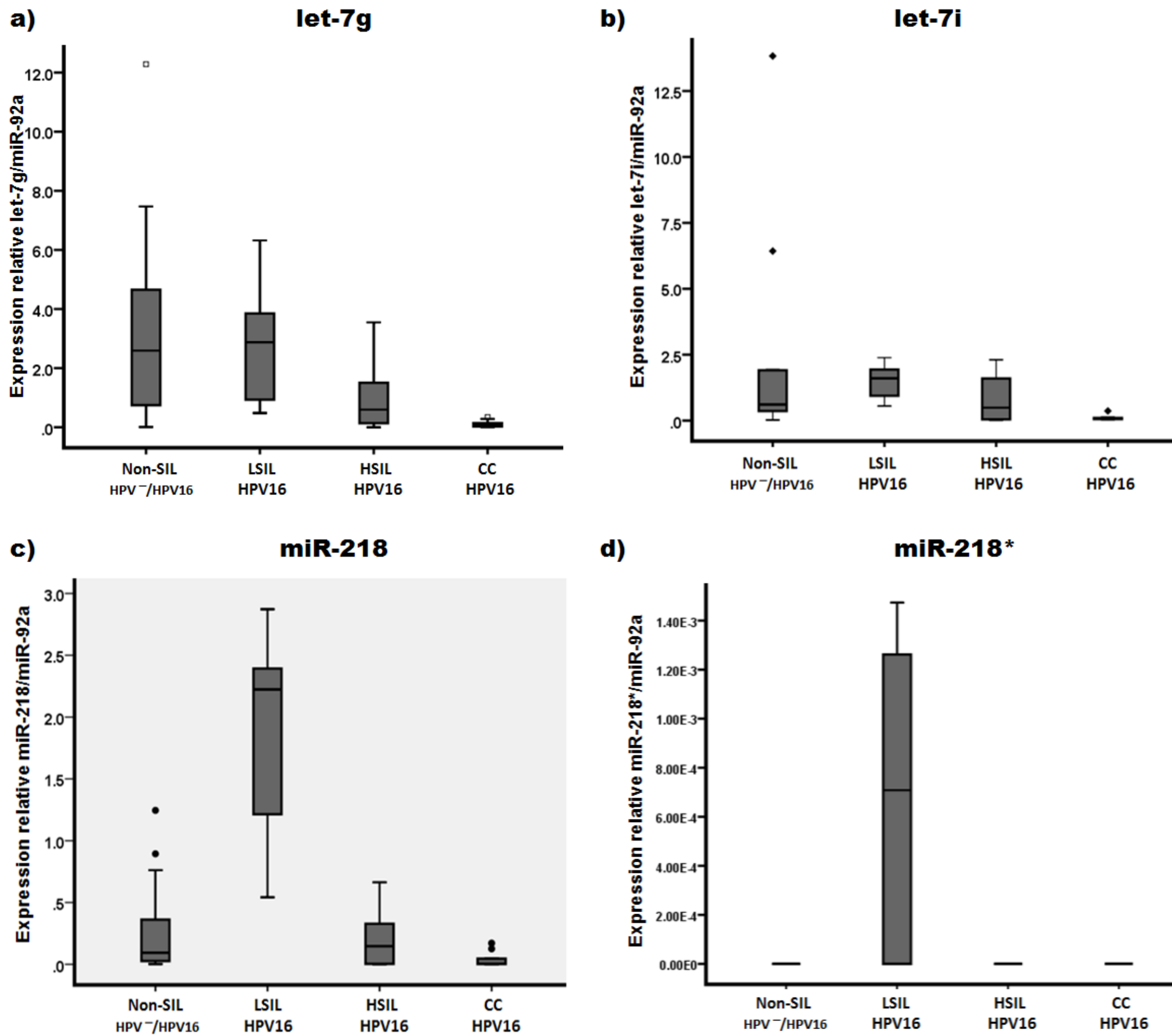

Figura 1. Expresión de let-7g, let-7i, miR-218 y miR.218* en la carcinogénesis cervical a) Expresión de let-7g. b) Expresión de let-7i. c) miR-218. Expresión de miR.218*. Los datos fueron medidos por qPCR y calculado por el método de delta CT. La expresión de cada miRNA fueron normalizados por los niveles de miR-92a. Los valores son expresados como la media de dos cultivos independientes.