UNIVERSIDAD AUTÓNOMA DEGUERRERO

FACULTAD DE CIENCIAS QUÍMICO-BIOLÓGICAS

UNIDAD DE INVESTIGACIÓN ESPECIALIZADA EN MICROBIOLOGÍA

DOCTORADO EN CIENCIAS BIOMÉDICAS

Participación de miR-23b en la proliferación, migración e invasión celular a través de la regulación negativa de c-Met en Cáncer Cérvico Uterino

T E S I S

QUE PARA OBTENER EL GRADO DE

DOCTOR EN CIENCIAS BIOMÉDICAS

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ACTA DE APROBACIÓN DE TESIS

En la ciudad de Chilpancingo, Guerrero, siendo los 9 días del mes de julio del dos mil dieciocho, se reunieron los miembros del Comité Tutoral designado por la Academia de Posgrado del Doctorado en Ciencias Biomédicas, para examinar la tesis titulada **"Participación de miR-23b en la proliferación, migración e invasión celular a través de la regulación negativa de c-MET en Cáncer Cérvico Uterino"**, presentada por la alumna Gabriela Elizabeth Campos Viguri, 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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Ojalá algún día llegue a ser tan buena, como ustedes ya creen que soy.

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Gabriela Elizabeth Campos Viguri

Participación de miR-23b en la proliferación, migración e invasión celular a través de la regulación negativa de c-MET en Cáncer Cérvico Uterino

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I. Resumen

miR-23b-3p es un miRNA propuesto como supresor tumoral en cáncer cervico uterino (CaCU). La expresión disminuida de miR-23b-3p en CC, contrasta con los niveles de expresión reportados para c-Met en esta malignidad. **Objetivo:** Determinar si miR-23b-3p regula la expresión de c-Met y si la represión post-transcripcional de este receptor induce disminución en la activación de Fak y Gab1 asi como en la proliferación, migración e invasión de líneas celulares de CC. **Material y métodos:** La expresión de miR-23b-3p y c-Met se determinó por RT-qPCR en células C33A, CaSki y HaCaT, así como en tejidos cervicales. Los efectos de sobre-expresión de miR-23b-3p sobre la expresión de c-Met se determinaron por RT-qPCR y Western blot. Ensayos MTS, de cierre de herida y transwell fueron realizados para determinar los efectos de miR-23b-3p sobre la proliferación, migración e invasión celular. La determinación de la interacción miR-23b-3p:c-Met se validó por ensayos reporteros de luciferasa. La activación de moléculas rio abajo de c-Met se evaluó por Western-blot. **Resultados:** En células de CaCU, la expresión de miR-23b-3p y de c-Met se relacionan inversamente. La expresión de c-Met es mayor en tejido de CaCU con VPH16 que en tejido cervical sin lesión intraepitelial y células HaCaT. En células C33A y CaSki, miR-23b-3p disminuye significativamente la proliferación, migración e invasión celular, así como la expresión de mRNA y proteína de c-Met, mediante su interacción directa con la región 3'-UTR de c-Met. miR-23b-3p modifica el nivel de activación de moléculas rio abajo de c-Met en células CaSki. **Conclusiones:** miR-23b-3p regula post-transcripcionalmente la expresión de c-Met, y en consecuencia afecta la proliferación, migración e invasión de células C33A y CaSki. En CaSki, la disminución de c-Met por miR-23b-3p reduce la activación de Gab1 y Fak, moléculas rio abajo de c-Met. Estos resultados apoyan el papel supresor tumoral de miR-23b-3p y su participación en CC a través de la inhibición del receptor c-Met.

Palabras clave: c-Met, cáncer cervical, miR-23b-3p, proliferación, migración, invasión, VPH16.

II. Introducción

Los microRNAs (miRNAs) son pequeñas moléculas de RNA endógeno no codificante de 19-25 nucleótidos de longitud, que actúan como reguladores de la expresión de genes. En cáncer, la expresión aberrante de genes y la subsecuente alteración de funciones celulares, se atribuye en parte, a la desregulación global de la expresión de miRNAs (Wang *et al.,* 2008; Taby & Issa, 2010; Zhang *et al.,* 2011). Dado que en procesos carcinogénicos los miRNAs tienen función de oncogenes o de genes supresores de tumor (miRNAs supresores de tumor), el estudio de sus patrones de expresión es importante en el diagnóstico y pronóstico de tumores (Tian *et al.,* 2014).

El microRNA-23b (miR-23b-3p) forma parte de un clúster conservado de genes de miRNAs, situado en una unidad de transcripción no codificante denominada C9orf3 que se ubica en el cromosoma humando 9q22.32 (Zhang *et al.*, 2011; Wang *et al.,* 2018). Aunque este clúster está compuesto por miR-23b, miR-27b y miR-24-1, las secuencias maduras de estos miRNAs son expresadas diferencialmente, (Bang *et al.,* 2011) debido a su procesamiento individual en pri- y pre-miRNAs o a la estabilidad de sus secuencias maduras (Winter *et al.,* 2009). Además, algunos transcritos primarios, como el de miR-23b, están bajo el control de factores de transcripción clásicos como p53 (He *et al.,* 2007), c-Myc (Gao *et al,* 2009), NF-κB (Jin *et al.,* 201) y AP-1 (Pellegrino *et al.,* 2013).

Los niveles de expresión de miR-23b-3p varían entre tejidos cancerosos y tejidos normales (Chiyomaru *et al.,* 2015), y con base en los genes que regula, se le ha atribuido función de promotor o supresor de tumor en un contexto celular específico (Pack *et al.,* 2013) (anexo 1). Las funciones pleiotrópicas de miR-23b-3p, incluso en el mismo tipo de cáncer (Pellegrino *et al.,* 2013; Jin *et al.,* 2013), pueden ser influidas por el microambiente tumoral a través de señales reguladoras del medio, o de los tejidos circundantes y por la heterogeneidad de los tejidos (subtipos de cáncer) y células tumorales (Zhuang *et al.,* 2016).

Interesantemente, miR-23b-3p se ha definido como un regulador clave en etapas previas a la metástasis del cáncer de mama (Pellegrino et al., 2013), de ovario (Li

et al., 2014) de colón (Zhang et al., 2011) y de vejiga (Majid et al., 2013; Chiyomaru et al., 2015) debido a que reprime genes que participan y/o promueven la movilidad e invasión celular, entre ellos Pack1, Anxa1 (Pellegrino et al., 2013), Runx2 (Li et al., 2014), Zeb1 (Majid et al., 2013) and Plau (Au-Yeung et al., 2011), entre otros.

En acuerdo con los resultados reportados por Au Yeung *et al.,* (2011), nuestro grupo de trabajo ha evidenciado la expresión disminuida de miR-23b-3p en CC. En un estudio previo, demostramos que la expresión de miR-23b-3p es regulada en parte, por la metilación de su región promotora; y nuestros resultados sugieren que miR-23b-3p tiene un patrón de expresión similar al de un supresor de tumor en tejidos y líneas celulares de CC (Campos-Viguri *et al.,* 2015). No obstante, los blancos moleculares de miR-23b y los mecanismos en los que está involucrado en CC aún deben ser estudiados para confirmar a este miRNA como un supresor tumoral.

El CC es precedido por lesiones premalignas denominadas Lesiones Escamosas Intraepiteliales (LEI) cervicales, clasificadas de acuerdo a su grado de progresión en LEI de Bajo Grado (LEIBG) y LEI de Alto Grado (LEIAG). A pesar de que las infecciones persistentes por VPH de alto riesgo oncogénico (VPH-AR) son el principal factor etiológico del desarrollo de LEIs y de su progresión a CC, (Zur Hausen, 2000), otros factores, incluyendo las anormalidades geneticas y epigeneticas, están involucrados en la tumorigénesis cervical y en su progresión a carcinoma invasor (Saavedra et al., 2012; Bhat *et al.,* 2016).

Una vez que el DNA de VPH-AR se integra al DNA de la célula, la transcripción del genoma viral es activada por factores de transcripción celulares que inducen la sobre-expresión constitutiva de las oncoproteínas virales E6 y E7. La expresión de E6 y E7 está ligada a la expresión alterada de genes celulares (Wang *et al.*, 2008; McLaughlin-Drubin *et al.,* 2009).

Hay evidencia de que la sobre-expresión E6 y E7 induce, en tejidos y líneas celulares de CaCU, la sobre-expresión de miRNAs oncogénicos y la disminución de la expresión de miRNAs supresores tumorales, en comparación con tejido cervical normal y/o no infectado, (Lee *et al.,* 2008; Pereira *et al.,* 2010; Nagamitzu et al., 2015; Gao et al., 2016).

Los cambios característicos en los patrones de expresión de miRNAs durante las diferentes etapas de la carcinogénesis cervical y en respuesta a la infección por VPH-AR, se proponen como potenciales marcadores moleculares de utilidad en el diagnóstico temprano o el pronóstico de CaCU. Harden *et al.,* (2017) demostraron que en cultivos no diferenciados de queratinocitos de prepucio humano, la expresión estable de E6 y E7 de VPH16, modifica diferencialmente la expresión de 51 miRNAs celulares asociados a la regulación de 1,456 blancos potenciales de miRNAs. Estos mismos autores determinaron que las endonucleasas Drosha y Dicer incrementan su expresión en presencia de E6 y E7 (Harden *et al.,* 2017). Por otro lado, Au-Yeung *et al.*, (2011), propusieron que la degradación del factor de transcripción p53 inducida por E6 de VPH16, disminuye la expresión de miR-23b en células SiHa y CaSki, sugiriendo la regulación de miR-23b por p53 (Bisio *et al.,* 2013; Zheng *et al.,* 2007). Por otro lado, se ha demostrado que el silenciamiento de E6/E7 de VPH-18, disminuye significativamente la expresión de 10 de 52 miRNAs sobre-expresados en células HeLa, y mostraron que, por el contrario miR-23b-3p, un miRNA con expresión disminuida en estas células *per se*, incrementa su expresión después del silenciamiento de tales oncoproteínas virales (Honegger *et al.,* 2015).

Estudios funcionales sugieren que la expresión del receptor c-Met es desregulada por miR-23b-3p en líneas celulares de carcinoma hepatocelular (Salvi *et al.,* 2009), de cáncer de vejiga (Chiyomaru *et al.,* 2015) de carcinoma oral de células escamosas (Fukumoto et al., 2016) y recientemente, se evidenció su regulación en CaCU (Au-Yeung et al., 2017), sin embargo, los eventos moleculares en los que participa el eje c-MET:miR-23b en CaCU aún son desconocidos. c-MET es un receptor tirosin-cinasa transmenbranal de estructura heterodimérica que en condiciones normales regula el desarrollo y homeostasis de tejidos. (Blumenschein *et al.,* 2012). La señalización alterada, inducida por este receptor, está implicada en una amplia variedad de malignidades humanas (Husmann *et al.,* 2015), incluyendo cáncer (Tsai *et al.,* 2006; Peruzzi *et al.,* 2006; Mesteri *et al.,* 2014; Chiyomaru *et al.,* 2015; Husmann *et al.,* 2015; Cao *et al.,* 2015).

c-Met se expresa en células epiteliales, mientras que su único ligando natural, el factor de crecimiento hepatocítico (HGF), (Birchmeier & Gherardi, 1998; Stella & Comoglio, 1999) es secretado principalmente por células mesenquimales, (Rosario & Birchmeier, 2003). En cáncer, circuitos paracrinos, producidos por interacciones epitelio-mesénquima, son responsables de la activación de c-Met por HGF. Sin embargo, el receptor también puede ser activado mediante circuitos autocrinos aberrantes (Venepalli *et al.,* 2013), que pueden ser resultado de tres eventos: el primero corresponde a la generación de la proteína de fusión TPR-MET por rearreglo cromosomal inducido por carcinógenos, que se ha descrito en líneas celulares de sarcoma osteogénico (Peschard *et al.,* 2007); el segundo puede deberse a la generación de variantes del transcrito de c-Met por splicing alternativo, como las descritas en diferentes subtipos de cáncer pulmonar (Onozato *et al.,* 2009); y el tercero, puede ser resultado de amplificaciones o mutaciones puntuales en el gen de c-Met, observadas frecuentemente en carcinoma papilar renal y otros tumores, (Leiser *et al.,* 2014).

La interacción c-MET:HGF induce la homo-dimerización y auto-fosforilación de c-MET en los residuos de tirosina Y1234 y Y1235 ubicados en su dominio de activación y, subsecuentemente, la fosforilación de los residuos Y1349 y Y1356 de la región c-terminal (Peruzzi *et al.,* 2006), este último evento activa al receptor y genera un sitio de acoplamiento multifuncional que recluta proteínas adaptadoras intracelulares, a través de dominios de homología 2 de src, entre ellas Gab1 (proteína Aglutinante Asociada a Grb2) a la que a su vez, se unen proteínas sustrato-cinasas como Grb2 (Proteína 2 de Unión al Receptor), p85 (subunidad reguladora de PI3K) y SOS, entre otras (Ponzetto et al., 1994; Benvenuti & Comoglio, 2007; Venepalli et al., 2013). Después del reclutamiento y activación de las proteínas adaptadoras, éstas transducen la señal rio abajo para activar diversas cinasas, como PI3K que induce la activación de FAK, involucrada en invasión de células epiteliales de tumores sólidos (Blumenschein *et al.,* 2012 Venepalli *et al.,* 2013; Husmann *et al.,* 2015); y cinasas de la vía MAPK (ERK y PAK) cruciales en procesos de transformación, proliferación, desprendimiento y migración celular en cáncer, todos estos eventos asociados a metástasis tumoral (Venepalli *et al.,* 2013;

Factor *et al.,* 2010; Dummler *et al.,* 2009). Adicionalmente, pueden ser activadas PLCγ y Shp2, ambas proteínas inductoras de morfogénesis, STAT3 que induce tubulogénesis y NF-κB que promueve la transformación y proliferación de células tumorales (Ariyawutyakorn *et al.,* 2016).

Al igual que en otros tipos de cáncer (Chau *et al.,* 2011; Chiyomaru *et al.,* 2015), en CaCU la expresión y activación de c-Met, correlacionan con la progresión tumoral, el grado y tamaño del tumor, la baja sobrevivencia de pacientes y con la recurrencia de la enfermedad, (Baykal *et al.,* 2003). En un panel heterogéneo de lines de CC, Miekus *et al.,* (2015), encontraron que el silenciamiento de c-Met mediante un siRNA, induce la disminución del crecimiento y diferenciación tumoral, en condiciones normales y bajo estrés (Miekus *et al.,* 2015). Por otro lado, estudios en población han mostrado que los niveles de expresión elevados de c-Met se relacionan significativamente a la severidad de las LEIs con VPH-AR, en comparación con LEIs negativas a VPH (Walker *et al.,* 2003; Horn *et al.,* 2012). Por otro lado, en ensayos *in vitro* se ha sugerido que la desregulación del receptor c-Met disminuye la expresión de SLUG, un regulador positivo de la transición epitelio-mesénquima (EMT), e incrementa la expresión de E-caderina, eventos que conllevan, a establecer un fenotipo epitelial en líneas celulares de CaCU (Miekus et al., 2015). Estos datos sugieren que la inhibición de la expresión y/o actividad de este receptor tirosina-cinasa, puede representar una estrategia potencial para el tratamiento del CC.

La heterogeneidad en los patrones de invasión reconocidos en distintos tipos de carcinomas, son el reflejo de diferentes grados de disociación entre las células del tejido tumoral (Horn *et al.,* 2006). En pacientes con CaCU en estadio IB y IIB, la elevada disociación de células tumorales, morfológicamente caracterizada por patrones de invasión similares a aerosol, correlaciona con disminución de las tasas de sobrevivencia total y de sobrevivencia libre de recurrencia, (Horn *et al.,* 2012), y probablemente estos casos culminen en CaCU invasivo, razón por la cual la búsqueda de nuevos marcadores pronósticos y el desarrollo de estrategias terapéuticas efectivas son necesarios.

Aunque actualmente se conoce que 1) c-Met es sobre-expresado en tejido de CaCU (Baykal *et al.*, 2003; Horn *et al.,* 2012) en comparación con tejido de cérvix normal (Walker *et al.,* 2003) 2) análisis *in silico* y ensayos reporteros han evidenciado la presencia de sitios de afinidad para miR-23b-3p en la región 3'-UTR del transcrito de c-Met (Salvi *et al.,* 2009; Chiyomaru *et al.,* 2015; Fukumoto et al., 2016) y 3) c.MET participa en un amplio espectro de procesos biológicos. Por lo tanto, es probable que el estudio de la regulación de procesos de proliferación, migración e invasión mediada a través de la regulación de c-MET por miR-23b, aporte nueva información de la participación de este miRNA sobre la modulación de los efectos biológicos inducidos por la señalización aberrante de c-MET en CaCU.

En el presente estudio, las líneas celulares C33A y CaSki derivadas de CaCU, fueron usadas con el objetivo de investigar si miR-23b-3p reprime directamente la expresión del receptor c-Met, y sí esta represión modifica la proliferación, migración e invasión de las células. Asimismo, evaluamos la activación de Gab1 y Fak en respuesta a la expresión ectópica de miR-23b-3p. Por otro lado, investigamos el nivel de expresión de mRNA y proteína de c-Met en tejidos de CC positivos a VPH16. En conjunto nuestros resultados demuestran que c-Met es un gen blanco de miR-23b-3p y que la sobreexpresión ectópica de este miRNA disminuye significativamente la proliferación, migración e invasión de células C33A y CaSki. En células CaSki, encontramos reducción significativa en la activación de Gab1 y Fak en respuesta a la sobreexpresión de miR-23b-3p. Además, nuestros hallazgos evidencian la expresión heterogénea de c-Met entre tejidos de CC positivos a VPH16.

III. Resultados

CAPITULO I

MiR-23b-3p suppresses the proliferation, migration and invasion of cervical cancer cells lines by decrease expression of the receptor tyrosine-kinase c-MET

Cover letter

Chilpancingo, Guerrero, México, 27th, January 2019

Anna Treadway, PhD
Head of Scientific Reports
Richard White, PhD
Chief Editor
Editorial office
The Macmillan Building
4 Crinan Street, London N1 9XW

Dear Sirs PhD. Treadway and PhD. White:

We submit the manuscript "**MiR-23b-3p suppresses proliferation, migration and invasion of cervical cancer cells lines by decrease expression of the receptor tyrosine-kinase c-Met"** for your consideration for peer review and its possible publication in Scientific Reports.

We affirm that the manuscript has been prepared in accordance with the instructions for authors.

All authors have been read the manuscript and they agreed to its content. The authors affirm that the content of this manuscript has not been submitted for its publication elsewhere.

All authors have sufficiently contributed to data generation, analysis and manuscript preparation. We have not financial interest or conflict of interest in association with this work.

