UNIVERSIDAD NACIONAL AUTÓNOMA DE MEXICO
DOCTORADO EN CIENCIAS BIOMÉDICAS
INSTITUTO DE INVESTIGACIONES BIOMÉDICAS

“EFECTO DE ANTIOXIDANTES Y LA ACTIVACIÓN DE LA VÍA Keap1/Nrf2 EN EL AUMENTO DE LOS NIVELES DE p53 EN LÍNEAS CELULARES DERIVADAS DE CaCU”

TESIS

QUE PARA OPTAR POR EL GRADO DE:

DOCTOR EN CIENCIAS BIOMÉDICAS

PRESENTA:

M en C. Carlos César Patiño Morales

DIRECTOR DE TESIS:
Dr. Alejandro García Carrancá
Instituto de Investigaciones Biomédicas, UNAM.
Unidad de Investigación Biomédica en Cáncer, INCAN

COMITÉ TUTOR:
Dr. Emilio Rojas del Castillo
Instituto de Investigaciones Biomédicas, UNAM.

Dr. Alfredo Hidalgo Miranda
Facultad de Medicina, UNAM.
Instituto Nacional de Medicina Genómica

Ciudad Universitaria, Cd. Mx., Noviembre de 2019.

Dedicatorias

A mis abuelos:

Elvira y Heladio los amo y estoy seguro que desde el cielo
están felices de ver que sí pude, fueron parte importante para que hoy yo esté aquí.

A mi madre:

Maricela, gracias a ti he podido llegar tan lejos, tus ejemplo, tus consejos, tu amor, tu paciencia, tu dándome ánimos, tu guiándome a cada pasó que doy, tu apoyándome en todo momento, todo lo que soy es gracias a ti, te amo.

A todos aquellos que trabajan muy duro para materializar sus sueños, todos aquellos que creen en que los sueños sí se convierten en realidad.

Sing with me, sing for the years
Sing for the laughter, sing for the tears
Sing with me, just for today
Dream on, dream on, dream on
Dream until your dreams come true

Dream on by Steven Tyler

AGRADECIMIENTOS

A mi Ángel muchas gracias por cuidarme, por estar ahí siempre guiándome y gracias por nunca abandonarme, gracias a tu apoyo yo he podido lograr esto.

A la Universidad Nacional Autónoma de México, el Instituto de Investigaciones Biomédicas y el Doctorado en Ciencias Biomédicas, gracias por ser parte de mi formación por todos los momentos tan felices que pasé ahí.

Al Instituto Nacional de Cancerología ha sido un sueño haber estudiado en unos de los mejores institutos del país gracias por enseñarme lo maravillosa que es la ciencia.

A la Universidad Autónoma Metropolitana por darme la oportunidad de crecer y por creer en mi trabajo, por permitirme seguir haciendo investigación y demostrarme que no me equivoqué al elegir la ciencia.

Al Dr. Alejandro García Carrancá, gracias por darme un espacio en su laboratorio, por creer en mi y por creer en el proyecto, gracias por todo lo que me enseñó.

Al Dr Emilio Rojas del Castillo y el Dr Alfredo Hidalgo Miranda por su paciencia, apoyo y dedicación en el desarrollo del proyecto,

Al Dr. Ernesto Soto Reyes, la Dra Elena Arechaga Ocampo y la Dra. Claudia Haydée Gonzalez de la Rosa, son personas increíble y un ejemplo de investigadores gracias por todo su apoyo, los admiro muchísimo.

A mi familia que aunque estamos lejos sé que siempre están ahí así como las estrellas. Tripis, Toño, Mary, Diana, Manuelito(bebé). Tía Lupita, Karen, David, Alex,.

A mis compañeros y amigos del laboratorio somos una gran familia y estoy muy feliz de haberlos conocido y compartido con ustedes estos años: Vero A, Vero M y Vero B, Peter, Karen, Mariel, Marco, Helga, Mike, Leny, Lalo, Silvia, Heriberto, Ivette.

A Miriam y Rocío y la Dra Elizabeth Ortiz, gracias por todo lo que me enseñaron, forman parte de un equipo de trabajo maravillosos en el laboratorio de Virus y Cáncer

A mi Coach Angie Herrera por enseñarme que soy más fuerte de lo que me imaginaba.

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Resumen:

El cáncer continua siendo un problema importante de salud y por tanto las investigaciones sobre nuevas alternativas preventivas y terapéuticas para disminuir la mortalidad del mismo son muy importantes. Aunque la quimioterapia y la radioterapia son los tratamientos por excelencia, estos disminuyen la calidad de vida de los pacientes, por lo tanto ha cobrado mucho interés en varios grupos de investigación el uso de compuestos naturales que puedan coadyuvar en el tratamiento rutinario de los pacientes con cáncer. Los antioxidantes naturales presentan una alternativa prometedora a nivel preventivo y terapéutico contra el cáncer. Entre estos compuestos, la curcumina tiene además de propiedades antioxidantes, propiedades antineoplásicas, incluida la modulación de p53. La regulación de p53 se ha sugerido como una estrategia importante en la terapia contra el cáncer. El objetivo de este estudio fue determinar el mecanismo por el cual la curcumina restaura los niveles de p53 en las líneas celulares de cáncer humano. Las células HeLa, SiHa, CaSki y MDA-MB-231 fueron expuestas a la curcumina y se realizaron ensayos de pulso y caza e inmunoprecipitación. Aquí mostramos que la curcumina aumenta la vida media de p53 promoviendo su interacción física con la proteína NQO. Curiosamente, el ensayo de viabilidad celular después del tratamiento con curcumina mostró que la actividad citotóxica fue selectivamente mayor en las células de cáncer de cuello uterino que contenían p53 silvestre pero no en las células de cáncer de mama que contenían p53 mutado. El efecto citotóxico de la curcumina en las células de cáncer de cuello uterino se relacionó con el complejo p53-NQO1 que evita la interacción entre p53 y su regulador negativo, la proteína asociada a ubiquitin ligasa E6 (E6AP). Finalmente, demostramos que en las células PANC1 (provenientes de carcinoma epitelial pancreático nulas para NQO1) el restablecimiento de la expresión de NQO1 puede estabilizar p53 en presencia de curcumina. En conjunto, nuestros hallazgos mostraron que la curcumina es necesaria para promover la interacción NQO1-p53, por lo tanto, aumenta la vida media de p53 y permite el efecto citotóxico de la curcumina en las células tumorales que contienen p53 silvestre.

2. Introducción:

2.1 Cáncer

La Organización Mundial de la Salud, define al cáncer como “un término genérico que designa un grupo de enfermedades que se caracterizan por la multiplicación de células anormales que invaden partes adyacentes del cuerpo o se propagan a otros órganos. El cáncer sigue siendo un problema de salud importante, según la Organización Mundial de la Salud es considerado como la segunda causa de muerte a nivel mundial ya que una de cada seis defunciones se debe a esta enfermedad. (https://www.who.int/en/news-room/fact-sheets/detail/cancer).

Las características distintivas del cáncer comprenden capacidades biológicas adquiridas durante el desarrollo tumoral incluyendo alta capacidad proliferativa, evasión de las señales supresoras del crecimiento, resistencia a la apoptosis, inducción de angiogénesis, metástasis, inestabilidad genómica e inflamación. En la última década se han agregado dos características emergentes: la alteración del metabolismo energético y la evasión de la respuesta inmune. Además de las células cancerosas, los tumores contienen gran cantidad de células que no son tumorales (es decir son “normales”) pero que tienen funciones alteradas, este grupo de células contribuyen a la adquisición de rasgos distintivos del cáncer al crear el "microambiente tumoral" (Hanahan & Weinberg 2011).

Existen múltiples factores de riesgo relacionados con cáncer, entre los cuales pueden destacarse: el consumo de tabaco y de alcohol, algunas infecciones virales o bacterianas, la inactividad física, la mala alimentación y la predisposición genética. (Athreya & Xavier 2017). En los últimos años la alimentación y su relación con el cáncer ha tomado interés por un gran número de investigadores, la vitamina E y C, el selenio, carotenoides, polifenoles, licopeno, y extractos de té verde son algunas de las sustancias que han sido estudiadas con detalle no solo por sus efectos antioxidantes si no también por sus propiedades antiinflamatorias y antitumorales (Chamberlin *et al*. 2019).

Los tratamientos actuales para el cáncer incluyen cirugía, radioterapia, quimioterapia, terapia hormonal, inmunoterapia y terapias dirigidas (Palumbo *et al*.

2013), no son efectivos en todos los casos, además debido a la cantidad de efectos secundarios que provocan, se sigue en la búsqueda de nuevos tratamientos que no disminuyan la calidad de vida de los pacientes. Los antioxidantes y compuestos naturales han sido estudiados por poseer este potencial terapéutico. Los efectos benéficos del consumo de antioxidantes se han reportado desde hace tiempo, en 1951, en un estudio *in vitro*, se pudo demostrar el efecto del α-tocoferol en la reducción de tumores hepáticos (Euler 1951), Chan & Black 1975 realizaron un trabajo en ratones expuestos a la luz UV y encontraron que los ratones alimentados con antioxidantes mantuvieron tasas más bajas de fotooxidación de macromoléculas como el colesterol, los autores concluyeron que los antioxidantes de la dieta inhibían el inicio de tumores y la progresión de las lesiones precancerosas.

2.1.1 Antioxidantes y cáncer

Un antioxidante se define como una molécula o elemento que tiene la capacidad de donar un electrón a un radical libre y neutralizarlo, reduciendo así su capacidad de dañar macromoléculas como ADN, proteínas o lípidos. Estos antioxidantes retrasan o inhiben el daño celular a través de su propiedad de eliminación de radicales libres (Halliwell 1986).

Los antioxidantes pueden ejercer su efecto sobre los sistemas biológicos mediante diferentes mecanismos, incluida la donación de electrones, la captura de iones metálicos o la regulación de la expresión génica. (Kancheva 2009). También existen varios sistemas que el organismo produce y que eliminan los radicales libres, en los alimentos, los antioxidantes que principalmente encontramos son la vitamina E (α-tocoferol), la vitamina C (ácido ascórbico) y el caroteno (Rahman 2007). La Organización Mundial de la Salud recomiendan el consumo de al menos 5 raciones diarias de frutas y/o verduras con la finalidad de aportar la ingesta adecuada de antioxidantes naturales principalmente vitaminas, carotenoides y otros compuestos fenólicos presentes en estos para mejorar el estado de salud general (Prior 2003).

El estrés oxidante se define como el desequilibrio entre la generación de especies reactivas oxígeno y su neutralización por el organismo. El estrés oxidante ha sido implicado en el desarrollo de numerosas enfermedades crónicas y el envejecimiento (Whiteman *et al.* 2004).

Un exceso de estrés oxidante en las células tienen como resultad la oxidación de ADN, lípidos y proteínas, lo que se relaciona con cambios en su estructura y funciones. Las especies reactivas de oxígeno (ROS) y nitrógeno (NOS); como el anión superóxido, el oxígeno singulete, el peróxido de hidrógeno, el radical hidroxilo y el óxido nítrico así como sus metabolitos biológicos juegan un papel importante en la carcinogénesis. Las ROS y NOS inducen daños en el ADN que incluye la modificación de las bases, ruptura de la cadena y formación de enlaces cruzados en proteínas y el ADN. Numerosos investigadores han propuesto la participación de radicales libres en la generación de mutaciones y la transformación celular. Los antioxidantes pueden disminuir la carcinogénesis inducida por el estrés oxidante mediante neutralización directa de ROS o regulando genes que permiten la expresión de proteínas con efecto antioxidante (Mut-Salud *et al.* 2016).

Antioxidantes naturales como el β-caroteno tienen propiedades fotoprotectoras y se ha reportado que protege a las células contra el daño inducido por la luz ultravioleta. Existe evidencia de que otros antioxidantes como la vitamina C también tiene un efecto antitumoral (Halabi *et al.* 2018) no solo por sus efectos antioxidantes (es decir su capacidad de neutralizar especies reactivas de oxígeno), si no también por su papel en la inhibición de la formación de nitrosaminas y el mejoramiento de la respuesta inmune. La vitamina E, es otro antioxidante del cual se ha reportado un efecto antitumoral, desempeña un papel importante al aumentar la respuesta inmune humoral, y la inmunidad celular, la producción del factor de necrosis tumoral y la reparación ADN (Das Gupta & Suh 2016).

Debido a que la prevención de enfermedades crónicas constituye una mejor estrategia que su tratamiento, reducir el riesgo de enfermedades como el cáncer ha acaparado la atención entre los profesionales de la salud, científicos y

personas dedicadas a la industria alimentaria (Liu 2003). Por esta razón, muchos alimentos funcionales (como el huevo rico en selenio) son diseñados actualmente con el objetivo de proporcionar una ingesta elevada de antioxidantes y reducir el riesgo de enfermedades asociadas al estrés oxidante. (Rafter 2002).

Entre los alimentos que contienen antioxidantes podemos encontrar; las frutas rojas y bayas que son ricas en compuestos fenólicos, principalmente flavonoides, los cuales se caracterizan por su actividad anticarcinogénica, antiinflamatoria, antiaterogénica y antimicrobiana (Moure et al. 2001).

Hay evidencia epidemiológica de que una dieta rica en frutas y verduras podría reducir el riesgo de ciertos tipos de cáncer. El efecto se ha atribuido principalmente a polifenoles naturales. Estructuralmente los polifenoles se caracterizan por tener al menos un anillo aromático con uno o más grupos funcionales hidroxilo unidos, están presentes en frutas, verduras, especias, soya, nueces, té y vino. Con base en su estructura química, los polifenoles se pueden dividir en cinco clases: flavonoides, ácidos fenólicos, lignanos y estilbenos (Santos-Buelga *et al*. 2019) y se ha comprobado que los polifenoles tienen efecto en la activación o en la inhibición de diferentes vías de señalización celular.

3. Curcumina

La curcumina es un compuesto polifenólico ampliamente conocido por su uso en la cocina asiática e Indú, pero también por sus propiedades antioxidantes, antimicrobiana, inmunomoduladoras y antitumorales. La curcumina también llamada: 1,7-bis-(4-hidroxi-3-metoxifenil)-1,6-heptadieno-3,5-diona, es un polvo cristalino de color amarillo o anaranjado, de fórmula molecular $C_{21}H_2O_6$ y un punto de fusión 183 °C, soluble en alcohol y ácido acético. A pH 3 la curcumina tiene color amarillo y a pH 10 tiene color anaranjado ó marrón-rojizo, es estable al calor, pero sensible a la luz. Este ingrediente activo comprende 2-5% de la cúrcuma (*Curcuma longa*). La curcumina ha sido usada como condimento y agente colorante durante cientos de años pero también se considera que tiene propiedades antitumorales (Kunnumakkara *et al*. 2017). Estudios en humanos han demostrado que la curcumina es generalmente reconocida como segura y tolerada

a dosis muy altas. Un ensayo clínico Fase I mostró que las dosis orales de hasta 8 g/día durante 3 meses no tenían ningún efecto tóxico (Cheng *et al*. 2001).