We believe that this manuscript should be published in Scientific Reports because:

1. The topic of this paper is among those that are the scope for Scientific Reports.
2. In our knowledge this is the first paper that researches the miR-23b-3p role on proliferative, migration and invasion of C33A and CaSki cells.
3. Our results suggest that decrease in proliferation, migration and invasion of CaSki cells are related to decreased activation of Gab1 and Fak in response

to c-Met repression by miR-23b-3p. We present data that validate to miR-23b-3p as a tumor suppressor in cervical cancer cells.

4. We present data that validate to miR-23b-3p as a tumor suppressor in cervical cancer cells.

None of the authors of the manuscript has had conflicts with the members of the board editorial of Scientific Reports about this work.

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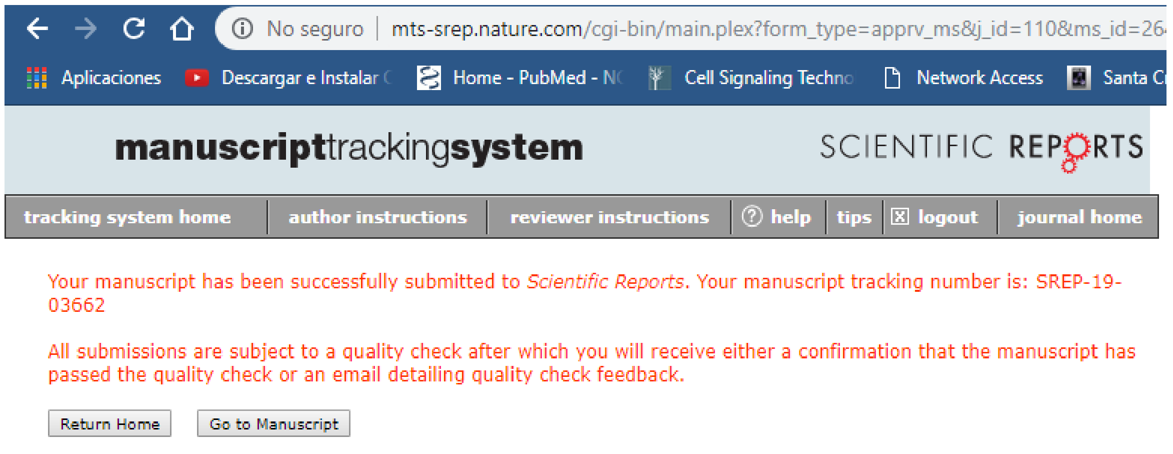
Envío y estatus del artículo



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Abstract	miR-23b-3p is a miRNA proposed as tumor suppressor in cervical cancer (CC). The low expression of miR-23b-3p in CC contrasts with the expression levels reported for c-Met in this malignancy. The aim of this investigation was to determine if miR-23b-3p regulates c-Met expression and if the post-transcriptional repression of this receptor decreases the activation of Fak and Gab1 as well as the proliferation, migration and invasion of CC cell lines. In CC cells, there is an inverse relationship between miR-23b-3p and c-Met expression. The expression of c-Met is higher in HPV positive CC tissues than in cervical smears from patients without squamous intraepithelial lesions and HaCaT cells. In CC cells, miR-23b-3p overexpression significantly decreases cell proliferation, migration, invasion and the expression of c-Met mRNA and protein. Our results show that miR-23b-3p regulates to c-Met expression through its binding to the 3'-UTR region of c-Met. miR-23b-3p modifies activation of molecules downstream of c-Met in CaSki cells. Therefore, miR-23b-3p post-transcriptionally regulates c-Met expression, and affects proliferation, migration and invasion of CC cells. In CaSki, the decrease of c-Met by miR-23b-3p reduces Gab1 and Fak activation. These results support the tumor suppressive role of miR-23b-3p and its participation in CC through c-Met inhibition.

Title Page

MiR-23b-3p suppresses proliferation, migration and invasion of cervical cancer cells lines by decrease expression of the receptor tyrosine-kinase c-Met

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Running Title: MiR-23b-3p regulates to c-Met in cervical cancer cells

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Abstract

miR-23b-3p is a miRNA proposed as tumor suppressor in cervical cancer (CC). The low expression of miR-23b-3p in CC contrasts with the expression levels reported for c-Met in this malignancy. The aim of this investigation was to determine if miR-23b-3p regulates c-Met expression and if the post-transcriptional repression of this receptor decreases the activation of Fak and Gab1 as well as the proliferation, migration and invasion of CC cell lines. In CC cells, there is an inverse relationship between miR-23b-3p and c-Met expression. The expression of c-Met is higher in HPV positive CC tissues than in cervical smears from patients without squamous intraepithelial lesions and HaCaT cells. In CC cells, miR-23b-3p overexpression significantly decreases cell proliferation, migration, invasion and the expression of c-Met mRNA and protein. Our results show that miR-23b-3p regulates to c-Met expression through its binding to the 3'-UTR region of c-Met. miR-23b-3p modifies activation of molecules downstream of c-Met in CaSki cells. Therefore, miR-23b-3p post-transcriptionally regulates c-Met expression, and affects proliferation, migration and invasion of CC cells. In CaSki, the decrease of c-Met by miR-23b-3p reduces Gab1 and Fak activation. These results support the tumor suppressive role of miR-23b-3p and its participation in CC through c-Met inhibition.

Key words: c-Met, cervical cancer, miR-23b-3p, proliferation, migration, invasion, HPV16.

Main text

Introduction

Cervical cancer (CC) is the fourth most common malignancy among women around the world. Annually, an approximate of 560,505 new cases of CC are diagnosed and 284,923 deaths are due to this malignancy[1]. In Mexico, CC is the second cause of death by cancer in women. In 2014, 13,960 new cases of CC were registered[2], with an incidence rate of 19.2-23.3 cases /100,000 women. Failure in the early detection of CC is the main cause of the high prevalence of this malignancy and increased risk of metastasis in cases diagnosed in advanced stage. On the other hand, the low efficacy of treatment is related to increase in recurrence risk and low survival rate. One third of CC patients are diagnosed in advanced stages and survival rate at 5 years of the patients diagnosed with metastatic CC is 16.5%, while that patients diagnosed with *in situ* carcinoma is 91.5%[3].

Metastasis of CC cells results in local and distal extension of tumor, and in lymphatic and hematogenous dissemination[4]. The invading CC cells spread to nearby organs such as the bladder, peritoneum and rectum; while distal metastasis affects liver, lung and bone[5]. From the CC cases reported annually, 371,000 correspond to invasive CC[6], this scene determines the need to search markers of progression and prognosis and the design of new and more effective therapeutic strategies.

In addition to the close etiological relationship between the persistent infections by high-risk human papillomaviruses (HR-HPVs) and CC[7], other molecular mechanisms, such as genetic and epigenetic abnormalities, contribute to cervical carcinogenesis and to its malignant progression[8-9]. The aberrant expression of microRNAs (miRNAs) is an epigenetic abnormality associated with CC[9]. miRNAs regulate gene expression at the posttranscriptional level and they participate in the regulation of various cellular processes[10]. miRNAs are non-coding RNAs of 19-25 nucleotides that induce mRNA degradation and/or translation repression of protein, trough their total or partial binding to complementary sequences located in the 3'-UTR region of a target transcript[10]. miRNAs expression profile is altered in cancer, for this reason are an attractive objective to investigate the modulation of gene expression and response to drugs in different cancer types.

Malignant transformation and cancer progression are associated with altered expression of multiple miRNAs, but whether these alterations are a cause or a consequence of malignant transformation is unclear. Based on their expression levels and the biological function they regulate, miRNAs are classified into oncomiRs and tumor suppressor miRNAs. OncomiRs are characterized by their increased expression in tumours and by regulating tumor suppressor genes; on the contrary,

tumor suppressor miRNAs have diminished expression in cancer cells and generally regulate oncogenes[11]. Due to the diversity of their target genes and the biological processes they regulate, miRNAs have been proposed as potential biomarkers of early diagnosis, progression and prognosis of malignant tumors, and they also represent attractive targets for gene therapy.

miRNAs modulate processes such as proliferation, metabolism, differentiation, apoptosis and, in general, all the hallmarks of cancer[10,12,13]. The decrease of miR-23b-3p is associated with several cancer types[14,15,16], including CC[17], but there are few target genes and biological processes studied. Recently, some targets of miR-23b-3p have been identified by different research groups. Functional analyzes suggest that miR-23b-3p represses cell growth, migration, invasion and tumor angiogenesis, through the regulation of oncogenes FZD70, MAP3K1, PAK2, TGFβR2, RRAS2 and uPA in colon cancer[15]. In ovarian cancer, miR-23b-3p inhibits proliferation and carcinogenesis through RUNX2[16]. These and other results suggest that miR-23b-3p is a tumor suppressor miRNA with important functions in prior stages to cancer metastasis[15,16,18]. Previously, in our research group we found that miR-23b-3p expression level is decreased in CC tumours and CC derived cell lines, due to changes in the methylation of its promoter region[17] and, on the other hand, miR-23b-3p expression increases when p53 expression is restored due to the silencing of E6 of HPV16[19]. These evidences strengthen the hypothesis that miR-23b-3p acts as a tumor suppressor in CC. In bladder cancer[18] and oral squamous cell carcinoma[20], miR-23b-3p expression is diminished and its ectopic expression represses c-Met expression, a tyrosine-kinase receptor, and significantly decreases migration and cell invasion. c-Met repression by miR-23b-3p promotes the apoptosis of SiHa cells from CC[21].

The hepatocyte growth factor receptor, also known as c-Met, is a transmembrane protein tyrosine-kinase involved in the regulation of embryonic development, tissue repair and normal cells regeneration. Expression and increased activation of c-Met in cancer favors proliferation, survival, mobility and cell invasion[22], and is recognized as an oncogene in different cancer types[18,20,23,24], including CC[25]. Although the c-Met interactions with other tyrosine kinase receptors are widely studied, the biological and molecular events involved in miR-23b-3p/c-Met axis in CC are still unknown.

In the present study, C33A and CaSki cell lines derived from CC were used to investigate whether miR-23b-3p directly represses c-Met expression, and if this repression modifies proliferation, migration and invasion of cells. We also evaluated Gab1 and Fak activation in response to the ectopic expression of miR-23b-3p. Finally, we investigated the expression level of mRNA and protein of c-Met in CC tissues with HPV16. Together our results demonstrate that c-Met is a target gene of miR-23b-3p and that ectopic overexpression of this miRNA significantly decreases proliferation, migration and invasion of C33A and CaSki cells. In CaSki cells, we

found significant reduction in Gab1 and Fak activation in response to overexpression of miR-23b-3p. In addition, our findings demonstrate heterogeneous expression of c-Met between CC tissues with HPV16.

Methods

Patients samples

A total of 13 RNA and 6 protein samples from tissues of patients cytopathologicaly and histopathologicaly diagnosed as squamous intraepithelial lesions (non-SIL) or CC with infection by HPV16 were studied. Of these samples, 10 RNA and 6 protein samples were of CC with HPV16, and 3 RNAs samples were of non-SIL. The samples were obtained from Clinical Research Laboratory of the Autonomous University of Guerrero (Chilpancingo, Guerrero, Mexico). At the time of tissue uptake, all patients signed an informed consent to participate in the study.

Cell lines and culture conditions

The CC cell lines CaSKi (HPV16) and C33A (non-transformed with HPV) as well as HaCaT cell (human epidermal primary keratinocytes); were obtained from the American Type Culture Collection (ATCC). CC cells were cultured in DMEM medium (Invitrogen, Carlsbad, CA), while HaCaT cells were cultured in DMEM F12 medium (Invitrogen, Carlsbad, CA), both supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin/100 µg/mL streptomycin, 2 mM L-glutamine, 250 ng/mL fungizone, and maintained at 37°C in a humidified atmosphere containing 5% CO_2.

miRNA expression plasmids for hsa-miR-23b-3p

miR-23b-3p overexpression was induced using the microRNAs expression vector pSilencer 1.0-U6 (Applied Biosystems, Foster, CA) which contains the U6 RNA Pol-III promoter to generate small RNA transcripts, to obtain the pMIR23B-3p plasmid. The pMIR23B-3p plasmid was generated by cloning a DNA insert that encodes miR-23b-3p [miRBase: MIMAT0000418] into pSilencer 1.0-U6. The sequence of the primers used for the generation of DNA insert are shown in table 1. The specificity of the oligonucleotides was confirmed by comparison with other sequences in GenBank™ using Nucleotide BLAST and the NEBcutter 2.0 software (New England Bio Labs Inc.) to rule out the presence of additional sites for enzyme restriction. The DNA insert was generated using an alignment buffer for oligonucleotides (30 mM HEPES pH 7.4, 100 mM potassium acetate, 2 mM magnesium acetate and 4% DMSO). The alignment reaction was incubated at 95°C for 5 min and then 37°C for 1 h. The DNA insert was cloned into the restriction sites for *Apa I* and *Eco RI* of pSilencer 1.0-U6 plasmid and was isolated with PureYield plasmid midiprep system (Promega, Madison, WI). The integrity of the pMIR23B-3p plasmid was verified by

DNA sequencing in Genetic Analyser 3500xl equipment (Applied Biosystems, Foster, CA).

Table 1. Oligonucleotides used in this study

Assay type	Oligonucleotide names and sequences	
RT-qPCR	c-Met-F (sense): 5'-TAT TTC CCA GAT CAT CCA TTG CA-3’ c-Met-R (antisense): 5'-AAT GTA GGA CTG GTC CGT CAA AA-3’.	
	GAPDH-F (sense): 5'-GGT GAA GGT CGG TGT GAA CG-3’ GAPDH-R (antisense): 5'-CTC GCT CCT GGA AGA TGG TG-3'.	
Generation of pMIR23B-3p plasmid	MIR23-3PF (sense): 5'-ATC ACA TTG CCA GGG ATT ACC TTT TTT-3' MIR23-3PR (antisense): 5’-AAT TAA AAA AGG TAA TCC CTG GCA ATG TGA TGG CC-3’	
Generation of reporter plasmids	pMRE23cMETLuc1 plasmid	pMRE23cMET557F (sense): 5'-CTA GAC TAG TCC AGG GCT GTA GTG CAG TGG TGAT CAT AGA AGC TTG TG-3' pMRE23cMET577R (antisense): 5'-CAC AAG CTT CTA TGA TCA CAC CAC TGC ACT ACA GCC CTG GAC TAG TCT AG-3'.
	pMRE23cMETLuc2 plasmid	pMRE23cMET1004F (sense): 5'-CTA GAC TAG TGA TGC TAC TCT GAT CTA ATG AAT GTG AAC ATG TAA GCT TGT G-3’. pMRE23cMET1027R (antisense): 5'-CAC AAG CTT ACA TGT TCA CAT TCA TTA GAT CAG AGT AGC ATC ACT AGT CTA G-3'.
	pMRE23cMETLuc3 plasmid	pMRE23cMET2043F (sense): 5'-CTA GAC TAG TTT GTA TAT ACA TTC TTG AGA ACA CTG CAA TGT GAA AAT CAA AGC TTG TG-3'. pMRE23cMET2072R (antisense): 5'-CAC AAG CTT TGA TTT TCA CAT TGC AGT GTT CTC AAG AAT GTA TAT ACA AAC TAG TCT AG-3'.

Transfection assays with miRNA expression plasmids

The cells were transiently transfected with pMIR23B-3p plasmid to over-express miR-23b or pSilencer 1.0-U6 plasmid (without DNA insert), using FuGENE HD transfection reagent (Promega, Madison, WI) according to the manufacturer’s instructions. Briefly, 48 h before the transfection assay, the cells were plated at a density of $4X10^4$ cells/well in a 24-well plate that contained 500 µL of DMEM with 10% FBS and penicillin/streptomycin. At the time of transfection, the plasmids and FuGENE reagent were diluted in FBS free DMEM and incubated for 15 min at room temperature. All assays were carried out with 5 µg of each plasmid. Cells were incubated with plasmid/FuGENE complexes for 4-6 h, rinsed and medium was replaced by DMEM with 10% FBS. After 48 h of transfection, the cells were

harvested and RNA isolation was carried out for quantitative real-time RT-PCR assays. Cellular protein isolation was performed for Western blot assays. Transfection assays were repeated at least three times independently.

RT-qPCR analysis for miR-23b-3p and c-Met

Isolation of total RNA from transfected and non-transfected (N/T) cells, was carried out with TriPure isolation reagent (Sigma-Aldrich, EE. UU). For analysis of miR-23b-3p expression, real time RT-qPCR analysis were performed using TaqMan microRNA assays (AUC ACA UUG CCA GGG AUU ACC; Assay ID: 000400; Applied Biosystems, Foster City, CA) following the manufacturer's instructions. The expression of miR-92a (UAU UGC AUU GUC CCG GCC UGU; Assay ID: 000431; Applied Biosystems, Foster City, CA) was used as an internal reference for the expression of miR-23b-3p. The Ct values were analyzed to determine the significant differences of miR-23b-3p expression in cells transfected with pMIR23B-3p plasmid or N/T cells. Relative expression was calculated using the $2^{-\Delta Ct}$ method. All RT-qPCR were performed in triplicate.

Expression of c-Met mRNA was determined by RT-qPCR before and after of transfection cells. The measurement was done in a BioRad C1000/CFX96 real-time system, with the One-Step SYBR® PrimerScripTM RT-PCT kit II (Takara, Clontech, Japan) according to the manufacturer's instructions. In each RT-qPCR reaction, 200 ng of total RNA and specific oligonucleotides to c-Met were used. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene expression was used as endogenous control. Primer sequences are described in table 1. The amplification program consisted of an initial step of cDNA synthesis at 42°C for 5 min; followed by an inactivation step of the RT at 95°C for 5 min; and 40 cycles of 95°C for 3 s, 60°C for 30 s, and 72°C for 30 s and finally a dissociation step at 72°C for 2 min. Changes in c-Met expression were calculated using the Ct comparative method. Each assay was repeated three times independently.

Western blot assays

Forty eight hours after transfection assays, cells were harvested and proteins were isolated for Western blot assays using TriPure isolation reagent (Sigma-Aldrich, EE. UU). The proteins of organic phase of TriPure were precipited with acetone overnight and were centrifuged at 12,000 rpm at 4°C for 10 min. The precipitate was washed three times with guanidine hydrochloride [0.3 M] in 95% ethanol, homogenized, centrifuged at 9000 rpm at 4°C for 7 min and incubated 20 min at room temperature. The proteins were finally washed with absolute etanol and dried in a fume hood. Proteins were diluted in 4% SDS. Total proteins were quantified using the Pierce® BCA protein assays kit (Pierce, Rockford, IL). Thirty micrograms of total proteins were separated by 10% SDS-PAGE and transferred to 0.45 µm nitrocellulose membrane in a wet system. Biotinilated and pre-stained molecular

weight marker was included. Blots were blocked with 5% non-fat milk in Tris-buffered saline that contained Tween 20 (TBST) for 1-2 h at room temperature, washed three times in TBST and incubated at 4°C overnight with the primary antibody corresponding (table 2); GAPDH was used as charge control. Membranes were incubated with HRP-coupled secondary antibodies (table 2). The proteins detection was made by enhanced chemiluminescence using the detection system Clarity™ Western ECL substrate (170-5060, Bio-Rad, Germany) in a ChemiDoc™ MP Imaging System (Bio-Rad, Germany). Relative protein expression was analyzed and represented as problem protein/GAPDH density ratio. Each assay was carried out three separate times.