3.1 Efecto antitumoral de la curcumina

Se ha descrito que la curcumina, sola o en combinación con otros agentes, es utilizada para la prevención y el tratamiento del cáncer, entre los que destacan el colorrectal, el pancreático, cáncer de mama, cáncer de próstata, mieloma múltiple, cáncer de pulmón y cáncer oral (Anand *et al*. 2008). Las propiedades antitumorales de la curcumina se atribuye a mecanismos proapoptóticos, antiproliferativos, antioxidantes y antiinflamatorios, la curcumina tiene afecto en múltiples vías de señalización; por un lado inhibiendo la proliferación celular y por otro regulando la apoptosis, se ha reportado la participación de la curcumina en la modulación de la apoptosis, la señalización de p53, la vía del factor nuclear-kB (NF-κB), la vía de la proteína quinasa activada por mitógeno (MAPK), la Ruta de Akt, la ruta de señalización de Notch-1, la ruta de factor nuclear 2 (Nrf2), la vía de Wnt/β-catenina, vía de señalización, la vía JAK/STAT , la vía AMPK/COX-2, (Ravindran *et al*. 2009)

Estructuralmente la curcumina posee un alto nivel de metoxilación y un bajo nivel de hidrogenación, que le permiten mejorar la eliminación de radicales libres. La estructura de la curcumina es en parte responsable de los efectos antitumorales y antiinflamatorios (Kocaadam & Sanlier 2017).

Aunque se conoce que la curcumina tiene diversos blancos, en el tratamiento contra el cáncer, el uso de antioxidantes es controversial, y los efectos que la curcumina y otros antioxidantes puedan tener a nivel celular dependen ampliamente de la dosis. Aunque se espera que tenga un efecto benéfico, se ha reportado que la curcumina tiene un efecto no benéfico en pacientes con cáncer ya que en algunos casos promueve el crecimiento del tumor por ejemplo en los casos de melanoma (Lawenda et al. 2008). Por lo que se puede afirmar que la ingesta de antioxidantes dietéticos o naturales siempre es mejor a nivel preventivo, la modulación de antioxidante y la actividad de radicales libres es una interacción compleja que tiene una apariencia limitada durante experimentos *in vitro*; por lo

tanto, el resultado del uso de antioxidantes naturales o dietéticos en pacientes es en ocasiones incierto, puede depender de el tipo de cáncer que se esté tratando, el grado de progresión y definitivamente la dosis de antioxidantes que se consuma (Pathak *et al*. 2003, Heber *et al*. 2001) .

Algunos estudios demuestran que hay una disminución en el riesgo y la mortalidad después de la ingesta dietética de antioxidantes o suplementos alimenticios con antioxidantes en pacientes con cáncer (Turley *et al.* 1997) otros estudios afirman que el uso de antioxidantes pueden promover el crecimiento tumoral (Sayin *et al*. 2014) Existen reportes que demuestran que, las altas dosis de antioxidantes se han correlacionado con el crecimiento de células cancerosas (Prasad *et al*. 1999).

4. Antioxidantes y la vía Keap1/Nrf2

Como ya se ha mencionado el estrés oxidante es una de los factores que contribuye en el inicio y la progresión de diversas enfermedades crónicas (Sies 2015) y aunque existen muchos factores cotidianos que generan ROS las células poseen mecanismos para contrarrestar las especies reactivas de oxígeno (ERO) y de nitrógeno (ERN) que el organismo genera diariamente, la reducción o eliminación tanto de ERO como de ERN se lleva a cabo en dos fases:

En la fase I, participan enzimas que metabolizan carcinógenos y xenobióticos, principalmente enzimas de la familia de los citocromos P450. En la fase II, se reducen los metabolitos de carcinógenos y xenobióticos producidos en la fase I, algunas de las enzimas que participan en la fase II son la NADPH:quinona oxidorreductasa 1 (NQO1), la glutatión s-transferasa (GST), las UDP-glucuronosil transferasas, la hidrolasa epóxica, la γ-glutamil cisteína sintetasa (γ-GCS), la ferritina y la hemo oxigenasa 1 (Lu *et al*. 2016).

Se han descrito más de 100 genes involucrados en la respuesta antioxidante entre los que destacan el factor nuclear κB (NF-κB), la proteína activadora 1 (AP1) y el factor relacionado al factor nuclear eritroide-2 Nrf2 (Nuclear Factor Erythroid 2-related factor), Nrf2 es un factor de transcripción que se activa en respuesta a cambios en el estado redox, se expresa de manera constitutiva en las células pero se encuentra regulado negativamente por la proteína semejante a Kelch (Keap1) la cual promueve su degradación por la vía de ubiquitina proteosoma (Kaspar *et*

al. 2009). Cuando la vía Keap1/Nrf2 se activa, Nrf2 pierde interacción con su regulador negativo Keap1 y se transloca al núcleo donde actúa como factor de transcripción uniéndose al promotor de enzimas antioxidantes de fase II, el análisis genómico de los promotores de estas enzimas ha revelado la presencia de secuencias específicas que comparten entre si, estas secuencias se conocen como ARE (Antioxidant Response Element), 5'-TGACnnnGCA3' (Kobayashi et al. 2004). Nrf2 posee un dominio de "zipper de leucinas (bZip) en la región C-terminal. Así mismo posee una región homóloga a la proteína "cap´n´collar" (CNC) de Drosophila. Venugopal y Jaiswal en 1996 reportaron por primera vez evidencia del papel de Nrf2 en la protección contra el estrés oxidante (Venugopal & Jaiswal 1996), ellos demostraron que la sobreexpresión de Nrf2 aumentaba le expresión de enzimas de fase II como NQO1 en respuesta a antioxidantes y xenobióticos.

Keap1 es un regulador negativo y suprime la translocación nuclear de Nrf2 y por lo tanto su actividad transcripcional. Se ha demostrado que la sola interacción de Keap1 con Nrf2 es insuficiente para mantener a Nrf2 en el citosol y se ha reportado que Keap1 se asocia al mismo tiempo con el citoesqueleto. Keap1 forma un complejo tanto con Nrf2 como con los filamentos de actina es decir; el complejo Keap1-Nrf2 se forma y se retiene en el citosol mediante interacciones con el citoesqueleto (Kang *et al.* 2004) .

4.1 Regulación de la vía Keap1/Nrf2

La degradación de Nrf2 es dependiente de Keap1 ya que funciona como un adaptador para dirigir a Nrf2 al proteasoma. En cuanto al mecanismo de degradación de proteínas por el proteosoma, es importante mencionar que su selectividad y especificidad están dadas por una proteína de 76 aminoácidos llamada ubiquitina (UBQ) que interacciona con las proteínas que serán degradadas. Las proteínas ubiquitinadas (o poliubiquitinadas) son reconocidas por un complejo de proteasas conocido como proteosoma 26S que las digiere. El proceso de poli-ubiquitinación y reconocimiento requiere de la participación de varias enzimas: las E1 para la activación de la UBQ, las E2 para la conjugación de la UBQ y las E3 para su unión. Existen una gran cantidad de ligasas E3 y destaca la familia de ligasas denominadas "Cullin-based" (Cul). Se ha demostrado que

Keap1 funciona como un adaptador que une a Nrf2 con la ligasa Cul-3 (Cullinan *et al.* 2004). Por lo tanto la activación de Nrf2 y su translocación al núcleo dependen directamente de su disociación de Keap1, esta perdida de interacción entre Keap1-Nrf2 es fomentada por el estrés oxidante. Debido a que Keap1 tiene una región rica en cisteínas, posee una función dual, por un lado como adaptador de la ligasa Cul-3 para la degradación de Nrf2 y al mismo tiempo un sensor de estrés oxidante (Revisado en Furukawa & Xiong 2005). Se ha sugerido a la cisteína 183 como o las cisteínas 273 y 288 como responsables de censar los niveles de estrés oxidante. En general se han propuesto 7 residuos de cisteína en Keap1 que se oxidan o se modifican covalentemente permitiendo la disociación del complejo Nrf2-Keap1. Adicionalmente, la actividad de Nrf2 puede ser modulada por modificaciones postraduccionales, como las fosforilaciones en serinas y treoninas por cinasas como fosfatidilinositol 3-cinasa (PI3K), proteína cinasa C (PKC), la cinasa c-Jun y la cinasa ERK. La fosforilación por estas enzimas promueve la disociación de Nrf2 de Keap1 y su translocación al núcleo. Como respuesta al estrés oxidante, la cascada de señalizaciones mediada por PI3K produce la despolimerización de los filamentos de actina, facilitando la translocación de Nrf2 (Revisado en Richardson *et al*. 2015)

Una vez que Nrf2 ha logrado translocarse al núcleo, forma dímeros con las proteínas MafG, MafK y MafF, con c-Maf u otras como c-Fos, Fra1, p45-NF-E2, Bach1 y Bach2. Se ha llegado a la conclusión de que Nrf2, requiere asociarse a las proteínas Maf de manera obligatoria para lograr unirse al sitio promotor específico de ARE que se encuentra en el promotor de enzimas antioxidantes de fase II y promover su transcripción (Copple *et al.* 2014) .

Algunos compuestos naturales, en su mayoría antioxidantes como la curcumina o el sulforafano, son inductores químicos fuertes de las enzimas citoprotectoras de fase II dependientes de ARE, es decir son activadores de la vía Keap1/Nrf2 (Lin *et al.* 2019).

Se han clasificado alrededor de 90 activadores químicos Nrf2 entre los que se encuentran: (1) aceptores Michael; (2) fenoles y quinonas oxidables; (3) isotiocianatos y sulfoxitiocarbamatos; (4) ditioletionas y sulfuros de dialilo; (5)

arsenicales trivalentes; (6) dimercaptanos; (7) compuestos que contienen selenio; (8) polienos; (9) hidroxiperóxidos; (10) metales pesados y complejos metálicos; y (11) inductores diversos (Magesh *et al.* 2012). Por ejemplo se sabe que la curcumina posee aceptores de Michael que se conjugan directamente con los residuos de cisteína (Cys) en Keap1 y estas modificaciones conducen a un cambio conformacional, lo que resulta en la liberación de Nrf2 (Deny *et al.* 2016). Existen más de 20 residuos de cisteína encontrados en Keap1, entre los que destacan Cys-151, Cys-273 y Cys-288 que se han identificado como sensores críticos de estrés oxidante (Suzuki *et al.* 2019).

Se sabe que los compuestos naturales como los antioxidantes tienen un efecto benéfico en la salud humana, es interesante resaltar que todos los compuestos naturales con aceptores de Michael como la curcumina pueden modificar directamente a Keap1 y promover la expresión de enzimas antioxidantes de fase II, el estudio de estos compuesto ha cobrado interés en diversos grupos de investigación para poder describir sus mecanismos quimiopreventivos (Paunkov *et al*. 2019).

5. Generalidades de p53

La proteína p53, fue descubierta a finales de la década de los 70 e identificada como una fosfoproteína celular de 53 kDa capaz de enlazarse al antígeno T SV40, (Brown 1997). Una importante función de p53 es el control del ciclo celular en el paso de la fase G1 a la fase S. Cuando se han producido lesiones en el ADN, p53 arresta el ciclo celular para permitir que actúen los sistemas de reparación del ADN y de esta forma asegurar la integridad genómica, en caso de que el daño ocasionado al ADN no sea reparable, p53 regula el proceso de apoptosis. Mientras la forma silvestre de p53 actúa como un gen supresor de tumor, las formas mutantes tienen características oncogénicas es por ello que las mutación en p53 son una característica frecuentemente encontrada en diferentes tumores humanos (Meng *et al.* 2014). p53 está presente normalmente en cantidades muy pequeñas, pero cuando las células son expuestas a estímulos genotóxicos, los niveles de p53 se incrementan rápidamente (Sionov & Haupt 1999).

El gen p53 está localizado en el brazo corto del cromosoma 17, (17p13.1), y tiene una longitud aproximada de 20 kb. La proteína *wild type* normalmente se encuentra en el núcleo celular y tiene una vida media muy corta de aproximadamente 20 minutos (Binayke et al. 2019). Esta proteína está siendo producida constantemente pero es rápidamente degradada (Chao 2015). p53 tiene varios dominios, tales como la región amino terminal (N-terminal) que es esencial para la transactivación, el dominio core que contiene una secuencia de unión al ADN y el dominio carboxilo terminal que tiene propiedades reguladoras. Las modificaciones postraduccionales de la región carboxilo terminal por acetilación, fosforilación y O-glicosilación generan cambios conformacionales en p53 que regulan la unión específica a secuencias del ADN así como el reconocimiento de ADN dañado. Aproximadamente el 50% de los cánceres poseen mutaciones en p53 que pueden impedir la oligomerización y formación de complejos tetraméricos capaces de unirse a secuencias específicas del ADN. El espectro de las mutaciones de p53 en los cánceres proporciona claves sobre la etiología y patogénesis molecular de las neoplasias ya que los cánceres con mutaciones en p53 tienen un mal pronóstico, en cánceres con p53 *wild type* como el cervicouterino, p53 sigue siendo un blanco terapéutico importante (Zhou *et al.* 2016)

5.1 Regulación negativa de p53

El mecanismo principal para el control de la estabilización y activación de p53 (en células que no están infectadas con el virus de papiloma humano VPH) depende de su interacción y ubiquitinación por MDM2. Es importante destacar que MDM2 es el producto de un gen inducible por p53 (Wade *et al*. 2010). Por lo tanto, las dos moléculas están relacionadas a través de un ciclo de retroalimentación negativa con el objetivo de mantener niveles bajos de p53 en ausencia de estrés. MDM2 es una fosfoproteína de 491 aminoácidos que interactúa a través de su dominio terminal con el dominio de transactivación de p53 (Wang *et al.* 2019). La unión de MDM2 al dominio de transactivación de p53 bloquea su actividad transcripcional y además, MDM2 funciona como la ligasa E3 que ubiquitina p53 para su

degradación. La estructura cristalina del complejo p53-MDM2 ha sido resuelta. La interacción directa entre las dos proteínas se ha localizado en un dominio hidrofóbico relativamente pequeño (aa 25-109) en el extremo NH2 de MDM2 y un péptido anfipático 15-aa en el extremo NH2 de p53, los residuos Gly58, Glu68, Val75 o Cys77 en MDM2 son indispensables para la unión con p53 (Xu *et a*l. 2015). Un requisito básico pero insuficiente para la degradación de p53 es la interacción directa entre p53 y MDM2 a través de su región aminoterminal. Los requisitos adicionales sobre p53 incluyen que su región de tetramerización esté intacto ya que mejora la degradación posiblemente favoreciendo la unión con MDM2, se ha descrito que mutaciones en el sitio de tetramerización de p53 provocan resistencia a la degradación por MDM2 (Kastan *et al*. 1991).