<table>
<tr><th colspan="5">Table 2. Antibodies used in the study</th></tr>
<tr><th rowspan="2">Primary antibodies</th><th rowspan="2">Catalog number / distributor</th><th rowspan="2">dilution</th><th colspan="2">Secondary antibodies
(catalog number / distributor)</th></tr>
<tr><th>Anti-rabbit IgG-HRP
7075P2 / CST, EE. UU.</th><th>Anti-mouse IgG-HRP
115-035-003 / JIR, EE. UU.</th></tr>
<tr><td>Anti-c-Met</td><td>D1C2 / CST EE. UU.</td><td>1:1000</td><td>1:3000</td><td rowspan="5">---</td></tr>
<tr><td>Anti-Gab1</td><td>3232 / CST, EE. UU.</td><td>1:1000</td><td rowspan="4">1:5000</td></tr>
<tr><td>Anti-phospho Gab1(Tyr-307)</td><td>3234 / CST, EE. UU.</td><td>1:3000</td></tr>
<tr><td>Anti-Fak</td><td>D2R2E-13009 / CST, EE. UU.</td><td>1:3000</td></tr>
<tr><td>Anti-phospho Fak (Tyr-397)</td><td>D20B1-8556 / CST, EE. UU.</td><td>1:1000</td></tr>
<tr><td>Anti-GAPDH</td><td>(6C5)-sc-32233 / SCBT, EE. UU.</td><td>1:1000</td><td>---</td><td>1:20,000</td></tr>
</table>

CST: Cell Signaling Technology; SCBT: Santa Cruz Biotechnology; JIR: Jackson ImmunoResearch

Reporter plasmids and luciferase activity assays

Cells were transiently transfected with the reporter plasmids pMRE23cMETLuc1, pMRE23cMETLuc2 and pMRE23cMETLuc3 that contained the cloned sequences MRE23-1 (6mer), MRE23-2 (8mer) and MRE23-4 (8mer) (MRE=MicroRNA Response Elements) for miR-23b-3p, located at positions 4730 to 4750, 5177 to 5200 and 6216 to 6245 of c-Met 3'-UTR region, respectively. The *in silico* analysis of MRE23 sites was supported by reference nucleotide sequences database for the human c-Met gene (NCBI: NM_000245.3) and for hsa-miR-23b-3p (NCBI: NR_029664.1 and miRBase: MIMAT0000418). The prediction of the MRE23 sites was performed with the bioinformatic platforms TargetScan (http://www.targetscan.org/vert_71/); miRTarBase

(http://mirtarbase.mbc.nctu.edu.tw/php/index.php); microRNA.org (http://34.236.212.39/microrna/home.do); miRDB (http://mirdb.org/); RNA22 (https://cm.jefferson.edu/rna22/); and PicTar (https://pictar.mdc-berlin.de/). Each MRE23 site was included in a primer sequence, which was hybridized with a complementary primer to generate the DNA inserts, using an alignment buffer (30 mM HEPES pH 7.4, 100 mM potassium acetate and 2 mM magnesium acetate) and each reaction was incubated at 95°C for 10 min and then its temperature gradually decreased for 1 h. The sequence of the primers used to generation of all DNA inserts are described in table 1.

The DNA inserts were individually cloned into the *Spe I* and *Hind III* restriction sites of the reporter vector pMIR-Report-Luciferase (Life Technologies, EE. UU.), which contains firefly luciferase reporter gene downstream of the CMV promoter/termination system. All plasmids were purified with the PureYield plasmid midiprep System kit (Promega, Madison, EE. UU.) and the integrity was verified by DNA sequencing.

Co-transfection assays were carried out with pMIR23B-3p plasmid to induce miR-23b-3p expression. All transfection and co-transfection assays were performed with 1 µg of each plasmid, using the FuGENE HD transfection reagent agent (Promega, Madison, WI). The vector without DNA insert was used to normalize the transfection data. A total of $4X10^4$ cells/well were plated in 24-well plates and incubated at 37°C for 24 h before the transfection. The cells were transfected with the plasmids above mentioned and incubated for 4-6 h deprived FBS. Then, the medium was replaced by supplemented medium, and the cultures were incubed in the same conditions for 48 h. The cells were washed twice with 1X PBS pH 7.4 and were harvested and lysed with a lysis buffer (20 mM Tris-HCl pH 7.4, 10 mM NaCl, 10 mM KCl, 3 mM $MgCl_2$, 0.5% Triton X-100, 0.5% Nonidet P40). Protein extracts were collected by centrifugation and 50 µg of total proteins were used to determine luciferase activity with the Dual-Glo luciferase assay kit (Promega, Madison, WI) on a Glomax multi-detection system (Promega, Madison, WI, USA). Luminescence was calculated and normalized with respect to the pMIR-Report-Luciferase vector without DNA insert. All determinations were repeated in three independent times.

Cell proliferation assays

Cell viability was measured using the CellTiter 96 AQueous non-radioactive cell proliferation assay components (MTS) kit (Promega, Madison WI). Briefly, a total of $0.5X10^4$ cells/well (transfected previously with pMIR23B-3p plasmid or pSilencer 1.0-U6 plasmid without DNA insert and N/T cells) were plated in 96-well plates and incubated at 37°C. After incubating the cells for 24 h, 20 µL of MTS reagent and 100 µL of fresh DMEM with 5% FBS were added into each well (with transfected cells or N/T cells) avoiding exposure the plate to any light source, then the plates were incubated at 37°C for 4 h. MTS tetrazolium compound salt reagent is bioreduced by living cells into a colored formazan product wich is

soluble in culture medium. After incubation, the absorbance values were measured at 490 nm in the Glomax multi-detection equipment (Promega, Madison, WI). The colorimetric reactions were measured each 24 h in the same way until 96 h after transfection. Cellular proliferation rate was calculated as the percentage of MTS adsorption as follows: % survival = (mean experimental absorbance / mean control absorbance) X 100. N/T cells were used as a negative control. Each assay was carried out three separate times.

Cell migration assays

Cell migration capacity was assessed by wound healing assays. Cells were seeded in 6-well plates until reaching 100% confluency and then were transfected with 5 µg of the pMIR23B-3p plasmid or 5 µg the pSilencer 1.0-U6 plasmid without DNA insert. A scratch wound was created in confluent monolayer with a pipette tip of 200 µL. Cultures were washed twice with 1X PBS to remove detached and damaged cells and then incubated at 37 °C with FBS free medium for 48 h. The cells that migrated to the wound center were documented by microphotographs at 0, 24 and 48 h after the wound scratch was created. The assay was carried out in three independent replicates.

Cell invasion assays

For invasion assay, $1X10^5$ cells were transfected with 5 µg of pMR23B-3p plasmid or pSilencer 1.0-U6 plasmid without DNA insert, and 72 h post transfection cells were loaded on transwell 8.0 µm polycarbonate membrane inserts coated with matrigel. Cells were seeded in the upper side of the inserts in 24 wells plates with FBS free DMEM; DMEM 20% FBS was added in the lower chamber, then the plates were incubated at 37°C in a 5% CO_2 incubator. After 24 h, remnant cells were removed from upper side of the insert and cells that invaded through the membrane were fixed with cold paraformaldehyde at 4% for 10 min and stained with 0.1% crystal violet. The invasion was documented by microphotographs, and the cells that invaded were counted in 6 fields/insert. The assay was performed by triplicate.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.0 software (Inc, San Diego, CA). Histograms represent the mean values, and bars indicate standard errors of the mean. Student's *t*-test (two-tailed) was used to determine statistical differences between groups. The values of non-parametric quantitative variables were calculated by the Mann-Whitney tests. The values of $p<0.05$ were considered significant. The authors declare the data availability statement.

Results

miR-23b-3p expression level is decreased in cervical cancer cells lines

We analyzed miR-23b-3p expression in CC cell lines compared with HaCaT cell (normal keratinocytes) by RT-qPCR. We found that C33A and CaSki cells express lower levels of miR-23b-3p compared to HaCaT cells (figure 1a). To overexpress miR-23b-3p in HaCaT and CC cells, we generated the pMIR23B-3p plasmid, and its efficiency was proven in the three cell lines. The transfection assays showed that pMIR23B-3p plasmid significantly increase miR-23b-3p expression in C33A and CaSki cells (figure 1b).

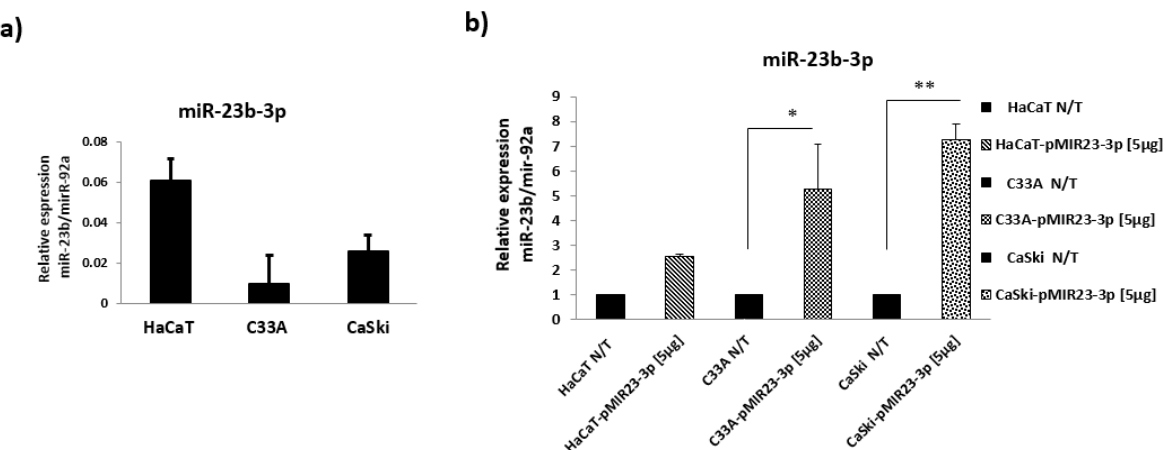


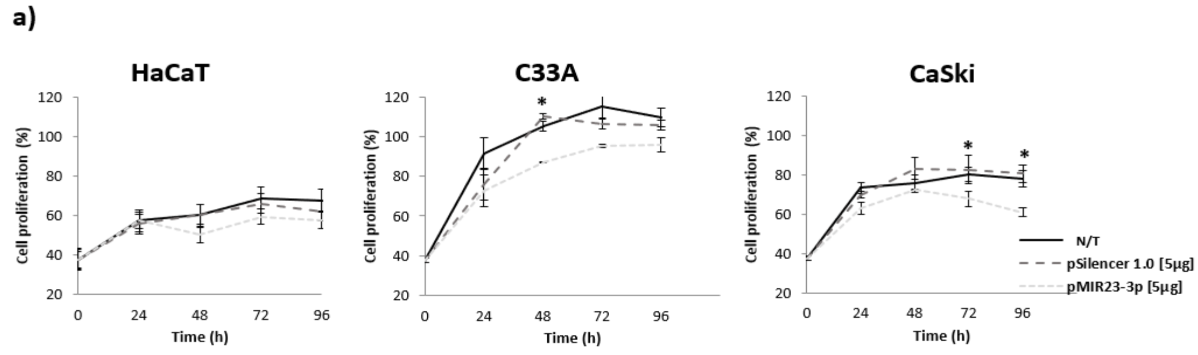
Figure 1. Overexpression of miR-23b-3p in CC cell lines. a) Quantification of miR-23b-3p expression levels in HaCaT, C33A and CaSki. The CC cells express lower miR-23b-3p level than HaCaT cell. **b)** pMIR23B-3p plasmid significantly increases the miR-23b-3p expression in CC cell lines. The expression level of miR-23b-3p was analyzed using the $2^{-\Delta Ct}$ method and normalized to miR-92a. To transfection assay were using 5 µg of pMIR23B-3p plasmid. N/T= Non-transfected. Data are means ± SEM * $p<0.05$, ** $p<0.01$.

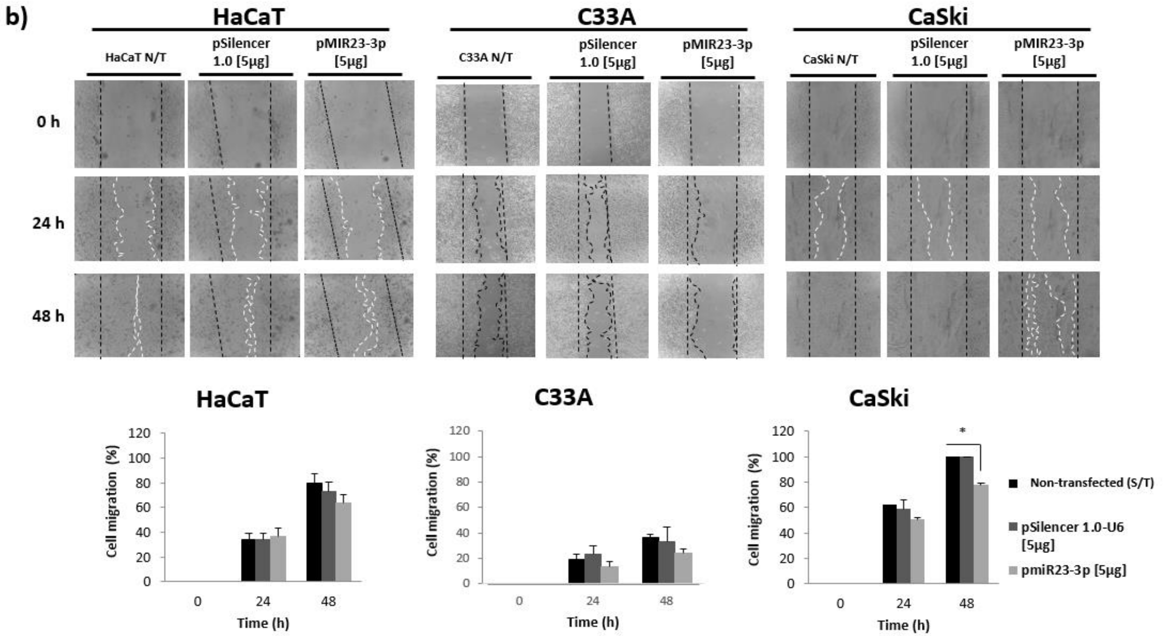
Effects of overexpression of miR-23b-3p on proliferation, migration and invasion of cervical cancer cells

To determine the effect of miR-23b-3p on the malignant behavior of CC cells, functional assays were performed. The MTS assay revealed that at 48 h post-transfection with pMIR23B-3p plasmid, C33A cell proliferation decreased 20%; while that CaSki cell proliferation declined in a 15% at 72 h after transfection with pMIR23B-3p plasmid and continued decreasing until 96 h (figure 2a).

Because cell mobility is key in cancer metastasis, we analyzed the effect of miR-23b-3p on the cell migration through wound healing assays. The results show that miR-23b-3p

overexpression, decreased significantly the CaSki cells migration 48 h after the transfection with the pMIR23B-3p plasmid (figure 2b). Therefore, transwell invasion assays were carried out to assess the miR-23b-3p role on cell invasion. Figure 2c shows that in transfected cells with pMIR23B-3p plasmid, the invasion rate is significantly lower compared with the controls. Together these data show that miR-23b-3p decreases proliferation, migration and invasion of CC cells.





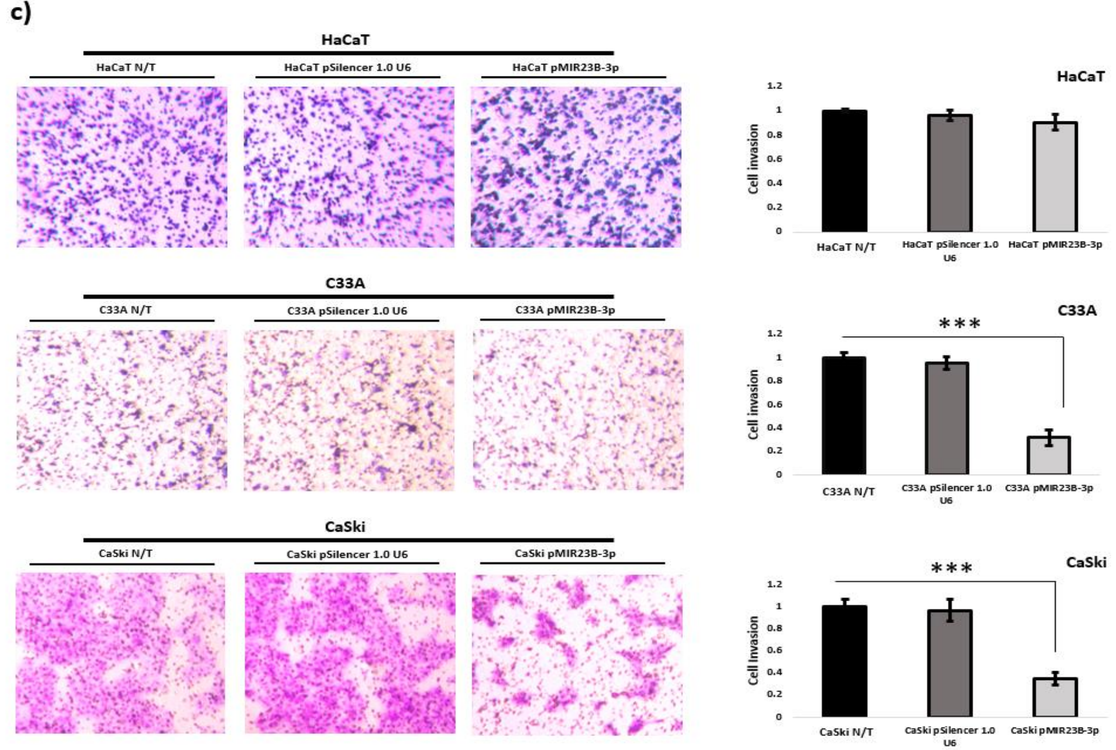


Figure 2. Effect of miR-23b-3p on proliferation, migration and invasion of C33A and CaSki cells. a) Proliferation of CC cell and HaCaT cells was evaluated by MTS assays. C33A and CaSki proliferation decrease at 48 and 72 h after the transfection with pMIR23B-3p plasmid, respectively. **b)** Cell migration was mesuared using a wound-healing assay. Wound images were captured at 0, 24 and 48 h after wounding. CaSki migration significantly decreases at 48 h post-transfection with pMIR23B-3p plasmid. **c)** Cell invasion was measured with transwell assays. Cells that invaded were stained with 0.1% crystal violet, imaged and counted under a microscope. The miR-23b-3p overexpression significantly decreases C33A and CaSki invasion. Five micrograms of pMIR23B-3p plasmid and pSilencer 1.0 U6 plasmid without DNA insert were using to each transfection assay. N/T= Non-transfected. Data are means ± SEM * p<0.05, *** p<0.001 versus respective negative control.

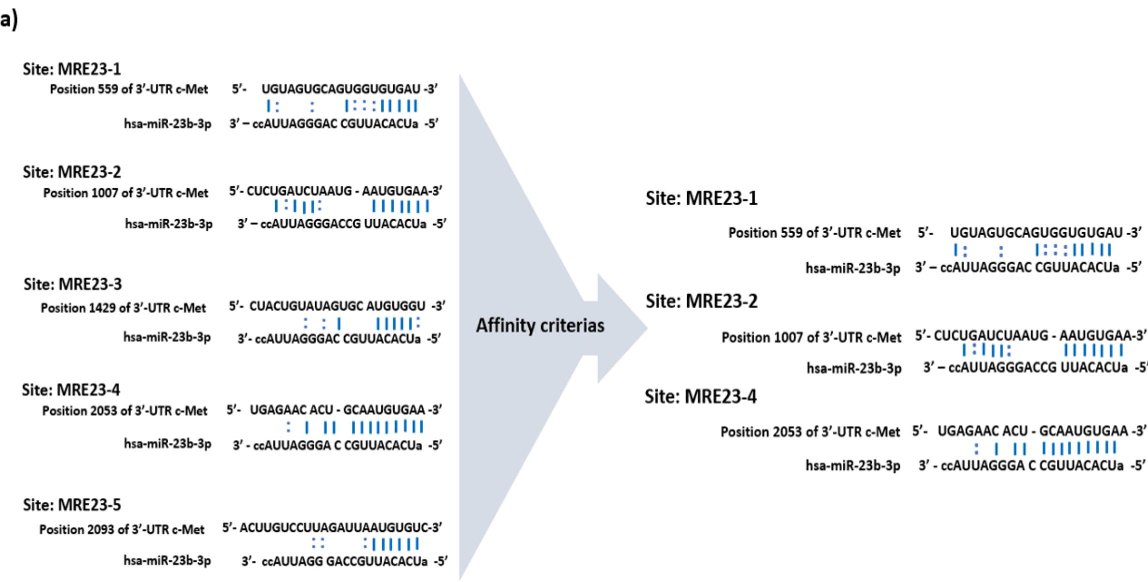
C-Met is a direct target of miR-23b-3p in cervical cancer cells

It has been shown that c-Met is a target of miR-23b-3p in CC derived SiHa cells and in other cancer types. To verify target sequences within of 3'-UTR region of c-Met an *in silico* analysis was carried out using the TargetScan, miRTarBase, microRNA.org, miRDB, RNA22 and PicTar algorithms. We found five sites of binding to miR-23b-3p inside the 3'-UTR region of c-Met; and the affinity criterias such as the score and hybridization type indicated that

the MRE23-1, MRE23-2 and MRE23-4, have the highest probability to hybridizing with miR-23b-3p (figure 3a).

The information available on the interaction between c-Met and miR-23b-3p suggests that the cellular microenvironment influences the probability of recognition and efficiency of its hybridization. To verify if the three MRE23 sites are recognized by miR-23b-3p in the intracellular context of HaCaT, C33A and CaSki, we generated the pMRE23cMETLuc1, pMRE23cMETLuc2 and pMRE23cMETLuc3 plasmids that containing the sequences MRE23-1, MRE23-2 and MRE23-4, and then we carried out reporter assays. For the luciferase reporter assays, the plasmids generated were transfected alone or they were co-transfected with the pMIR23B-3p plasmid in C33A, CaSki and HaCaT cells. The results show that miR-23b-3p hybridizes with MRE23-1, 2 and 4 sites, and significantly decrease the relative luciferase activity of the three reporter plasmids in C33A and CaSki cells (figure 3b). miR-23b-3p hybridizes with the three MRE23 sites but functional effect varies between MRE sites and between cell lines.

In adition, we evaluated the c-Met expression level in CC cell lines and HaCaT cells. Figure 3c shows that c-Met expression was higher in C33A.



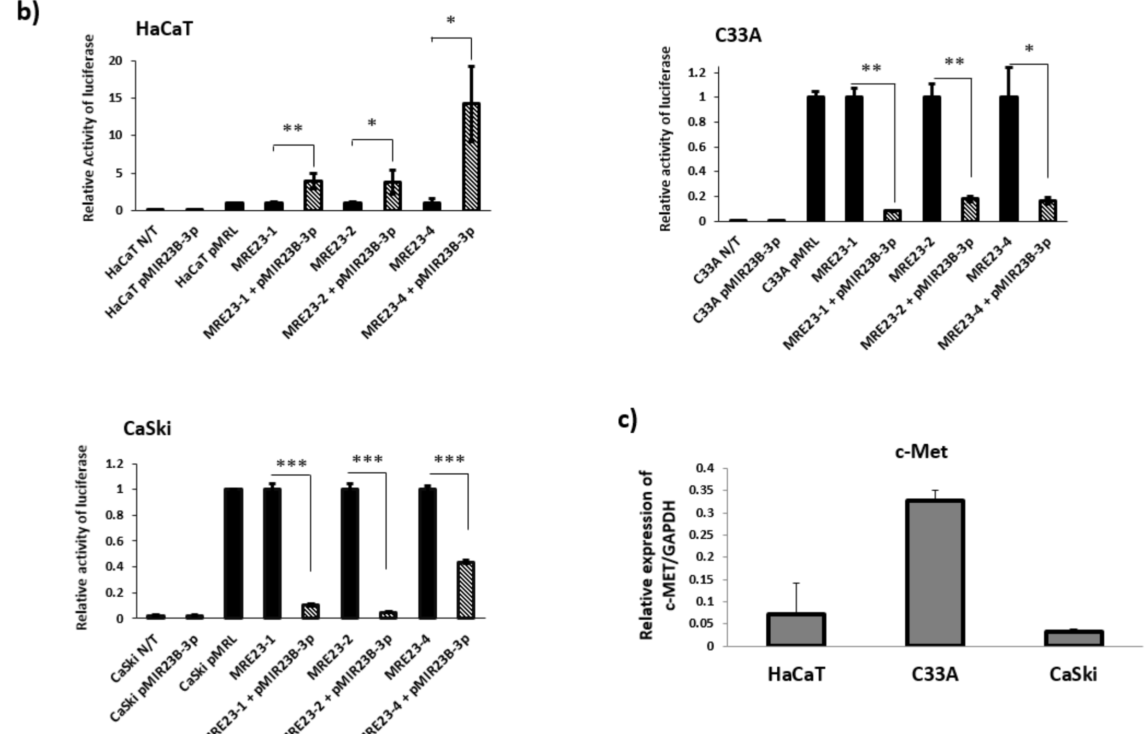


Figure 3. miR-23b-3p represses to c-Met through its interaction with three MRE sites within 3'-UTR region of c-Met in C33A and CaSki cells. a) MRE23 sites located in 3'-UTR region of c-MET with more probability of interaction with miR-23b-3p. **b)** Relative luciferase activity of C33A, CaSki and HaCaT cells that were co-transfected with reporter constructs and pMIR23B-3p plasmid. The binding miR-23b-3p to the MRE23-1, MRE23-2 and MRE23-4 sites located in 3'-UTR of c-Met, inhibits luciferase activity. Relative luciferase activity was detected at 48 h after transfection. *$p<0.05$, **$p<0.01$, ***$p<0.001$ versus simple transfection. N/T=Non-transfected; pMRL=pMiR-Report-Luciferase without DNA insert. **c)** c-Met expression was determined by RT-qPCR. The expression level of c-Met was normalized with GAPDH and analyzed using the ΔCt method. Data are means ± SEM.

On the other hand, through of RT-qPCR and Western blot assays, we found that the over-expression of miR-23b-3p significantly decreases the mRNA and protein levels of c-Met in C33A and CaSki cells (figure 4).

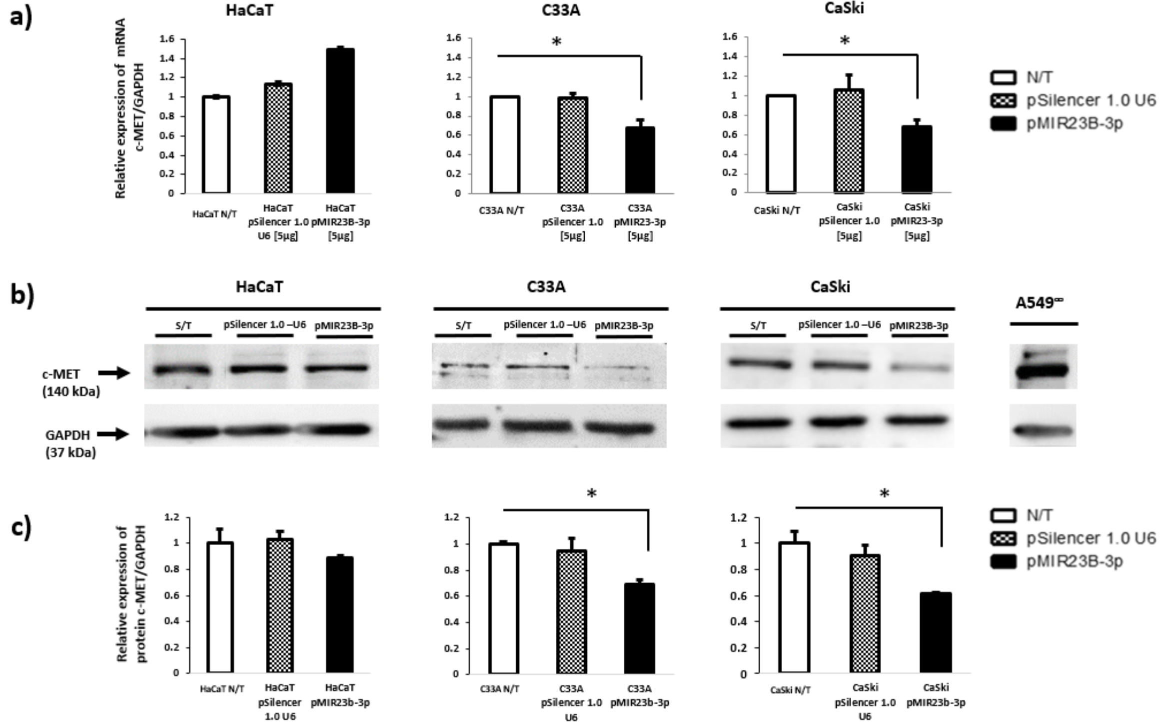
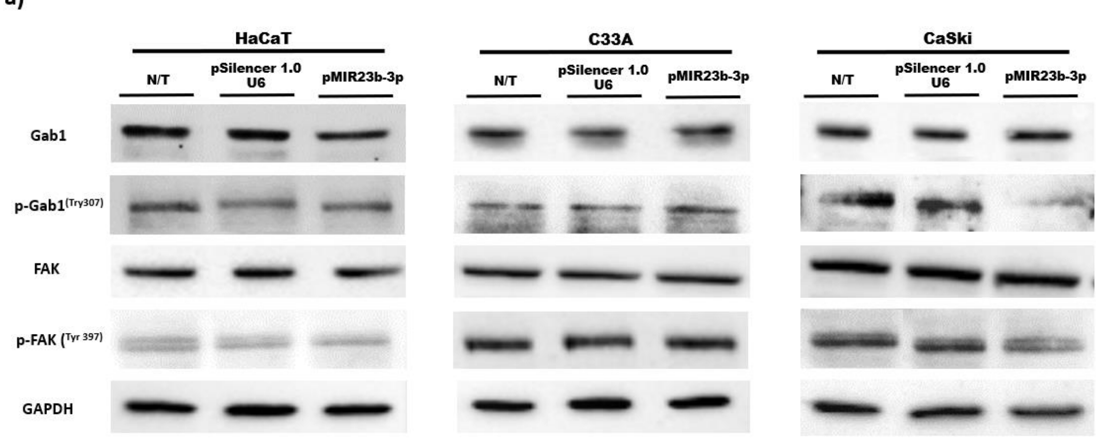


Figure 4. Effect of miR-23b-3p overexpression on c-Met expression in C33A and CaSki cells. a) miR-23b-3p significantly decreases c-Met mRNA expression. c-Met expression was determined by RT-qPCR. The expression level of c-Met was analyzed by the $2^{-\Delta Ct}$ method; **b)** miR-23b-3p significantly decreases c-Met protein expression. The mRNA and protein levels were normalized with GAPDH expression. ∞A549 cell line was used as positive control of c-Met expression. **c)** Densitometric analysis of c-Met expression. N/T= non-transfected. *p<0.05. Data are means ± SEM.

To determine if miR-23b-3p effect on proliferation, migration and invasion of CC cells is related to the activation of proteins downstream of c-Met, we evaluated the activation of the adapter protein Gab1 and the Fak kinase, who participate in pathways stimulated by c-Met. Only CaSki cells showed a significant decrease in Gab1 and Fak activation (figure 5).



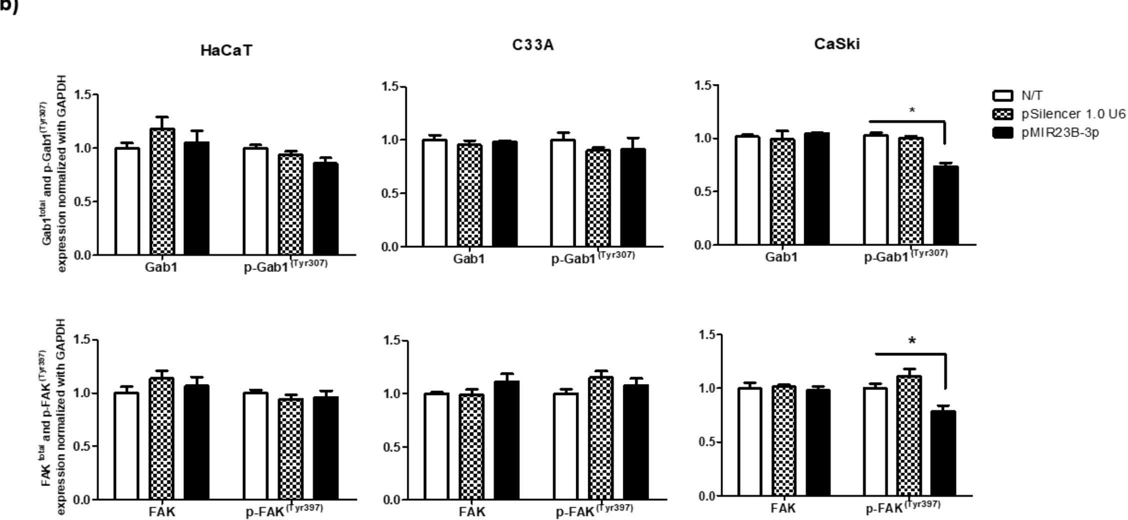


Figure 5. Effect of miR-23b-3p on the activation of downstream molecules of c-Met. a) miR-23b-3p significantly decreases Gab1 and Fak activation in CaSki cells. The activation was determined by detection of p-Gab1 (Tyr307) and p-Fak (Tyr397) proteins. **b)** Densitometric analysis of the activation of Gab1 and Fak. The protein expression was normalized with the GAPDH charge control. N/T=non-transfected cells. *p<0.05. The data are expressed as means ± SEM.

c-Met is overexpressed in cervical cancer tissues

With the purpose of to know the c-Met expression levels in CC tissues in which low expression levels of miR-23b-3p were previously determined; the mRNA expression of c-Met was quantified by RT-qPCR assays in 10 CC samples, in 3 samples from non-SIL and in normal keratinocytes (HaCaT cells). c-Met expression was significantly higher in cancer

tissues than in HaCaT cells. No significant differences were found in the expression level of c-Met between cancer tissues and non-SIL samples (figure 6a).

To analyze c-Met protein levels in CC tissues, we evaluated c-Met protein levels in six samples from CC and we compared them with the c-Met protein levels from HaCaT cells. c-Met protein expression was higher in CC tissues than in HaCaT cells, however, these differences were not significant.

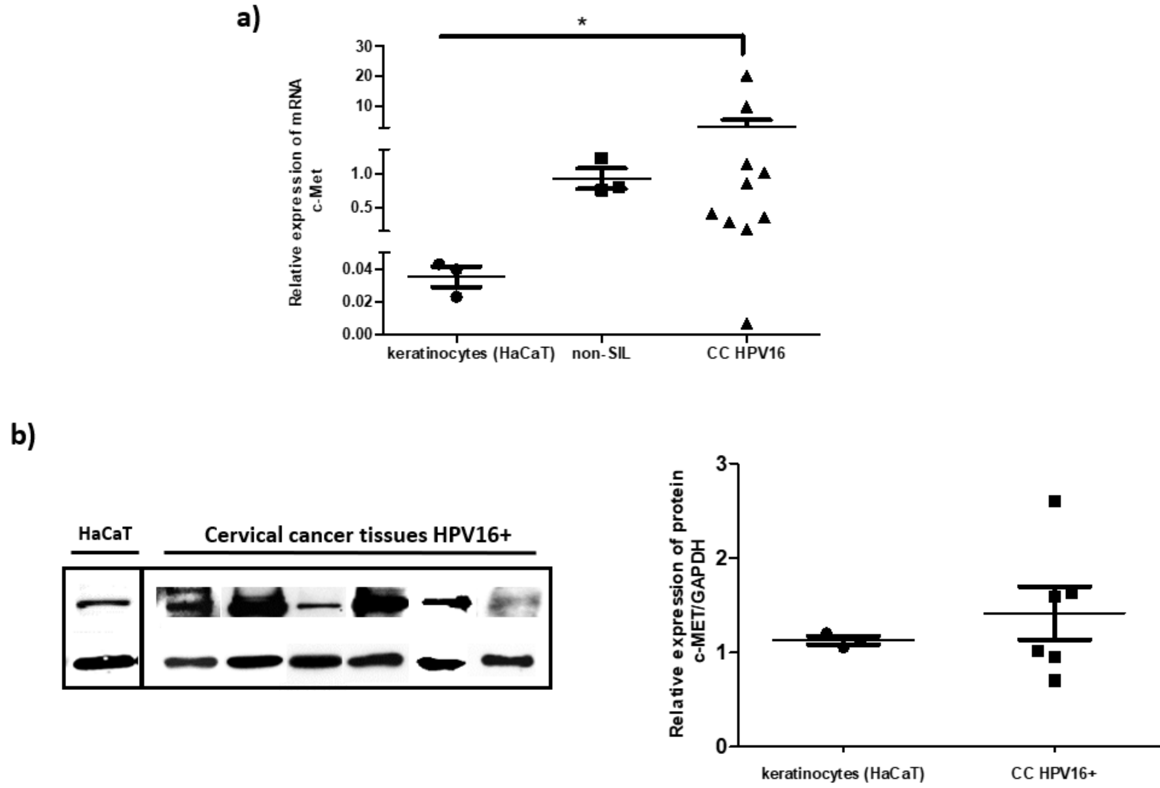


Figure 6. c-Met expression levels in CC tissues. a) qRT-PCR assays were carried out to quantification of c-Met in keratinocytes (HaCaT cells), non-SIL and CC tissues. In CC tissues, c-Met mRNA expression is higher than in non-SIL tissues and in HaCaT cells. The expression level of c-Met was normalized with GAPDH and analized using the ΔCt method. Data are means ± standard deviation; *p<0.05. The data analysis was made using Mann Whitney test. **b)** c-Met protein expression was higher in CC tissues than HaCaT cells. The c-Met expression was normalized with GAPDH expression. Data are means ± standard deviation.