5.2 Virus del papiloma humano (VPH) y la degradación de p53:

El VPH es un virus de ADN circular de doble cadena cuyo genoma está dividido en tres regiones, una región temprana E , (que codifica para las proteínas virales E1, E2, E4, E5, E6 y E7), una región tardía L que codifica las proteínas L1 y L2; y una región reguladora (LCR). Cuando el virus infecta a la célula, puede permanecer en estado latente (episomal o no integrado) o integrar su genoma al de la célula huésped y este es un paso importante en el desarrollo de cáncer cervicouterino. Cuando el VPH integra su genoma al de la célula huésped se produce la sobreexpresión de los oncogenes virales E6 y E7 debido a la falta del represor E2 (Harden & Munger 2017). El proceso de integración puede ser inducido por diferentes mecanismos; por ejemplo, la ruptura del cromosoma sitios frágiles (Thorland *et al.* 2000), modificaciones epigenéticas (Groves *et al.* 2016), una disminución en el actividad de maquinaria de reparación de ADN (Winder *et al*. 2007)o estrés oxidante (Chen Wongworawat *et al*. 2016). Se ha descubierto que la expresión de oncoproteínas virales juega un papel importante en el estado redox celular y por consiguiente en la carcinogénesis cervical, por ejemplo E6 disminuyen la expresión y la actividad de la catalasa y aumenta el daño del ADN mediado por especies reactivas de oxígeno (ROS) (Cruz-Gregorio *et al*. 2018).

El gen E6 del virus del papiloma humano codifica para una proteína de 150 aminoácidos y contienen dos motivos dedos de zinc, caracterizados por la presencia del motivo Cys-X-X-Cys, (Prati *et al*. 2018). El gen E6 se expresa de forma temprana durante el ciclo viral, lo que le permite bloquear la apoptosis, regular la transcripción viral, abatir la diferenciación celular y las interacciones célula-célula, e incrementar la inestabilidad genómica (Graham 2017). La proteína E6, puede asociarse con p53 y marcarlo para su degradación vía ubiquitina proteasoma, en el caso del cáncer cervicouterino las células contienen p53 silvestre, es decir la vía de p53 se encuentra intacta pero está enmascarada por efecto de la proteína E6. p53 es considerado el guardián del genoma ya que se activa en condiciones de estrés celular como radiación UV, hipoxia o infecciones virales: la cantidad de la proteína puede ser incrementada por estabilización (interaccionando con otras proteínas como NQO1) o por modificaciones post-transcripcionales, produciendo un bloqueo celular en la fase G1. Este bloqueo, permite a la célula reparar el daño al DNA antes de que el ciclo celular continúe (Revisado en Joerger & Fersht 2016). p53, es un factor de transcripción que promueve la expresión de genes involucrados en la regulación del ciclo celular y apoptosis. E6 se une a p53 y la conduce a su degradación a través de la vía de la ubiquitina. En este proceso participa la proteína celular asociada a E6 (E6AP), que actúa como una ubiquitín ligasa. Para que la degradación de p53 sea efectiva es necesario que se forme el complejo E6-E6AP-p53. E6 al unirse a p53 también puede producir su retención en el citoplasma, bloqueando su translocación hacia el núcleo e inhibiendo su función (Doorbar 2005).

Moléculas estabilizadoras de p53:

Además de MDM2 existen otras proteínas que contribuyen a la estabilización de p53 en respuesta a diferentes estímulos de estrés. La prevención de la ubiquitinación de p53 al reducir su interacción con MDM2 es una forma de estabilizar p53, otra posibilidad es mediante la eliminación de las ubiquitinas, por ejemplo HAUSP es una enzima que puede causar estabilidad de p53 mediante este proceso (Li *et al*. 2004). Se ha demostrado que la sobreexpresión de HAUSP

estabiliza p53 causando arresto en el ciclo celular y apoptosis (Cummins & Vogelstein 2004).También se ha sugerido que la degradación de p53 ocurre de manera independiente de MDM2 y la ubiquitinación. Posteriormente se demostró que este proceso está mediado por el proteasoma 20S y está regulado por la NAD(P)H quinona oxidorreductasa 1 (NQO1). (Asher & Shaul 2005). Sin embargo también se ha reportado que p53 interactúa con NQO1 y provoca su estabilidad, la cantidad de unión de p53 a NQO1 aumentó en respuesta al daño en el ADN y esto fue evitado por dicoumarol, un compuesto que compite por la unión de NADH a NQO1 es decir que NQO1 debe estar funcionalmente activa para poder interaccionar con p53. Parece probable que NQO1 se asocia con p53 de una manera dependiente de NADH para protegerlo de la degradación (Asher *et al*. 2002).

Varias proteínas distintas de MDM2 regulan la estabilidad de p53, algunas al influir en la interacción entre MDM2 y p53 y otras por mecanismos independientes de MDM2, por ejemplo la fosforilación de p53 inducida por el daño del ADN aumenta su asociación con los coactivadores transcripcionales CBP/p300 y, como consecuencia, aumenta la acetilación de p53 lo que conduce a una mayor estabilidad. (Barlev *et al.* 2001) también se ha reportado que una proteína asociada de p300, strap incrementa los niveles y la vida media de p53 (Demonacos *et al*. 2004). En respuesta al daño en el ADN, strap es fosforilada por ATM que conduce a su translocación al núcleo, asociación con p300 y acetilación de p53. La proteína nuclear p33ING2 también aumenta la acetilación de p53 en Lys382, induciendo el arresto en la fase G1 del ciclo celular y apoptosis (Nagashima *et al.* 2001). El nucleolo y las proteínas nucleolares también impactan en la estabilización de p53. En un trabajo se demostró que el daño en el ADN *per se* no estabiliza p53, sino que también requiere la interrupción nucleolar. Este modelo está respaldado por las observaciones de que la proteína nucleolar, nucleofosmina, deja el nucleolo en respuesta al daño en el ADN, se une a p53 y lo estabiliza y activa. La capacidad de nucleofosmina para interactuar con MDM2 y actuar como un regulador negativo del complejo p53-MDM2 también es clave para esta regulación. (Kurki *et al*. 2004). Existe evidencia de varias proteínas quinasas

influyen en la estabilización de p53. La sobreexpresión de la proteína quinasa 2 (HIPK2) aumenta la estabilidad de p53, que se potencia aún más en presencia de doxorrubicina. Si bien el mecanismo exacto de protección no es conocido, la fosforilación de p53 inducida por HIPK2 en ser46 puede ayudar a disociar p53 de MDM2. (Rui *et al*. 2004). La proteína PARP es un componente esencial de la reparación de la escisión bases. Se ha observado que PARP se puede unir a p53 y se sugiere que esto podría influir en la estabilidad y actividad de p53. En células que sobrexpresan PARP se observó un arresto en la fase G1 y se observó inducción de apoptosis (Wieler *et al.* 2003). Por lo tanto, PARP se une a una larga lista de proteínas que influyen en la estabilización y / o activación de p53.

En este trabajo se estudió la influencia de la curcumina en la estabilidad de p53, la curcumina promueve la interacción entre p53-NQO1 y a su vez promueve la pérdida de interacción entre p53 y su regulador negativo E6AP (en células infectadas con el virus del papiloma humano y con p53 *wild type*) lo que se traduce en el aumento de la vida media de p53 y la reactivación de la vía.

6. Hipótesis

La curcumina restablece los niveles de p53 de una manera dependiente de la activación de la vía Keap1/Nrf2 en líneas celulares derivadas de cáncer cervical.