Together, these results indicate that in CC, miR-23b-3p participate in the modulation of cell proliferation, migration and invasion through of regulation of c-Met receptor. On the other hand, they support the role of miR-23b-3p as a tumor suppressor in CC.

Discussion

Previous results from our group and other authors indicate that methylation in the promoter region of miR-23b-3p is partially responsible of its decreased expression in CC and, based on this expression pattern, it has been proposed that miR-23b-3p is a tumor suppressor in CC[19,21]. The specific functions of miR-23b-3p in CC are still unknown. In this study we investigated the effect of miR-23b-3p overexpression on proliferation, migration and cell invasion processes and demonstrated that c-Met is target of miR-23b-3p in CC cell lines. Additionally, we verified if the miRNA overexpression is related to Gab1 and Fak activation.

The most significant findings were: 1) miR-23b-3p expression is decreased in CC cell lines; 2) miR-23b-3p overexpression significantly decreases the proliferation, migration and invasion of C33A and CaSki cells; 3) c-Met mRNA has five binding sites for miR-23b-3p in its 3'-UTR region; 4) c-Met is a direct target of miR-23b-3p in C33A and CaSki cells; 5) miR-23b-3p overexpression significantly decreases the expression of mRNA and protein of c-Met in C33A and CaSki; 6) in CaSki cells, Gab1 and Fak activation levels decrease in response to miR-23b-3p overexpression and 7) c-Met expression is heterogeneous among CC tissues.

In agreement with it reported by Lui *et al.,* (2007) and Li *et al.,* (2018), our results confirm that miR-23b-3p expression is decreased in C33A and CaSki cells but is lower in C33A. The diminished expression of miR-23b-3p in C33A and CaSki cells may be due, in part, to tumor cells characteristics and cancer type from which they are derived (carcinoma *in situ* or metastatic), to differential expression of transcription factors for miR-23b-3p and to multiple mechanisms that regulate gene expression in these cells.

In addition to the differences in histological type of cancer from which C33A and CaSki cells derive, these cells also differ in the HPV16 presence. In CC positive to HPV16, E6 and E7 oncoproteins modulate the expression of cellular miRNAs through the aberrant methylation of their promoters[28], proteins overexpression involved in the processing of miRNAs, such as DROSHA and DICER endonucleases[29]; and commitment of the normal function of transcription factors that modulate the transcription of miRNAs, including p53 and E2F[30]. p53 partially regulates miR-23b-3p expression by *cis* regulation[31].

The differences between miR-23b-3p expression levels in C33A and CaSki cells, may be due to 1) that DROSHA and DICER expression is higher in CaSki than in C33A cells[29] and, it is possible that expression levels highest of these endonucleases are related to greater processing and concentration of the precursor and mature miR-23b-3p molecules in CaSki cells; 2) that p53 modulates miR-23b-3p expression and C33A expresses a mutated form that lacks function as a transcriptional factor[32], contributing to diminished expression of miR-23b-3p in C33A; and 3) that the percentage of methylated copies of miR-23b-3p

promoter varies between both cell lines[17]. On the other hand, miR-23b-3p expression level in HaCaT cells, non-tumor epithelial cells, was higher than in CaSki and C33A. In this way, its strengthened the hypothesis that this miRNA is a tumor suppressor in CC[17,21], as in colon cancer[15], in oral squamous cell carcinoma[20] and in bladder cancer[18,33].

miR-23b-3p regulates biological processes characteristic of CC. The proliferation of C33A and CaSki cells significantly decreased after 48 and 72 h of miR-23b-3p overexpression. These data are similar to those reported by Au-Yeung *et al.,* (2017), who found that miR-23b-3p restoration is related to induction of apoptosis of SiHa cells after 48 h[21]. The decrease in the proliferation of C33A and CaSki may be due to the fact that c-Met, target of miR-23b-3p, regulates survival, apoptosis and cell proliferation, through the activation of Akt, Fas and Ras[22]. miR-23b-3p participates in the regulation of tumor cell mobility. In HeLa cells, the miR-23b/MAP1K axis modulates growth and cell migration[27]. In colon cancer, miR-23b-3p overexpression is related to decreased expression of vimentin, a mesenchymal marker, and to increase of E-cadherin in the cell membrane. These events contribute to the generation of a stable epithelial phenotype and decrease migration and cell invasion, characteristic functions of metastatic cells[15]. In gastric cancer miR-23b-3p inhibits the expression of molecules of the Wnt/β-catenin pathway involved in cell motility[34]. We found that in CaSki cells, miR-23b-3p overexpression significantly decreases migration and cell invasion, whereas in C33A cells only significant changes in invasion were observed. CC can have an invasive behavior and spread to lymph nodes and, for tumor cells to carry out these processes, changes in the expression of molecules such as vimentin and E-cadherin, markers of epithelial-mesenchymal transition (EMT), which is related to invasion and metastasis, are required[35]. EMT, mobility, migration and cell invasion are regulated by signals induced by c-Met, which activate proteins such as Fak[22]. Thus, miR-23b-3p overexpression induces a decrease in c-Met expression and in signals emitted by this receptor, which partially explains the decrease in the migration and invasion of C33A and CaSki cells. It is also likely that the results of proliferation, migration and invasion observed in this study are product of synergistic function of other targets of miR-23b-3p, such as the PLAU transcript, whose protein participates in regulation of these processes in SiHa cells[19]. miR-23b regulates migration and EMT in breast cancer throug ANXA2 and PAK2[36] that are overexpressed in CC[37-38], and that can be recognized by miR-23b-3p.

To invade the extracellular matrix (ECM) and surrounding tissues, tumor cells can develop the pattern of collective or individual invasion. Cancer cells can migrate following the mesenchymal phenotype, in which cells move slowly; or the amoeboid phenotype, that is more efficient[35]. Our observations suggest that C33A could present patterns of individual invasion and mesenchymal migration, characteristics that explain the low number of cells that migrate to center of stria in the migration assays and the lower number of invading

cells in the transwell assays. On the other hand, the pattern of invasion observed in CaSki could be due to the collective type with amoeboid migration, since that these cells show greater mobility in groupings. Our results suggest that miR-23b-3p modulates different migration and invasion types in CC. The characterization of the invasive and migratory patterns of C33A and CaSki require more detailed studies and the determination of specific markers and mechanisms.

miR-23b-3p is a tumor suppressor that participates in the regulation of proliferation, migration and cell invasion, prior processes to metastasis of CC. One of the pathways that regulates such processes is directed by the receptor tisosin-kinase, c-Met. The altered expression and function of c-Met is a common event in solid tumors[39]. Recently, Au-Yeung *et al*., (2017) described to c-Met as a miR-23b-3p target gene in SiHa cells, these investigators studied a binding site (MRE site) for miR- 23b-3p by means of assays with reporter plasmids[21].

Through *in silico* analysis, we found that the 3'-UTR region of c-Met contains 5 potential MRE sites for miR-23b-3p; one MRE site more than previously reported them[40]. The bioinformatic prediction algorithms are generated based on experimental models that include a cell type but the results are proposed for different cell types[41-42], without considering the variations in each transcriptional repertoire. According to Nam *et al.,* (2014), the study of isoforms of 3'-UTR regions using bioinformatic algorithms significantly improves the prediction of MRE sites between cell types[43]. Salvi *et al.,* (2009) used two bioinformatic platforms for analysis of MRE sites in the 3'-UTR region of c-Met[40], and we accessed to six platforms for the same analysis.

It is likely that the prediction of the fifth MRE site in c-Met is product of constant updating of databases and the number of bioinformatic algorithms used for the search. Three of the five MRE sites of c-Met with highest affinity and stability of the c-Met:miR-23b-3p complex *in silico* were evaluated by luciferase reporter assays. MRE sites studied were of type 6mer (MRE23-1) and 8mer (MRE23-2 and 4). The MRE23-1 site was evaluated by Au-Yeung *et al.,* (2017) in SiHa cells[21], and the three MRE23 sites that we selected were evaluated by Salvi *et al.,* (2009) in SKHep1C3 cells. In this latter study, the hybridization of c-Met:miR-23b-3p reduced the luciferase activity by 26% for the MRE23-1 site, in 20% for MRE23-2 and in 10% for MRE23-4[40]. In contrast, we found that the luciferase activity is reduced between 50 and 95% in the three MRE23 sites evaluated in C33A and CaSki cells transfected with pMIR23B-3p plasmid. It is likely that the cellular context is determining the recognition, the stability of the hybridization and c-Met repression by miR-23b-3p. The decrease in luciferase activity in C33A and CaSki cells confirms that c-Met is a direct target of miR-23b-3p in these cells. It was verified that the three MRE23 sequences of c-Met evaluated, interact with miR-23b-3p in C33A and CaSki cells. However, the reduction in luciferase activity may result from the

interaction of other endogenous miRNAs that recognize the same MRE sequences in c-Met. A search in the TargetScan database indicates that miR-23a-3p, miR-23c and miR-130a-5p recognize the same MRE sequences of c-Met as miR-23b-3p. In contrast, the c-Met:miR-23b-3p complex is not formed in HaCaT cells and, surprisingly, the luciferase activity increased by co-transfecting the MRE23 sequences and miR-23b-3p-inducing plasmid. This result could be due to the fact that the MRE sequences were cloned in a vector that contains a CMV promoter; which has variable activity, depending on the cellular context[44-45] and regulatory mechanisms, such as methylation[46]. On the other hand, in HaCaT cells, the repression of the protein Argonauta 2 (AGO2) by miRNAs, decreases the formation and efficiency of the miRISC complex. Disabling of miRISC complex allows normal translation of proteins regulated by miRNAs[47]. It is possible that mechanisms regulating of the biogenesis of miRNAs in HaCaT cells *per se*, facilitate the expression of luciferase in reporter assays. It is also likely that, in HaCaT cells, miR-23b-3p overexpression induces activation of transcription factors that bind to the promoter of reporter plasmids used and exacerbates luciferase expression levels.

c-Met expression level varies between C33A and CaSki cells, and is inversely related to miR-23b-3p expression found in the same cells. In CaSki cells lower levels of c-Met were detected than in C33A and HaCaT cells. The highest levels of c-Met mRNA were found in C33A cells. Qian *et al.,* (2016), found that by silencing E6 from HPV16 in CaSki cells, p53 expression is restored and level of transcript and protein of c-Met is reduced[39]. In cells SKOV-3 and OSN2, Hwang *et al.,* (2011) determined that c-Met repression is due to the binding of p53 to consensus sites in the receptor promoter[48]. It is possible that the low level of c-Met mRNA found in CaSki cells with respect to C33A is due to residual p53 functionality or other transcription factors with repressor function. In contrast, C33A cells expressed the highest c-Met mRNA levels, suggesting that the mutated form of p53 present in these cells does not inhibit c-Met expression. On the other hand, the decrease in c-Met transcript level is partially mediated by miRNAs, such as miR-23a-3p, miR-23c, miR-130a-5p (predicted by TargetScan), miR-34a[49], miR-138[50] and miR-23b-3p. Thus, it is possible that the difference in c-Met expression between C33A, CaSki and HaCaT is explained by the abundance and activity of different miRNAs, including miR-23b-3p.

We have shown that miR-23b-3p overexpression significantly decreased the expression of mRNA and protein of c-Met in C33A and CaSki cells, and that this effect is not observed in HaCaT cells. These findings are in agreement with that reported by Au-Yeung *et al.,* (2017), who demonstrated that miR-23b-3p ectopic expression decreases c-Met expression at the level of mRNA and protein in SiHa cells[21]. These results and those of the reporter assays, validate to c-Met as a direct target of miR-23b-3p in C33A and CaSki cells.

In addition, we found that miR-23b-3p overexpression is related to the activation of adapter protein Gab1 and Fak kinase in CaSki cells. These results indicate that miR-23b-3p deregulates processes of proliferation, migration and invasion in CaSki cells through the inhibition of the signal pathway activated by c-Met. The effect of miR-23b-3p overexpression depends of cells intrinsic characteristics. It is probable that part of the metastatic potential of CaSki cells is related to the activity of the c-Met/Gab1/Fak pathway, while the same receptor possibly activates other signaling pathways in C33A cells, and therefore no changes were observed in these cells.

Previously, we determined that miR-23b-3p expression levels are decreased in CC tissue[17]. In this study, we evaluated the expression of c-Met mRNA in cervical tissues and found that the expression of c-Met mRNA is higher in CC tissue than in the smears from patients non-SIL and in normal keratinocytes (HaCaT cells). The level of c-Met protein in tissues of patients with CC is higher than in HaCaT cells. Our results indicate that c-Met expression is heterogeneous among CC tissues. These variations in the level of c-Met can be the result of the interindividual differences generated by different microenvironments, and of the different mechanisms of c-Met regulation, such as the paracrine regulation through its ligand, HGF. It has been described that the kinase activity, constitutive of c-Met, may be related to the absence or presence of amplifications of c-Met gene[22]. The heterogeneous expression of c-Met between patients can also be a product of the activity of the transcription factors that control the expression of c-Met; of epigenetic regulation at chromatin and post-transcriptional level, by means of miRNAs; as well as of presence of mutations or SNPs in c-Met gene.

On the other hand, if the E6 oncoprotein of HPV16 induces c-Met overexpression, it is likely that the viral load and the integration of HPV16 DNA into the cellular genome are factors that modulate the regulatory mechanisms of c-Met expression between the patients.

Conclusions

In conclusion, our results indicate that miR-23b-3p is a tumor suppressor in CC and that it contributes to regulation of proliferation, migration and invasion of C33A and CaSki cells through of direct regulation of c-Met. In CaSki cells, miR-23b-3p influences the signal pathway activated by c-Met. The regulation of c-Met by miR-23b-3p is given by the binding of the miRNA to three MRE sequences located in the 3'-UTR region of c-Met, inducing a decrease in expression levels of mRNA and protein of this receptor in C33A and CaSki cells.

Our results show that in CC, some biological mechanisms characteristic of cancer are regulated post-transcriptionally by modulation of c-Met oncogene and demonstrate the participation of miR-23b-3p in the regulation of CC progression.

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Author Contributions

GFT, HJW, GECV, and OPZ conceived and designed the experiments and coordinated the study and wrote the draft; GECV and AELG performed the experiments; GECV discussed the data and wrote the paper; MRC contributed to performed the experiments; MCLC and ECS advised the experiments and review the paper; MAJL did the cytological and histopathological diagnosis; DNMC carried out the calculation study. All authors reviewed the final manuscript.

Competing interests

The authors declare no competing interests.

IV. Discusión

Estudios de perfiles de expresión de miRNAs han mostrado que la expresión aberrante de estos RNAs pequeños, conduce a alteraciones en la expresión normal de genes que regulan procesos celulares, asociados a varios tipos de cáncer. Existe evidencia *in vivo* e *in vitro*, de que los miRNAs presentan las funciones oncogénicas y de supresores de tumor.

De acuerdo con los reportes previos, miR-23b-3p es un miRNA con funciones duales en tejido tumoral (Pellegrino et al., 2013). El algoritmo TargetScan predice un aproximado de 1,332 transcritos blancos para miR-23b-3p en humanos, entre los cuales se encuentran genes que codifican para oncogenes y para genes supresores de tumor, algunos validados como genes blanco de miR-23b-3p, mediante enfoques estándar en diferentes tipos de cáncer. En consecuencia, miR-23B-3p se ha asociado a la promoción y supresión de tejido tumoral.

Análisis funcionales sugieren que miR-23b-3p reprime el crecimiento, migración e invasión celular, así como de la angiogénesis a través de la inhibición de los oncogenes FZD70, MAP3K1, PAK2, TGFβR2, RRAS2 y uPA en cáncer de colon (Zhang *et al.,* 2011). En cáncer de próstata, miR-23b-3p regula la proliferación, el arresto del ciclo celular, apoptosis, migración e invasión celular a través de la represión de la cinasa Src y Akt (Majid *et al.,* 2012). Además, en cáncer de próstata, miR-23b-3p reduce los niveles de activación de la GTPasa Rac1, eventualmente incrementa los niveles de E-caderina y disminuye el comportamiento metastásico en este tejido (Ishteiwy *et al.,* 2012). Mientras que en cáncer de vejiga, miR-23b-3p regula procesos asociados a metástasis a través de la represión de Zeb1 (Majid *et al.,* 2013), (anexo 1).

En este estudio, se encontró que la expresión de miR-23b-3p esta disminuída en células C33A y CaSki, pero es menor en C33A. Estos resultados están en acuerdo con lo reportado por Lui *et al.,* (2007) y Li *et al.,* (2018). La expresión disminuida de miR-23b-3p y su variación entre éstas células de CaCU, puede deberse a las características propias de las células tumorales y al tipo de cáncer del que provienen (carcinoma *in situ* o metastásico), a la expresión diferencial de factores de

transcripción para miR-23b-3p y a mecanismos regulatorios específicos de la expresión de genes, y a la presencia de VPH16.

En CaCU con infección por VPH16, se ha observado que las oncoproteínas E6 y E7 modifican la expresión de miRNAs celulares a través de la metilación aberrante de sus promotores (Au-Yeung *et al.,* 2010). E6 y E7 también pueden inducir daños genómicos que, a su vez, pueden conducir a la sobre-expresión o expresión disminuida de miRNAs celulares, por amplificación o deleción de regines genómicas en las que se alojan estos miRNAs y que son cercanas a sitios frágiles donde el DNA del VPH puede integrarse al genoma celular (Diaz-González et al., 2015). Aunque para miR-23b-3p no se ha reportado que la integración del DNA de VPH al genoma celular, puede cambiar su expresión, la expresión de las oncoproteínas virales, principalmente de E6, si se ha asociado a la expresión disminuida de este miRNA. La sobre-expresión de las endonucleasas DROSHA y DICER (involucradas en el procesamiento de miRNAs) también puede modular la expresión de miRNAs (Harden and Munger, 2017). Además, el compromiso de la función normal de factores de transcripción, entre ellos p53 y E2F, puede modular la expresión de miRNAs, (Bueno and Malumbres, 2011).