7. Objetivos

Objetivo general:
Determinar si la curcumina induce la estabilización de p53 y restablece sus niveles de manera dependiente de la activación de la vía Keap1/Nrf2 en líneas celulares derivadas de CaCU.

Objetivos particulares:

- Comprobar la activación de la vía Keap1/Nrf2 después del tratamiento con antioxidantes.

- Evaluar el efecto de la curcumina en la viabilidad de células provenientes de CaCU.

- Evaluar el efecto a nivel de mensajero y niveles de proteína de p53 después del tratamiento con curcumina.

- Analizar la estabilidad de p53 después del tratamiento con antioxidantes.

- Observar si el tratamiento con curcumina produce pérdida de interacción entre p53 y su regulador negativo E6AP.

8. Materiales y métodos:

Reactivos

Bromuro de 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolio (MTT), dicumarol, cicloheximida (CHX), dimetilsulfóxido (DMSO), curcumina (catálogo C1386), tabletas de cóctel inhibidor de proteasas libre de EDTA (S8830), proteína G sepharose (GE28), Trizma base (T1503), cloruro de sodio (NaCl S9888) se adquirieron de Sigma-Aldrich (St. Louis, MO, EE. UU). El kit de cuantificación de proteínas BCA Pierce (23225) y el reactivo de transfección lipofectamina y plus (15338100) fueron adquiridos de ThermoFisher (Waltham, MA, EE. UU.) El Nonidet P-40 (CAS 68412-54-4), anticuerpo monoclonal de ratón anti-p53 (DO-1), anticuerpo monoclonal de ratón anti-NQO1 (H9), anticuerpo monoclonal de ratón antiE6AP (E4), anticuerpo monoclonal de ratón anti-lamina A / C (2A1), anticuerpo policlonal de cabra anti-gliceraldehído-3-fosfato deshidrogenasa (GAPDH, L8), los anticuerpos secundarios, IgG-HRP anti-cabra (sc2020) y anti-ratón (HRP, sc-2005) se adquirieron de Santa Cruz Biotechnology (Dallas, TX, EE. UU.). Dulbecco's Modified, Eagle Medium High Glucose (DMEM 11965–084) y el suero fetal bovino (10500056) fueron adquiridos de GIBCO.

Cultivo celular:

Las líneas celulares HeLa, SiHa, CaSki, H1299 y PANC1 se cultivaron en DMEM suplementado con suero fetal bovino al 10%. Las células MDA-MB-231 se cultivaron en DMEM F12, (GIBCO, 11320–033) suplementado con suero fetal bovino al 10%. Todas las líneas celulares se cultivaron a 37 ° C y con una tensión de CO_2 al 5%.

Extracción del ARN y PCR en tiempo real (RT-qPCR)

La extracción de ARN de las líneas celulares usadas (confluencia 70–80%) tratadas o no tratadas con curcumina durante 24 h se realizó utilizando Trizol (Invitrogen, Cat. No. 15596026), el ARN extraído se trató con DNasaI (ThermoFisher Cat. No. EN0521) y se purificó utilizando el kit Direct-zol TM, RNA MicroPrep (Zymo Research, Cat. No. R2060). El ARN purificado se cuantificó

utilizando un sistema de espectrofotometría Epoch TM y se sometió a retro-transcripción con 2000 ng de ARN con el sistema de síntesis SuperScriptTM IV (Invitrogen, n.º de cat. 18091050) para obtener un cDNA. Se utilizó PCR en tiempo real, para determinar los niveles de expresión de p53 y GAPDH como gen de normalización. Se usaron los siguientes oligo-nucleótidos para el gen p53 (p53-F GGC AGT GGT ACG AGC CCT GGC CGT CTA) o (p53-R GTG CTC TGG TGG TGA GGA CAG CGT CTC A) y los siguientes para GAPDH (F-GAPDH: AAG GTC GGA GTC AAC GGA TTT G y R-GAPDH: CCA TGG GTG GAA TCA TAT TGG AA) como control. Se colocaron 100 ng de cDNA, 12.5 µL Master-mix SYBR y 10 pmol de cada oligonucleótido en un volumen total de 25 µL y se llevó a cabo la PCR en tiempo real. Las condiciones de reacción fueron 95°C durante 10 min para la desnaturalización inicial, 60°C durante 30 s para la alineación, 40 ciclos de 95°C durante 15 s para la extensión. La reacción se realizó en el equipo QIA-GEN RotorGene Q. Los niveles de expresión de los ARNm se determinaron a partir del ciclo umbral (Ct), y los niveles de expresión relativos se calcularon utilizando el método $2^{-\Delta\Delta Ct}$. Para la cuantificación de ARNm, los valores de Ct se normalizaron a la expresión del nivel del ARNm de GAPDH.

Western blot:

Los lisados celulares se extrajeron con un buffer de lisis compuesto por Tris 50 mM, pH 7,6, NaCl 150 mM, Nonidet P-40 al 1%, fosfato sódico 10 mM y un tableta de coctel inhibidor de proteasas por cada 100 ml de buffer, la concentración de proteínas se cuantificó usando un kit de ácido bicinconínico, se utilizó albúmina de sérica bovina como estándar. El extracto de proteína total se usó para realizar el *Western blot*. Para la electroforesis se cargaron cantidades iguales de proteína (30-60 µg de proteína) total en un gel de poliacrilamida al 10% en condiciones desnaturalizantes (SDSPAGE) y se transfirieron a una membrana de nitrocelulosa, seguido de incubación durante toda la noche a 4°C se usaron las siguientes diluciones de anticuerpos primarios: anti-p53 (1: 100), anti NQO1 (1: 1000), anti-MDM2 (1: 500), anti-E6AP (1: 1000), anti-lamina A / C (1: 500), anti-GAPDH (1: 1000) seguido por la incubación con el anticuerpo secundario en solución de

bloqueo 1 hora a temperatura ambiente, se utilizaron los anticuerpos secundarios goat anti-mouse (1: 10000), donkey anti-goat (1:20000) acoplados a HRP. Los niveles de expresión de proteína finalmente se visualizaron con el escáner Li-COR C-DiGit quimioluminiscencia y el sistema de imágenes UVP.

Ensayos de pulso y caza:

Las líneas celulares usadas se sembraron en placas petri p35 a una densidad de 1,5 x 10^5 células/placa y se trataron con curcumina a una concentración de 20 µM durante 24 h, se retiró el tratamiento con curcumina y las células se lavaron con PBS dos veces, se continuó con el tratamiento con CHX (la CHX es un inhibidor de la síntesis de proteínas) a una concentración de 50 µg/ml como se ha descrito (Yewdell *et al*. 2011; Hochstrasser 1995), inmediatamente después de la adición del compuesto se realizó una extracción de proteínas cada 10 minutos, comenzando en 0 minutos, 10 minutos, 20 minutos y así hasta completar 60 minutos, las células se lisaron con un tampón de buffer de lisis (descrito anteriormente). Se analizaron los niveles de proteína p53 en cada punto de tiempo, mediante Western blot como se ha descrito anteriormente.

Inmunoprecipitación de proteínas:

Las células se sembraron en placas p60 a una densidad de 2,5 x 10^5 células/placa y se trataron con curcumina a una concentración de 20 µM durante 24 h y luego se lisaron con el buffer descrito anteriormente, después de la centrifugación, se colectó el sobrenadante y se incubaron 400 µl, 2h a 4°C con 50 µl de proteína G sepharose, 1 µg de anticuerpos y 0.5 mg/ml de RNAse A, después de la incubación en rotación constante, las perlas de sepharose acopladas a la proteína G se lavaron 20 veces con buffer de lisis. La inmunoprecipitación se reveló mediante Western blot, se visualizaron los niveles de expresión de proteína con el escáner Li-COR C-DiGit (LI-COR Biosciences , Lincoln, Nebraska, USA).

Ensayos de viabilidad celular:

La viabilidad celular se determinó mediante el ensayo MTT (M2128) (Langdon 2003). Se sembraron células HeLa, SiHa, CaSki y MDA-MB-231 (5 x 10 4 células/pocillo) en una placa de 96 pocillos y se trataron con curcumina 10μM y 20μM (C1386) durante 24 h. Después de la adición de 10 μl por pocillo de solución de MTT (5 mg / ml), las células se incubaron a 37 ° C durante 4 h, los cristales de formazán se disolvieron utilizando 50 μl de DMSO. La viabilidad celular se determinó midiendo la absorbancia a 570 nm en un lector de microplacas Synergy H1 MultiMode (BioTek, Winooski, VT, EE. UU).

Transfección de células PANC1 con NQO1:

Las células PANC1 (nulas a NQO1 adquiridas de American Type Culture Collection (ATCC CRL-1469) se transfectaron de forma transitoria utilizando Lipofectamine plus con 1 μg de pCDNA3 NQO1 o 1 μg de pCDNA3 (vector vacío). Los resultados se obtuvieron de tres réplicas biológicas. Las células PANC1 transfectadas y el *wild type* (no transfectadas) se trataron con curcumina, se realizó un ensayo de pulso y caza. Los niveles de expresión de la proteína p53 se analizaron mediante *Western blot* como se describió anteriormente.

Análisis estadístico:

Los resultados se expresan como media ± DE. Las pruebas estadísticas se realizaron con GraphPad PRISM versión 6.0c. Se aplicó la prueba ANOVA con Tukey para comparar las medias entre los grupos, una $p < 0.05$ fue considerada como significativa.

9. Resultados

Curva dosis respuesta de curcumina en líneas celulares tumorales.

Para determinar la dosis adecuada de curcumina que se utilizaría en los experimentos, se realizó un curva dosis respuesta en diferentes líneas celulares; SiHa, HeLa, CaSki (líneas provenientes de cáncer cervicouterino y con p53 *wild type*) y MDA-MB-231 (proveniente de cáncer de mama con p53 mutado). Se determinó que la dosis de curcumina adecuada para trabajar, es de 10 μM y 20 μM durante 24 horas, tiempos más prolongados y dosis más altas implican demasiada muerte celular que no permitirían la obtención de proteínas o material genético (Figura 1). A la dosis de 20 μM se observa que no hay efecto en la viabilidad de MDA (células con p53 mutado), pero sí en la viabilidad de células HeLa, SiHa y CaSki (células con p53 wild type).

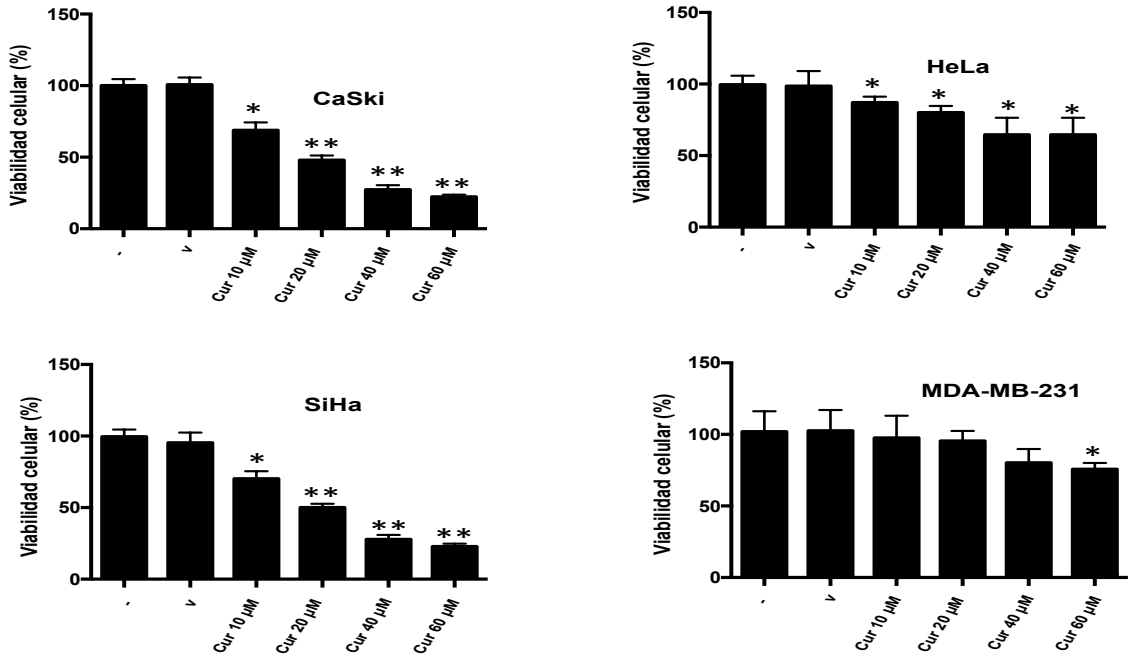

Figura 1. Curva dosis respuesta de curcumina en células CaSki, HeLa, SiHa y MDA-MB-231. Las células CaSki, HeLa y SiHa y MDA-MB-231 fueron tratadas con 10 μM, 20 μM, 40 μM y 60 μM de curcumina, la viabilidad celular fue evaluada usando MTT. Los valores se representan como la media ± DE de tres experimentos independientes. EL signo negativo (-) representa sin tratamiento, la V representa vehículo. Las diferencias estadÍsticas fueron determinadas usando ANOVA de una vía y el test de comparación de Tukey; (*) $p<0.005$, (**) $p<0.001$ (-) vs V, (-) vs 10 μM de curcumina, (-) vs 20 μM of curcumina, (-) vs 40 μM of curcumina, (-) vs 60 μM of curcumina.

El tratamiento con curcumina provoca la translocación de Nrf2 al núcleo.

Estudios previos han demostrado que la curcumina tiene la capacidad de activar la vía Keap1/Nrf2 y de esta manera aumentar la expresión de enzimas antioxidantes de fase II tales como NQO1 (Pulido-Moran *et al*. 2016). Para determinar la participación de la curcumina en la activación de esta vía en nuestro modelo, se evaluó la translocación de Nrf2 al núcleo. Observamos la inmunodetección de Nrf2 en el núcleo en células que fueron tratadas con 20 μM de curcumina (Figura 2).

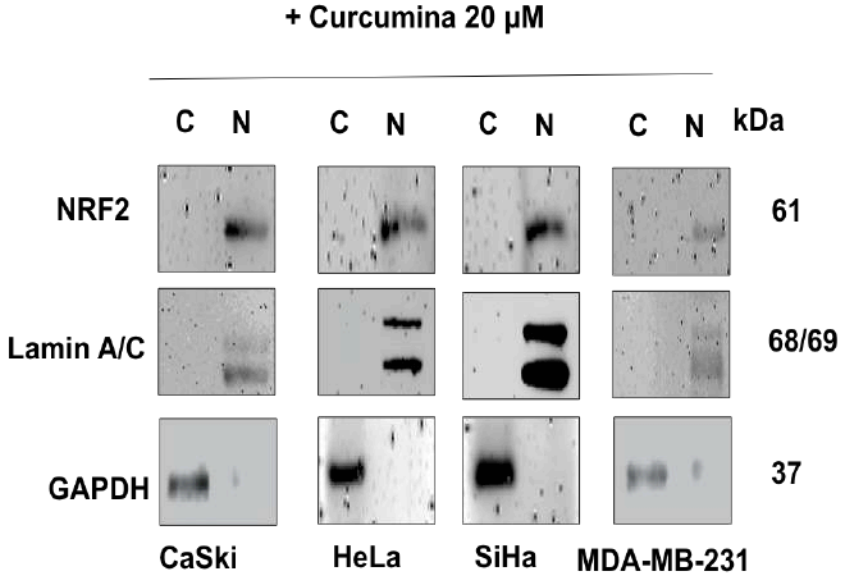

Figura 2. La curcumina activa la vía Keap1/Nrf2. Las células CaSki, HeLa y SiHa y MDA-MB-231 fueron tratadas con 20 μM curcumina y la inducción de la translocación de Nrf2 (61 KDa) al núcleo fue detectada por Western blot. C representa la fracción citoplasmática y N representa la fracción nuclear.