Entonces, las diferencias entre los niveles de expresión de miR-23b-3p en células C33A y CaSki, pueden deberse a 1) que el porcentaje de copias metiladas del promotor de miR-23b-3p varía entre ambas líneas celulares (Campos-Viguri *et al.,* 2015) y esto impacta en los niveles de transcripción del miRNA; 2) a que la expresión de DROSHA y DICER es mayor en células CaSki que en C33A (Harden and Munger, 2017), y es posible que estas diferencias, se relacionen con mayor procesamiento y concentración de las moléculas precursoras y maduras de miR-23b-3p en células CaSki; y 3) a que p53, un factor de transcripción que modula la expresión de miR-23b-3p (Bisio et al., 2013), en C33A expresa de forma mutada y que carece de función como factor transcripcional (Scheffner *et al.,* 1991), contribuyendo a la expresión disminuida de miR-23b-3p en C33A. Interesantemente, en CaCU se ha evidenciado la expresión aumentada de los

factores de transcripción c-Myc y NF-κB, ambos asociados a la represión de miR-23b-3p (Donadelli et al., 2013).

Por otro lado, el nivel de expresión de miR-23b-3p en células HaCaT, células epiteliales no tumorales, fue mayor que en CaSki y C33A. De esta forma, se fortalece la hipótesis de que este miRNA es supresor de tumor en CC (Campos-Viguri *et al.,* 2015; Au-Yeung *et al.,* 2017), como ocurre en cáncer de colon (Zhang et al., 2011), en carcinoma oral de células escamosas (Fukumoto et al., 2016) y en cáncer de vejiga (Chiyomaru et al., 2015; Majid et al., 2013).

Con la finalidad de sobre-expresar a miR-23b-3p, nosotros diseñamos y generamos un plásmido para inducir la expresión del miRNA (plásmido pMIR23B-3p), que fue transfectado en células HaCaT, C33A y CaSki. Los ensayos de eficiencia mostraron que el plásmido es capaz de inducir de 2 a 7 veces más expresión de miR-23b-3p en las células transfectadas comparado con células no transfectadas. Un ensayo similar fue realizado por Salvi *et al.,* (2009) en células SKHep1C3, en donde observaron que la expresión de miR-23b-3p aumentó hasta 9 veces más con su sistema de expresión ectópica.

De acuerdo a estudios previos, el oncogén c-Met, es un gen blanco de miR-23b-3p (Salvi *et al.,* 2009), con importantes funciones en el establecimiento y desarrollo de cáncer. Dentro de los mecanismos celulares alterados por efecto de la sobreexpresión y activación de c-Met, se encuentran la proliferación, migración e invasión celular. Tales mecanismos son dirigidos por diversas vías de señalización, como las mediadas por ERK, PI3K y Fak, entre otras.

Los resultados obtenidos en esta investigación indican que miR-23b-3p inhibe significativamente fenómenos biológico-malignos del CaCU. Los resultados muestran que la sobre-expresión de miR-23b-3p en células de CaCU, disminuye significativamente el comportamiento proliferativo de células C33A y CaSki en comparación con células HaCaT. Las células C33A presentaron una disminución significativa en su proliferación a las 48 h de sobre-expresar a miR-23b-3p, mientras que las células CaSki a las 72 h. Recientemente Au-Yeung *et al.,* (2017) encontraron que miR-23b-3p induce apoptosis de células SiHa, provenientes de CaCU. Nuestros

hallazgos sugieren que la disminución de la proliferación de células C33A y CaSki pueden deberse a las funciones regulatorias de c-Met sobre procesos de sobrevida, apoptósis y proliferación celular, mediante vías de señales que incluyen la activación de Akt, Fas y Ras (Blumenschein *et al.,* 2012).

Estudios funcionales sugieren que la expresión de miR-23b-3p puede regular la transición epitelio-mesénquima, un evento involucrado en las primeras etapas de la metástasis tumoral. En células HeLa, el eje miR-23b/ MAP1K modula el crecimiento y la migración celular (Li *et al.,* 2018). Independientemente de que miR-23b-3p reduce los niveles de activación de la GTPasa Rac1, y en consecuencia induce la expresión de E-caderina (Ishteiwy *et al.,* 2012), en cáncer de colon, miR-23b-3p disminuye la expresión del marcador mesenquimal vimentina e incrementa la localización en membrana de E-caderina (Zhang *et al.,* 2011), y en conjunto estos eventos les confieren un fenotipo epitelial estable a las células. Por otro lado, en cáncer gástrico, miR-23b-3p inhibe la expresión de moléculas de la vía Wnt/β-catenina involucrada en movilidad celular (Xian *et al.,* 2018).

El cáncer cervical se caracteriza por su propensión a invadir y a diseminarse a nódulos linfáticos. Para que las células tumorales lleven a cabo estos procesos, se precisan cambios en el perfil de expresión de moléculas de adhesión célula-célula y célula-matriz extracelular (ECM), además de la degradación de ECM y la migración celular (Krakhmal *et al.,* 2015). Los resultados muestran que en células CaSki, la sobre-expresión de miR-23b-3p disminuye significativamente la migración e invasión celular, mientras que en células C33A solo se observaron cambios significativos en la invasión celular.

Durante la invasión de ECM y tejidos circundantes, las células tumorales desarrollan dos patrones de invasión, conocidos como invasión colectiva e invasión individual; en los cuales se presentan dos fenotipos de migración, el fenotipo mesenquimal, en el que las células se movilizan lentamente; y el fenotipo ameboide que representa una migración más eficiente (Krakhmal *et al.,* 2015). En este sentido, nuestras observaciones sugieren que C33A podría presentar patrones de invasión individual y migrar de forma mesenquimal, debido a la baja cantidad de células que migran al

centro de la estría en los ensayos de migración y que fueron capaces de invadir en los ensayos transwell. Mientras que el patrón de invasión observado en CaSki podría obedecer al tipo colectivo con migración ameboide, dado que estas células muestran mayor movilidad en agrupaciones. Por lo tanto, estos datos sugieren que miR-23b-3p es capaz de modular diferentes tipos de invasión y migración en cáncer cervical. No obstante, la caracterización de los patrones invasivos y migratorios de C33A y CaSki requiere el estudio de marcadores y mecanismos específicos.

Nuestros resultados indican que miR-23b-3p es un miRNA supresor tumoral que regula proliferación, migración e invasión celular, y una de las vías de señales implicada en tales procesos es la dirigida por el receptor tisosina-cinasa c-Met, que en tejido de cáncer presenta expresión y funciones alteradas (Qian *et al.,* 2016).

Recientemente Au-Yeung *et al.,* (2017) describieron a c-Met como un gen blanco de miR-23b-3p en CaCU. En su investigación, estos autores evaluaron el reconocimiento de un sitio de unión para miR-23-3p (sitio MRE) mediante ensayos reporteros (Au-Yeung et al., 2017). No obstante, nuestos resultados *in silico* muestran que dentro de la región 3'-UTR de c-Met, hay 5 sitios MRE potenciales para miR-23b-3p; un sitio MRE más que lo reportado previamente en la literatura (Salvi *et al.,* 2009). Estos hallazgos podrían ser resultado del número y actualización de bases de datos para la predicción de miRNAs, así como de las diferencias entre los modelos celulares estudiados para la construcción de los algoritmos que emplean (Agarwal et al., 2015).

Los criterios para evaluar la probabilidad de unión, la afinidad y estabilidad del complejo c-Met:miR-23b-3p incluyeron el porcentaje y el tipo de complementariedad entre la región semilla de miR-23b-3p y la región 3'-UTR del mRNA de c-Met. Con base en estos criterios, seleccionamos 3 de las 5 secuencias MRE, para ser evaluadas mediante ensayos reporteros de actividad de luciferasa. Los ensayos arrojaron que la actividad de luciferasa disminuyó en células C33A y CaSki, confirmando que c-Met es un gen blanco directo de miR-23b-3p en estas células. Además, se comprobó que las 3 secuencias MRE evaluadas responden a miR-23b-3p en estas células. A pesar de que Salvi *et al.,* al evaluar los mismos sitios MRE,

encontraron que la actividad de luciferasa se redujo entre el 26 y 10% en su modelo de estudio (células SKHep1C3), nuestros resultados indican una reducción del 50-95%, lo que sugiere que las variaciones en el contexto celular, incluyendo moléculas endógenas de otros miRNAs, intervienen en la reducción de la luciferasa. Por el contrario, en células HaCaT la unión de miR-23b-3p:c-Met no fue favorecida y curiosamente, la actividad de luciferasa incrementó al co-transfectar las secuencias MRE con el plásmido inductor de miR-23b-3p. Este resultado podría ser explicado debido a que, las secuencias MRE fueron clonadas en un vector que contenía un promotor CMV; que presenta una actividad variable dependiendo del contexto celular (Qi *et al.,* 2010) y mecanismos de regulación (como su metilación) (Meilinger *et al.,* 2009). Por otro lado, la ausencia de la proteína Argonauta 2 (AGO2), un evento descrito en células HaCaT, permite la traducción normal de proteínas reguladas vía el complejo miRISC (Roberts *et al.,* 2013).

De acuerdo a nuestros resultados, el nivel de expresión de c-Met varía entre las líneas celulares de CaCU estudiadas. Las células C33A presentaron niveles más aumentados de mRNA de c-Met respecto a CaSki. Actual evidencia indica que la expresión de la oncoproteína E6 de VPH16 induce la sobre-expresión del receptor c-Met, mediante diferentes mecanismos de regulación transcripcional. Qian *et al.,* (2016), mostraron que al silenciar la expresión de E6 de VPH16 en células CaSki, se restaura la expresión de p53 y hay una reducción concomitante de la expresión de mRNA y proteína de c-Met. La represión de c-Met es resultado entonces, de la unión de p53 a sitios consenso en el promotor de c-Met (Hwang *et al.,* 2011). E6 de VPH16, también es capaz de inducir la degradación vía proteasoma de la demetilasa de histona KDM5A, resultando en la activación de un super-enhancer localizado en cercanías del promotor de c-Met, lo que conduce a la transcripción de c-Met (Chen *et al.,* 2018). Sin embargo, nuestras observaciones también pueden ser el resultado de otro nivel de regulación de c-Met, la regulación post-transcripcional mediada por miRNAs, como es el caso de miR-34a (Li *et al.,* 2009) miR-138 (Li *et al.,* 2016) y miR-23b-3p, así como de otros miRNAs que comparten homologia en las secuancias en las que potencialmente hibridan dentro de la 3'-UTR de c-Met (miR-23a-3p, miR-130a-5p y miR-23c). Entonces es posible que la

diferencia en la expresión de c-Met entre C33A, CaSki y HaCaT se explique, además, por la abundancia y actividad de miRNAs que regulen la expresión de este receptor, incluyendo miR-23b-3p.

De acuerdo con nuestras observaciones en los ensayos reporteros, la sobre-expresión de miR-23b-3p disminuye significativamente la expresión del mRNA y proteína de c-Met en células C33A y CaSki, mientras que en células HaCaT no se observa el mismo efecto. Estos hallazgos están en acuerdo al reporte de Au-Yeung *et al.,* (2017) quienes demostraron que la expresión ectópica de miR-23b-3p disminuye la expresión de c-Met a nivel de mRNA y proteína en células SiHa. En conjunto estos resultados validan a c-Met como un blanco directo de miR-23b-3p en células C33A y CaSki. Como parte de los efectos de la represión de c-Met por miR-23b-3p, se determinó que en células CaSki, la sobre-expresión de este miRNA también influye significativamente la activación de la proteína adaptadora Gab1 y de la cinasa Fak, sugiriendo que miR-23b-3p desregula los procesos de proliferación, migración e invasión de células CaSki a través de la inhibición de la vía dirigida por c-Met. Es posible que en células C33A, miR-23b-3p además de reprimir la expresión de c-Met, esté resregulando otros genes implicados en proliferación y movilidad celular, como TGFβR2 (Zhang *et al.,* 2011) y VCAN (Wei et al., 2017).

Finalmente, el nivel de expresión del mRNA de c-Met fue evaluado en tejidos cervicales, encontrando que la expresión del mRNA de c-Met es mayor en tejido de CaCU comparado con tejido de pacientes sin lesiones intraepiteliales y con células HaCaT (queratinocitos normales). Además, determinamos el nivel de proteína de c-Met en tejidos de pacientes con cáncer cervical y encontramos que la expresión de c-Met es mayor en estos tejidos comparados con células HaCaT. Nuestros resultados indican que la expresión de c-Met es heterogénea entre tejidos de cáncer cervical. Estas variaciones entre tejidos pueden vincularse a varios mecanismos de regulación de c-Met, incluyendo 1) la regulación paracrina de c-Met mediante su ligando HGF; 2) la regulación mediada por factores de transcripción; 3) la regulación

epigenética a nivel de cromatina y a nivel post-transcripcional (mediada por miRNAs) y 4) la presencia de mutaciones o SNPs en el gen de c-Met.

Dado que la expresión de la oncoproteína E6 de VPH16 induce la sobre-expresión de c-Met; seguramente la carga e integración del DNA viral al genoma celular, aunada a la expresión constitutiva de las oncoproteínas E6 y E7, juegan un papel importante en la expresión de c-Met.

En conjunto, los resultados obtenidos indican que c-Met es un blanco directo de miR-23b-3p, valiadan a este miRNA como un supresor de tumor y demuestran su papel como regulador de la proliferación, migración e invasión en cáncer cervico uterino.

V. Conclusiones

Los hallazgos de esta investigación indican que miR-23b-3p es un supresor de tumor en CaCU, que regula la proliferación, migración e invasión de células C33A y CaSki a través de la regulación directa de c-Met. En células CaSki, miR-23b-3p influye la vía de señales activada por c-Met. La regulación de c-Met por miR-23b-3p se da por la unión del miRNA al menos a tres secuencias MRE localizadas en la región 3'-UTR de c-Met, induciendo disminución en los niveles de expresión del mRNA y proteína de este receptor en células C33A y CaSki. Los mecanismos biológicos característicos del cancer son regulados post-transcripcionalmente por modulación del oncogén c-Met y demuestran la participación de miR-23b-3p en la regulación de la progresión del CaCU.

VI. Referencias

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VII. Anexos

Anexo 1. Estudios de miR-23b y sus blancos moleculares en cáncer.

Tabla 1. Papel y Blancos moleculares de miR-23b en Cáncer				
Papel Biológico de mir-23b	**Tipo de cáncer (Modelo de estudio)**	**Expresión de miR-23b**	**Gen blanco validado**	**Función del blanco**
OncomiR	Carcinoma Gástrico [Zhuang et al., 2016: Huang et al., 2015*]	Aumentada	Notch2*	Proliferación, crecimiento, migración, diferenciación y muerte celular programada
	Células Madre de Cáncer Ovárico Quimioresistentes [Pack et al., 2013]		No hay genes validados	
	Cáncer de mama [Jin *et al.,* 2013]		Nischarina	Remodelamiento del citoesqueleto
	Cáncer Renal [Zaman et al., 2012]		PTEN	Regulación del ciclo celular
Supresor metastásico	Cáncer de ovario [Li *et al.,* 2014]	Disminuida	*runx2*	Proliferación celular
	Cáncer de mama [Pellegrino *et al.,* 2013]		*Pak2*	Remodelamiento de citoesqueleto
Supresor de tumor	Cáncer de colon [Zhang *et al.,* 2011]		*FZD7* *MAP3k1*	Movilidad celular
	Cáncer de Próstata [Majid *et al.,* 2012*, Goto *et al.,* 2012**; Ishteiwy et al., 2012]		Src, Akt* GOLM1**	Proliferación migración e invasión
	Cáncer pancreático [Wang *et al.,* 2013]		*atg12*	Regulación de la autofagia.
	Células madre (hígado) [Ham *et al.,* 2012]		*Pka*	Diferenciación de células madre a condrocitos
	Líneas de C. Hepatocelular [Salvi *et al.,* 2009]		*u-pa,* *c-met*	Migración y proliferación celular.
	Cáncer de Vejiga [Majid et al., 2013*; Chiyomaru *et al.,* 2015**]		*Zeb1** *Egfr, c-met***	Proliferación, invasión Y Metástasis.

	Cáncer Cérvico Uterino^ [Au-Yeung et al., 2011*; Campos-Viguri et al., 2015]	*uPa**	Migración celular

^En este tipo tumoral no se ha confirmado la función de miR-23b como supresor de tumor

Anexo 2: Efecto de miR-23b-3p sobre la invasión de células CaSki: Modelo de onco-esferas.

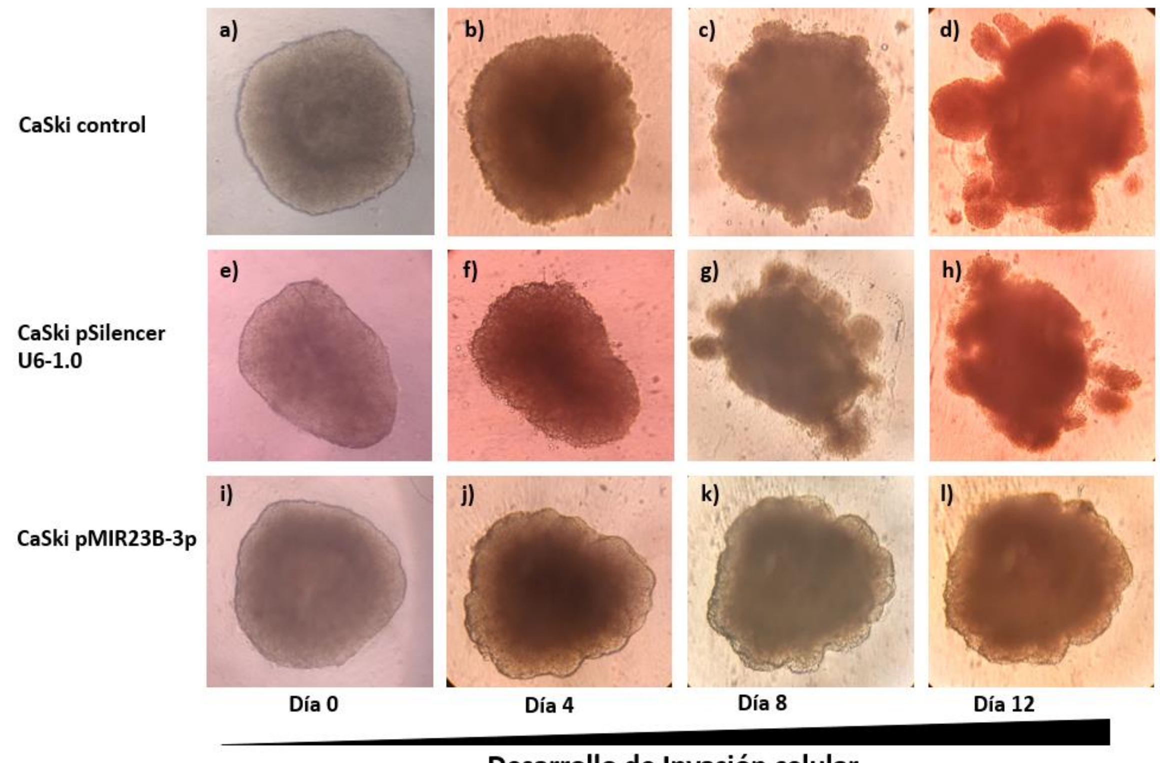

Figura 1. miR-23b-3p inhibe la invasión de células CaSki en un modelo de Onco-esferas. Métodología: Para los ensayos de invasión en onco-esferas, células CaSki transfectadas (2X10^4) 72 h antes del ensayo, con 5 μg del plásmido pMIR23B-3p ó del plásmido pSilencer U6-1.0 (sin inserto de DNA) y células no transfectadas (CaSki control), fueron sembradas en placas de 96 pozos de fondo cónico. Las células fueron incubadas por 24 h a 37°C para favorecer la formación de una esfera, y después, los eferoides fueron encapsulados en 100 μL de matrigel (dia 0). Las onco-esferas fueron monitoreadas por microfotografías diariamente durante 12 días después de la adición del matrigel; y durante ese tiempo, a cada pozo se le adicionaron 120 μL de medio DMEM con 10% de SFB cada tercer día.

Resultados: al cuarto día de la adición del matrigel, las células control (panel **b**) y las transfectadas con el plásmido pSilencer U6-1.0 (panel **f**) mostraron los primeros indicios de invasión y disrupción del borde de la onco-esfera. Al octavo día se presentó una marcada

invasión de células control y transfectadas con pSilencer U6-1.0 (paneles **c** y **g**) que incrementó hasta el día 12 (paneles **d** y **h**). Las células transfectadas con el plásmido inductor de miR-23b-3p, no mostraron invasión hacia el matrigel (panel **i-l**), y los primeros signos de disrupción del borde de la onco-esfera fueron observados hasta el día 12 del ensayo (panel **l**).

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RESEARCH ARTICLE
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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. The aim of this study was to evaluate the potential role of methylation in the silencing of miR-23b in cervical cancer cell lines and to determine its expression in stages of malignant progression and in cervical cancer tissues HPV16-positive.

Methods: The methylation of the miR-23b promoter was determined in HeLa, SiHa, CaSki and C33A cells using a Human Cancer miRNA EpiTectMethyl II Signature PCR Array®. The cells were treated with 5-Aza-2′-deoxycytidine, and the expression of miR-23b, *uPa, c-Met* and *Zeb1* was determined by qRT-PCR. miR-92a and GAPDH were used as controls. The expression of miR-23b was determined in cervical scrapes and biopsies of women without squamous intraepithelial lesions, with precursor lesions and with cervical cancer, all were HPV16-positive. The Fisher exact and Mann–Whitney tests were used to compare the differences of the expression of miR-23b, *uPa, c-Met* and *Zeb1* among cell groups, and the difference among patients, respectively. The association between the expression of miR-23b and cervical cancer was determined by logistic regression with a confidence level of 95 %. A value of $p < 0.05$ was considered statistically significant.

Results: In C33A, HeLa and CaSki cells, methylation was associated with decreased expression of miR-23b. After treatment with 5-Aza-CdR, the expression of miR-23b increased in all cell lines and the expression of *c-Met* decreased in HeLa cells, while *uPa* and *Zeb1* decreased in C33A and CaSki cells. In SiHa cells the expression of *uPa*, *c-Met* and *Zeb1* increased. The expression of miR-23b decreased in relation to the increase in the severity of the lesion and was significantly lower in cervical cancer. In women with premalignant lesions HPV16-positive, decreased levels of miR-23b increased the risk of cervical cancer (OR = 36, 95 % CI = 6.7-192.6, $p < 0.05$).

Conclusions: The results suggest that the expression of miR-23b is regulated by the methylation of its promoter and is possible that this microRNA influence the expression of *uPa, c-Met* and *Zeb1* in cervical cancer cells lines. In women with premalignant lesions and cervical cancer infected with HPV16, the expression level of miR-23b agree with a tumor suppressor gene.

Keywords: expression, miR-23b, DNA methylation, cervical cancer, HPV16, *upa*, *c-met*, *zeb1*

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Background

The high-risk human papillomavirus (HR-HPV) causes cervical cancer [1], and this infection is also associated with precancerous lesions of the cervix [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].

The miRNAs can regulate 60 to 90 % of the protein-encoding genes and a single miRNA can regulate, directly or indirectly, the expression of hundreds of target mRNAs [5–7]. The aberrant expression of miRNAs has been associated with the maintenance of the undifferentiated state of cancer cells [8].

miRNA biogenesis is highly regulated by multiple processes. Approximately 40 % of human miRNAs are organized in conserved clusters, with distances of at least 5000 bp between them [9], and these miRNAs are co-transcribed as discrete polycistronic pri-miRNAs [6, 10]. 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 [11, 12].

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, and is thus recognized as an oncogene [13]. In contrast, the expression of miR-23b in breast cancer increases the formation of focal adhesions and cell-cell junctions, thereby indicating a metastatic suppressor role for this microRNA [14]. Furthermore, the expression of miR-23b has been found to be decreased 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 [6, 7, 9].

The expression of miR-23b is decreased in HR-HPV positive cervical cancer tissue and cell lines [15], and it is associated with the overexpression of the urokinase-type plasminogen activator (uPA), which is target gene of this miRNA [16]. uPA has been directly associated with migration and invasion in cervical cancer [17]. Although it is proposed that the E6 oncoprotein of HR-HPV regulates the expression of miR-23b in cervical cancer, this mechanism has not been fully elucidated.

A significant number of miRNAs are embedded in CpG islands and that they are targets of epigenetic regulation [8, 17–19]. In human neoplasia, hypermethylation of CpG sites is associated with transcriptional silencing of tumor suppressor genes and genes that encode miRNAs [20]. There is evidence that miR-23b is subjected to epigenetic silencing in glioblastoma and prostate cancer [7, 8]. Interestingly, it has been suggested that, HPV16 E6 increases the levels of DNA methyltransferase 1 (DNMT1) by degradation of p53 in cervical cancer, causing hypermethylation of miRNA genes, among others [21].

Is proposed that in cervical cancer the inhibition of p53 expression by HR-HPV E6 contributes to the decreased expression of miR-23b. 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. Identifying the mechanism by which the expression of miR-23b is regulated in cervical cancer 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 cervical cancer cell lines and to determine the pattern of expression of this microRNA in cervical tissues of patients without squamous intraepithelial lesion (Non-SIL), with precursor lesions and HPV16 cervical cancer. In this study, we found decreased expression of miR-23b in cervical tissue with premalignant lesions in cervical cancer and in cervical cancer cell lines. Treatment with 5′-aza-2-deoxycytidine restored the expression of miR-23b in C33A, HeLa and CaSki cells.

Results

Model of the elements regulating the expression of miR-23b

An extensive review of the literature was conducted for the selection of miR-23b. We constructed a model of the regulatory region of the miR-23b gene based on the information found. Some elements that may contribute to the regulation of expression of this microRNA are highlighted in this model as follows:

a) The promoter region of miR-23b gene is located in CpG dinucleotide-rich area [7, 8].
b) In the first kilobase, there are two CpG islands with high CpG density upstream of the transcription start site (TSS) of miR-23b [7].
c) The regulatory sequence of miR-23b, contains four putative sites representing response elements for p53 at 1, 8, 10 and 28 kb upstream of the transcription start site of miR-23b [5, 22, 23] (Fig. 1).

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 was close to 100 % in the four cell lines (Fig. 2a).

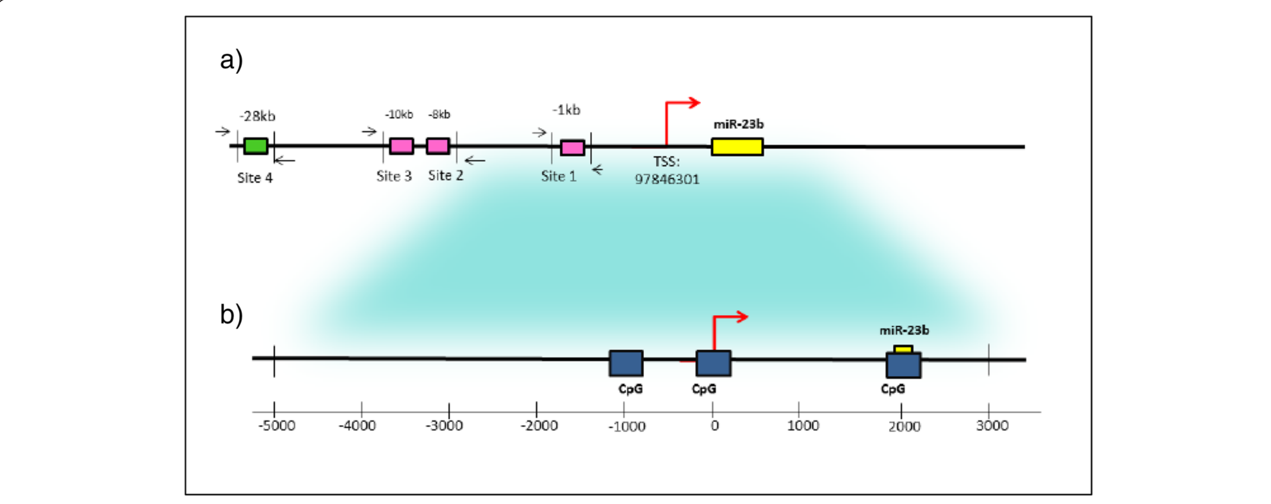

Fig. 1 Regulatory elements for the expression of miR-23b. **a** The diagram shows putative p53-binding sites in the miR-23b promoter. The yellow box represents the location of miR-23b precursor, and the green box symbolizes a binding site for p53 [22]. The pink boxes indicate putative binding sites for p53 [22, 23]. **b** CpG islands in the promoter region of miR-23b (blue boxes). The region containing CpG islands was amplified in this study to determine the methylation status of miR-23b promoter

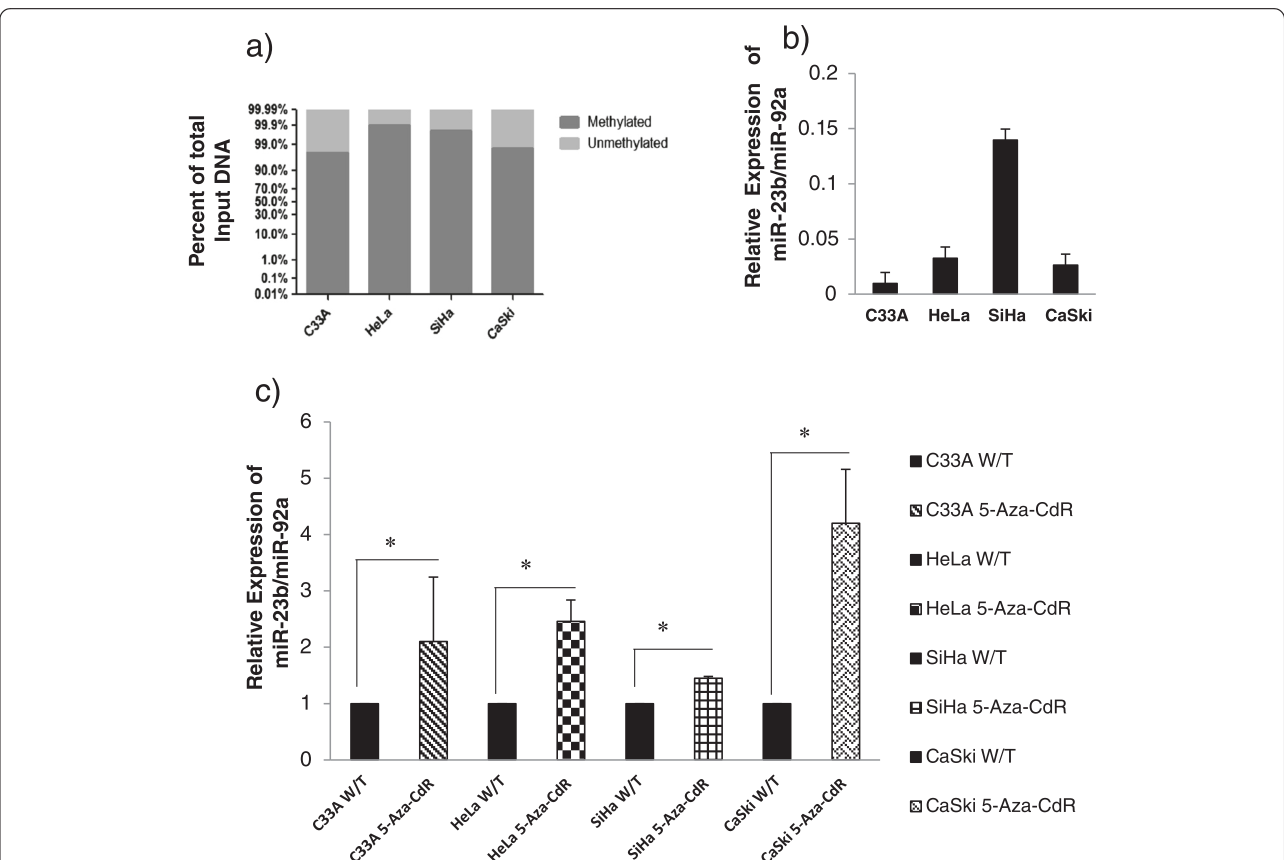

Fig. 2 miR-23b is deregulated by methylation of its promoter region in cervical cancer cell lines. **a** The analysis of the methylation status revealed the almost 100 % of total input DNA had methylated miR-23b gene promoter in C33A, HeLa, SiHa and CaSki cells untreated with 5′-Aza-CdR. **b** The relative expression levels of miR-23b were decreased in C33A, HeLa and CaSki cells compared to the levels observed in SiHa cells. **c** Treatment with 10 μM 5′-Aza-CdR significantly increased the expression of miR-23b in the four cell lines (weft bars) compared to untreated cells (black bars). The increased relative expression of miR-23b was ≥50 % over the initial expression (W/T) in C33A, HeLa and CaSki but not in SiHa cells. *Statistically significant difference ($p < 0.05$); W/T: Untreated cells

To investigate if 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 (Fig. 2b).

Furthermore, to confirm that methylation is a mechanism that affects the expression of miR-23b in HeLa, SiHa, CaSki and C33A cells, the cells were exposed to 5′-Aza-CdR, a compound that inhibits DNA methylation. Compared with untreated cells, the expression level of miR-23b was higher in all cell lines after treatment with 5′-Aza-CdR. The increase in the expression of miR-23b was statistically significant in C33A, HeLa, SiHa and CaSki cells ($p < 0.05$), (Fig. 2c).

Effect of exposure of cervical cancer cell lines to 5′-Aza-CdR on potential molecular targets of miR-23b

The information available in the literature and from the *miRanda* and *TargedScan* databases indicates that *uPa, c-Met* and *Zeb1* mRNAs contain miR-23b-binding sequences (Fig. 3a).

To verify that the observed changes in the expression level of miR-23b influence the expression of *uPa*, *c-Met* and *Zeb1,* which are likely targets of this microRNA, the relative mRNA expression of the three genes was determined in HeLa, SiHa, CaSki and C33A cells before and after exposure to 5′-Aza-CdR.

The hypomethylating treatment with 5′-Aza-CdR decreased the mRNA expression of uPa in C33A and CaSki cells but not in HeLa and SiHa cells. Furthermore, the expression of c-Met was significantly reduced only in HeLa cells after treatment. The mRNA expression of Zeb1 decreased only in C33A and CaSki cells, showing significant changes in both cell lines treated with 5′-Aza-CdR. In SiHa cells, the expression levels of uPa, c-Met and Zeb1 increased significantly after exposure to 5-Aza-CdR. The expression of these genes in SiHa cells may be dysregulated by epigenetic factors among other modulation mechanisms, (Fig. 3b, c and d).

Expression of miR-23b in scrapes from non-SILs, with premalignant lesions and cervical cancer

To investigate whether the expression level of miR-23b was similar in cell lines and in samples from the cervix, we analyzed cervical scrapes from non-SILs ($n = 18$) and tissues from patients diagnosed with LSILs ($n = 19$), HSILs ($n = 7$) or cervical cancer ($n = 28$), all patients were infected with HPV16.

The expression of miR-23b was significantly lower in non-SIL samples ($p < 0.05$) and in cervical cancer tissues ($p < 0.05$) than in tissues from patients diagnosed with LSIL and HSIL, (Fig. 4).

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 cervical cancer by 36-fold (OR = 36; 95 % CI =6.7-192.6; $p < 0.05$) (Table 1).

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 most significant findings of this study were as follows: 1) the miR-23b promoter is methylated in cervical cancer cell lines; 2) the expression of miR-23b is low in cervical cancer cell lines; 3) the expression of miR-23b increases significantly in HeLa, 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 cervical cancer, that is, it decreases as the grade of the lesion increases; 5) in biopsies positive for HPV16 cervical cancer, the expression level of miR-23b is similar to that found in HeLa, SiHa, CaSki and C33A cell lines, and 6) the expression of uPa, c-Met and Zeb1, which are likely targets for miR-23b, is different among cervical cancer 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 prostate cancer cell lines and tumor tissues. In cervical cancer, it is likely that the expression of miR-23b is also regulated by dinucleotides methylation in the CpG islands located upstream of the TSS within of its promoter.

Corresponding to the methylation level of the miR-23b promoter, which is close to 100 %, the expression of miR-23b was found to be low in cervical cancer cell lines, suggesting that methylation is the mechanism of regulation of the expression of miR-23b in these cells. Interestingly, we found that in CaSki and HeLa cells, which have a greater number of integrated viral copies, the expression of miR-23b was decreased more than in the SiHa cells with fewer integrated copies. While in C33A cells, which are HPV-negative, the lowest expression of miR-23b was detected. The difference in the expression level of miR-23b in the cervical cancer cell lines can be explained by the functional characteristics of each cell line and by the different molecular events as follows: a) tissue origin of each cell line; b) expression of miR-23b transcription factors; and c) multiple mechanisms of regulation for genetic expression.

In cells untreated with 5′-Aza-CdR, the miR-23b expression was higher in SiHa cells than in HeLa, CaSki and C33A cells, which can be explained, at least in

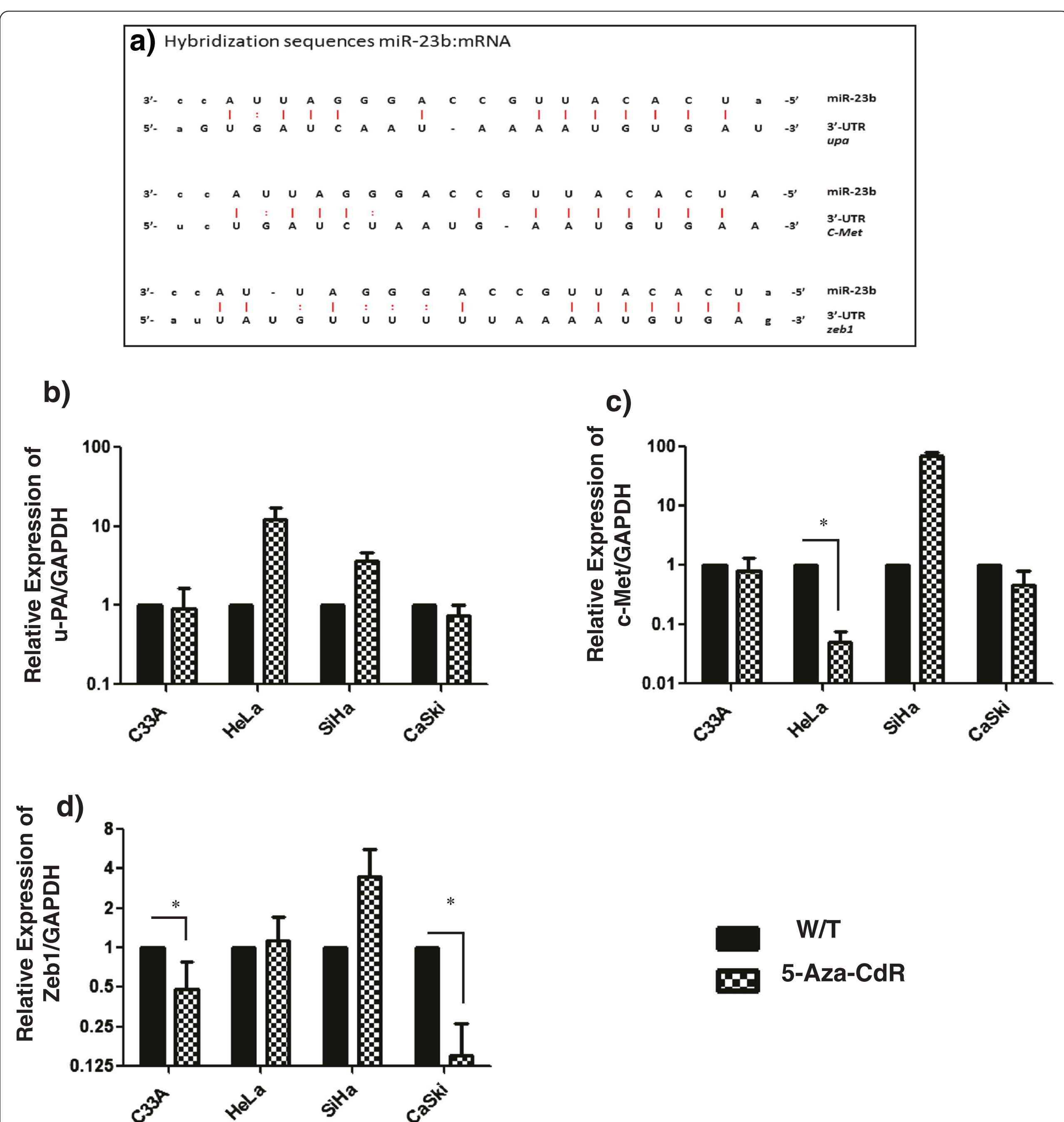

Fig. 3 Differential mRNA expression of uPa, c-Met and Zeb1 in cervical cancer cell lines treated with 5′-Aza-CdR. **a** Hybridization sequences between miR-23b and its potential target mRNAs. **b** After treatment with 5′-Aza-CdR, the mRNA expression level of uPa decreased in C33A and CaSki cells. **c** The expression of c-Met decreased in C33A, CaSki and HeLa cells after exposure to 5′-Aza-CdR and the changes in HeLa cells were significant. **d** Treatment with 5′- Aza-CdR significantly decreased Zeb1 expression levels in C33A and CaSki cells. The mRNA expression levels of GAPDH were used to normalize the data. *Statistically significant difference ($p < 0.05$); W/T: Untreated cells

part, by the differential expression of transcription factors that modulate the expression of miR-23b. SiHa cells express functional p53 protein at higher levels than in HeLa, CaSki and C33A cells [24]. p53 is a transcription factor [24] that modulates the expression of various genes. Bisio *et al.* found that miR-23b contains a consensus sequence with low-affinity binding for p53 upstream of the TSS. The authors concluded that cis regulation by p53 partially modulates the expression of miR-23b [22]. In SiHa cells, Au Yeung *et al.* found that p53 expression correlates with miR-23b expression when the HPV16 E6 oncoprotein is silenced [25]. The higher

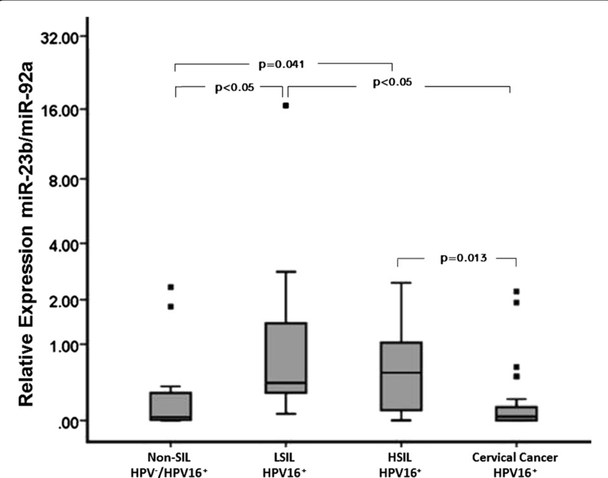

Fig. 4 Expression of miR-23b in cervical scrapes from non-SILs and in tissues from patients diagnosed with LSIL, HSIL and cervical cancer. The expression of miR-23b was significantly lower in cervical cancer biopsies compared with LSIL and HSIL tissues. miR-92a was used for normalization of the data. *Statistically significant difference ($p < 0.05$)

miR-23b expression level in SiHa cells can be a consequence of the p53 expression level.

In C33A cells, p53 is expressed at normal levels but is not functional because it has a point mutation at codon 273 in an evolutionarily conserved domain. This mutation results in the substitution of an arginine with a cysteine [24]. It is likely that mutated p53 is unable to transactivate miR-23b, which would explain the low levels of this miRNA in HPV-negative cervical cancer cells. Other factors that may be influencing the expression of miR-23b and other microRNAs are as follows: the HPV type; the number of viral genome copies integrated into the cell genome [2, 26]; genetic polymorphisms or mutations in the promoter of the transcription factors that modulate the expression of miR-23b [27, 28]; defects in the molecular machinery responsible for the biogenesis of miR-23b [29, 30]; and the degree of methylation of miR-23b [7, 8].

We found that the expression of miR-23b is higher in LSILs than in HSILs and cervical cancer, that is, the expression of this miRNA decreased as the grade of the lesion increased. The decreased expression of miR-23b in biopsies from patients with cervical cancer positive for HPV-16 was similar to that found in HeLa, SiHa, CaSki and C33A cells. miR-23b overexpression in LSILs suggested that miR-23b in cervical carcinogenesis is a tumor suppressor.

Table 1 Risk analysis for the expression of miR-23b in cervical tissues

Expression of miR-23b	Clinical diagnosis		OR (95 % CI)	Value of *p*
	SILs (n = 26)	Cervical Cancer (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*	

SIL LSIL + HSIL, *OR* Odds ratio
95 % confidence interval
*Reference value
The bold data indicate the frequency of the expression level found in premalignant lesions and cervical cancer.

The significantly lower miR-23b expression in biopsies of cervical cancer than in LSILs and HSILs, may be the result of gradual miR-23b promoter methylation and alterations of other gene expression regulatory mechanisms that are frequent in carcinogenesis. During tumorigenesis, cells undergoing epithelial-mesenchymal transition (EMT) are subject to metaplasia, which is characterized by the loss of E-cadherin (CDH1) expression [31, 32]. In hepatocellular carcinoma cell lines, decreased levels of miR-23b are correlated with the loss of expression of CDH1 [31, 32]. In endometrial carcinosarcoma, miR-23b inhibits the expression of mesenchymal markers [33]. Evidence indicates that CDH1 expression is downregulated by the snai1 and zeb1 transcription factors, which are molecular targets of miR-23b [31–33].

The low miR-23b expression in cervical scrapes can be explained by the origin of the sample, which determines the type and features of the cells present in the scrape. Cervical scrapes were obtained from the cell transformation zone (TZ). The transformation zone contains highly undifferentiated normal cells, such as metaplastic cells that generally lose cell-cell junctions due to the decreased expression of CDH1 [31, 32]. In endothelial cells, miR-23b expression is negatively associated with genes that regulate the cell cycle. During the cell cycle, phosphorylated pRb induces G1/S cell-cycle transition [34]. Low miR-23b expression is associated with the phosphorylation status of pRb and indirectly with increased E2F expression, thus promoting the proliferation of endothelial cells [35]. In metaplastic cervical cells, the decreased miR-23b expression may be correlated with continuous pRb activity and cell proliferation.

The similarity in miR-23b expression levels between cervical scrapes and biopsies of cervical cancer may be the result of the phenotypic similarity of squamocolumnar junction cells (also known as TZ) with cancer cells and HSIL cells. The subset of cuboidal cells in the squamocolumnar junction, which give rise to cancer, and the cancerous tissue maintain a common partial profile of gene expression [36, 37]. A high percentage of LSIL and HSIL disappear spontaneously [37] and is highly likely that in such cases the expression level of miR-23b is increased compared with Non-SIL. Thus, the expression level of miR-23b in SILs and cervical cancer agree with a tumor suppressor gene.

Evidence indicates that uPa, c-Met and Zeb1 are important promoters of tumor phenotype. uPA is a serine protease that modulates the turnover of the extracellular matrix and is related to metastatic tumor phenotypes

[38]. c-Met is recognized as a factor that induces cell migration, and Zeb1 is recognized as a factor that promotes epithelial-mesenchymal transition [32]. In highly malignant cervical cancer tissue, the expression of uPA, the loss of expression of CDH1 and nuclear expression of snai1 and zeb1 are strongly associated with advanced stages of cervical cancer and lymph node metastasis [32, 39].

In this study, the mRNA expression levels of uPa, c-Met and Zeb1, which are likely targets of miR-23b, were compared in HeLa, SiHa, CaSki and C33A cells with and without exposure to 5′-Aza-CdR. The expression levels of *uPa* decreased in CaSki and C33A cells after treatment. These findings partially agreed with those reported by Au Yeung *et al.*, who demonstrated that the overexpression of miR-23b decreases the expression of uPa in SiHa and CaSki cells. The increase in the expression of miR-23b after treatment with 5′-Aza-CdR may not be sufficient to inhibit the expression of uPa in SiHa and HeLa cells. In contrast, uPa expression is also influenced by the level of expression and activity of its transcription factors [40] as well as by methylation of its promoter region [41]. In cervical cancer tissue, the HR-HPV infection and subsequent alteration of miRNA expression contribute to deregulate the expression of uPa [25]. However, there is still insufficient evidence to state that miR-23b directly regulates the expression of uPa in this tissue.

The expression level of *c-Met* was significant lowest in HeLa cells after treatment with 5′-Aza-CdR, which agreed with data reported by Salvi *et al.* [38], who proposed that miR-23b is a regulator of c-Met expression. Although *zeb1* is a direct target of miR-23b in bladder cancer [42], there are no current studies that support this theory in cervical cancer. We found that the expression levels of *Zeb1* decrease significantly in C33A and CaSki cells after being treated with 5′-Aza-CdR, suggesting that miR-23b is involved in regulating the expression of *Zeb1* in these cells. Interestingly, in SiHa cells, the hypomethylating treatment resulted in increased expression of *uPa, c-Met* and *Zeb1*, thereby suggesting that other molecular mechanisms affected by changes in the levels of DNA methylation independent of miR-23b are involved in regulating these genes in SiHa cells.

To explain the differential expression of uPa, c-Met and Zeb1 in cervical cancer cell lines, it should be noted that miRNAs repress or stimulate gene expression in response to specific cellular conditions, sequences and cofactors. The biological outcome of the miRNA-mRNA interaction is influenced by the following factors: the percentage of base pairing between the miRNA and target site; the number and relative position of target sites for the same miRNA; accessibility of the site; sequences flanking the target site; and the mRNA secondary structure, which may influence the hybridization sequences [43]. Moreover, Vasudevan and Steitz reported that the gene repression mediated by a miRNA may be reversible [44]. Thus, the expression of uPa, c-Met and Zeb1, which are likely targets of miR-23b, can be the result of specific features of each cell type, but it is also likely that some of these mRNAs were translated into protein at the time of RNA collection. More detailed studies are needed to determine if uPa, c-Met and Zeb1 are genes regulated by miR-23b in cervical cancer.

Conclusions

In cervical cancer cell lines, miR-23b expression is regulated by its promoter methylation, and is likely the same process that occurs in cervical carcinogenesis. The decrease in miR-23b expression level is associated with cervical cancer, suggesting that this miRNA is a tumor suppressor in this cancer. The expression of uPa, c-Met and Zeb1 in cervical cancer cell lines is likely influenced by miR-23b, and the difference in the result of the miRNA-mRNA interaction is determined by factors related to the specific microenvironment of each cell type, with unique recognition sequences, cofactors and events that influence the biological outcome.

Material and methods

Culture of cervical cancer cell lines

The following cell lines were used: 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 cells were incubated at 37 °C in humidified atmosphere with 5 % CO_2 [15, 45].

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

Cells were seeded in 6-well plates (25×10^3 cells/well) and cultured for 72 h before treatment. The cells were treated with 10 μM 5-Aza-CdR dissolved in DMSO and added to fresh culture medium. The cultures were incubated at 37 °C in 5 % CO_2 for 24 h. The treatment was then repeated, and the incubation was continued for an additional 24 h under the same conditions [46, 47]. The assay was performed in triplicate for each cell line. Untreated cells were included as a control [45].

Patients and cell or cervical tissue samples

We studied 54 biopsies from women with cytopathological and histopathological diagnosis of squamous intraepithelial lesions (SILs) or cervical cancer with infection by HPV16. Of these biopsies, 19 were of low-grade

squamous intraepithelial lesions (LSILs), 7 were of high-grade squamous intraepithelial lesions (HSIL) and 28 were of cervical cancer. The samples were obtained during routine screening for detection of premalignant lesions or cervical cancer at the State Cancer Institute "Dr. Arturo Beltran Ortega" in Acapulco, Guerrero. We included 18 cervical scrapings from women who had cervical cytologies without squamous intraepithelial lesions (non-SILs) and who were infected with or without HPV16. These women attended the Immunohistochemistry and Cytopathology Laboratory of the Autonomous University of Guerrero (Chilpancingo, Guerrero, Mexico) for timely detection of cervical cancer.

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 performed with TRIzol reagent according to the manufacturer's instructions. The integrity of both nucleic acids was determined by electrophoretic shift in a 1 % agarose gel [48]. The DNA was stored at −20 °C, and the RNA was stored at −70 °C.

Detection and typing of HPV16

Detection and typing of HPV was performed using the 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 the 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 methylation-sensitive and methylation-dependent restriction enzymes. The DNA that remained after digestion was added to the matrix. The ABI 7500 system for real-time PCR was used to read the plates. The relative amount of methylated and unmethylated DNA was calculated using the standard ΔCt method, normalizing the amount of DNA in each digestion against the total amount of input DNA in a 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 an internal reference for the expression of miR-23b. The relative expression of both miRNAs was analyzed by the comparative Ct method.

Quantitative polymerase chain reaction (qPCR) assays of upa, c-met and zeb1

The qPCR assays were performed according to the manufacturer's instructions with a One-Step qRT-PCR kit (KAPA SYBR® FAST, Boston, Massachusetts, USA). Using qPCR assays, we assessed mRNA expression levels of the following genes: uPa (5′-GTCGTGAGCGACTCCAAA GGCA-3′ and 5′- GGGCAGTTGCACCAGTGAATG T-3′), c-Met (5′-TATTTCCCAGATCATCCATTGCA-3′ and 5′- AATGTAGGACTGGTCCGTCAAAA-3′) and Zeb1 (5′- GCACCTGAAGAGGACCAGAG-3′ and 5′- T GCATCTGGTGTTCCATTTT-3′). We used GAPDH (5′ GGTGAAGGTCGGTGTGAACG-3′ and 5′ CTCGCTC CTGGAAGATGGTG-3′) as the internal reference. The thermal cycling profile was as follows: cDNA synthesis step was performed using 200 ng of total RNA at 42 °C for 5 min, followed by an inactivation RT step at 95 °C for 5 min, and 40 cycles of a denaturation step at 95 °C for 3 s, an annealing/extension step at 60 °C for 30 s, and a dissociation step according to the instrument guidelines. The qPCR assay was independently repeated three times using the StepOne system for real-time PCR (Applied Biosystems, Foster City, CA). The relative expression of uPa, c-Met and Zeb1 was analyzed by the comparative Ct method.

Data analysis

Using the STATA statistical package (version 9.2), we determined the frequency of methylation of the promoter region of miR-23b, and the same statistical package was used to determine the expression changes of miR-23b, uPa, c-Met and Zeb1 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 miR-23b expression was determined, and the difference between groups was calculated by the Mann-Whitney's 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.

Abbreviations

HR-HPV: High-Risk Human Papillomavirus; microRNA: miRNA; uPA; *upa*: Urokinase-type Plasminogen Activator; DNMT1: DNA methyltransferase 1; 5′-Aza-CdR: 5′Aza-2-deoxycytidine; Non-SIL: Without squamous intraepithelial lesion; SIL: Squamous intraepithelial lesion; LSIL: Low-grade squamous intraepithelial; HSIL: High-grade squamous intraepithelial lesion; CDH1: E-cadherin; EMT: Epithelial-Mesenchymal Transition; Snai1: Zinc finger

protein SNAI1; ZEB1: Zinc finger E-box-binding homeobox 1 and 2; DMSO: Dimethylsulfoxide; DMEM: Dulbecco's modified eagle médium.

Competing interest
The authors declare no conflict of interest.

Authors' contributions
GFT, HJW conceived and designed the experiments and coordinated the study and wrote the draft; GECV performed the experiments and wrote the paper; GTA, DSF contributed to performed the experiments; LdelCAR, MAJL did the cytological and histopathological diagnosis; DHS, OPZ made the donation of cell lines. and contributed with reagents/materials; BIA contributed with molecular diagnosis of HPV and reagents/materials. All authors have been reading and approved the final manuscript.

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