Localización de un elemento de respuesta a antioxidantes (ARE) en el promotor de p53.

Para conocer si Nrf2 (una vez que se transloca al núcleo) podía unirse al promotor de p53 y promover su transcripción se realizó un análisis del promotor de p53 (número de acceso del *Gene Bank* J04238) y se localizó un elemento de respuesta a antioxidantes (ARE) que cumple con la secuencia base TGACnnnGC (Wasserman *et al*. 1997), la secuencia de este elemento es la siguiente: TGACTCTGC (Figura 3). Nrf2 reconoce estas secuencias (que generalmente se encuentran en los promotores de enzimas antioxidantes de fase II), se une a ellas y promueve la transcripción.

Figura 3. El promotor de p53 presenta un elemento de respuesta a antioxidantes ARE en su promotor. Secuencia de la región reguladora de p53. GenBank número de acceso J04238. La región resaltada en negro representa el elemento ARE (TGACTCTGCA). (Modificado de (Tuck & Crawford 1989)).

El tratamiento con curcumina no tienen efecto sobre el promotor de p53.

Para demostrar si el tratamiento con curcumina tienen un efecto sobre los niveles de p53, se transfectaron las células provenientes de cáncer de pulmón H1299 (nulas a p53) con el plásmido pGL2-356pb número 16292 addgene. Este plásmido contiene un fragmento del promotor de p53 en el cual se encuentra el sitio ARE, las células transfectadas se trataron con curcumina y se midió la actividad de luciferasa, en los resultados no se observa un cambio entre el control (-) y el tratamiento con curcumina (Figura 4A).

Para observar si el tratamiento con curcumina incrementa los niveles del mensajero de p53, se realizó un análisis de expresión por PCR en tiempo real con el RNA extraído de células SiHa con y sin tratamiento con curcumina (Figura 4B). Se observa que no existe una diferencia significativa en los niveles de RNAm de p53 en las células tratadas con curcumina comparadas con las células que no tienen tratamiento.

Para demostrar que el incremento de curcumina incrementa los niveles de proteína pero no del mensajero de p53 las células H1299 se transfectaron con un plásmido que expresa p53 completo pero en ausencia de su promotor, es decir este plásmido contiene el promotor de citomegalovirus, en los resultados se observa un incremento en los niveles de proteína de p53, los resultados en conjunto nos indican que el aumento de los niveles de p53 no sigue una regulación transcripcional (Figura 4C).

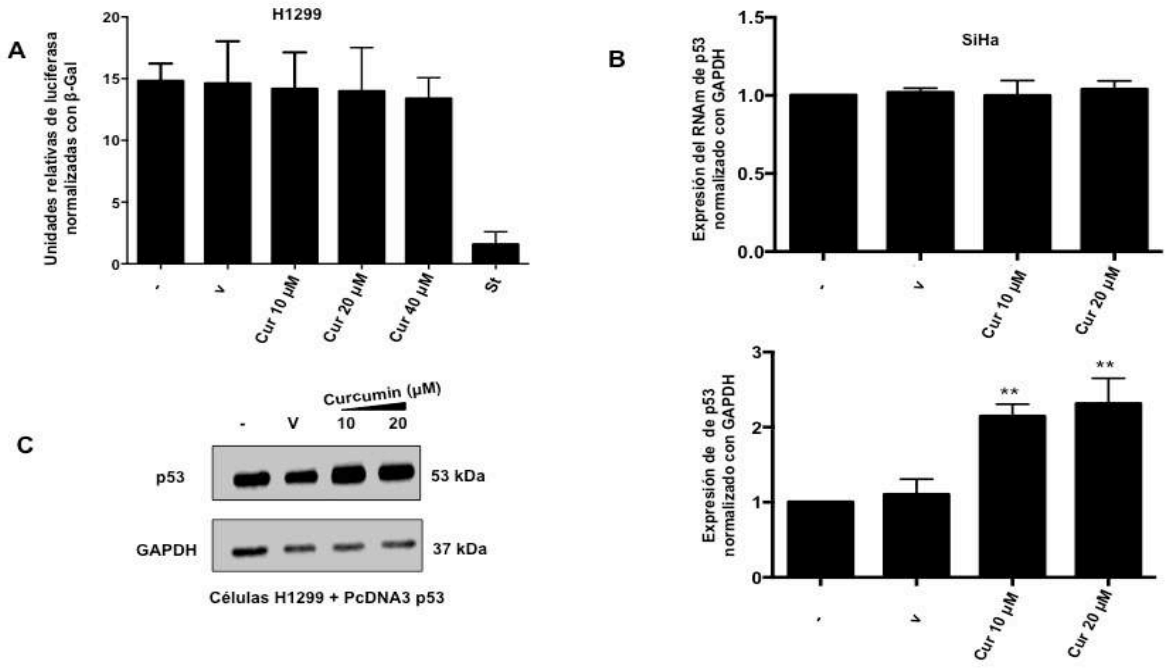

Figura 4. Efecto del tratamiento con curcumina sobre el promotor de p53. Las células H1299 provenientes de cáncer pulmonar (nulas a p53) se transfectaron con el plásmido pGL2-356pb número 16292 addgene o se dejaron sin transfectar (St), posteriormente las células trasnfectadas se dejaron sin tratamiento, (-), se trataron con el vehículo (V) o con las concentraciones indicadas de curcumina. (A) se realizó un análisis de PCR en tiempo real en las células SiHa con y sin tratamiento con curcumina (B) Las mismas células H1299 fueron transfectadas con un plásmido que no contiene el promotor de p53 pero sí tiene el gen para expresar la proteína completa. Las células fueron tratadas por 24 horas con curcumina (C). Los valores estan presentados como media ± DE de tres experimentos independientes.Las diferencias estadísticas fueron determinadas usando ANOVA de una vía y el test de comparación multiple de Tukey; (*) $p < 0.005$, (**) $p < 0.001$. (-) vs V, (-) vs 10 μM de curcumina, (-) vs 20 μM de curcumina y (-) vs 20 μM de curcumina.

La curcumina incrementa los niveles de p53 y de NQO1

Una vez que se determinó que la curcumina induce la translocación nuclear de Nrf2 (un evento que indica la activación de la vía Keap1/Nrf2) se midió la expresión de una proteína blanco de la vía, una enzima de fase II conocida como NQO1, se observó un incremento en los niveles de NQO1 lo cual es consistente con la translocación de Nrf2 al núcleo (Figura 5A). Además se observó un incremento en los niveles de p53 (Figura 5B). Los resultados indican un importante papel de la curcumina en la activación de la vía de p53, dado que la mayoría de las líneas utilizadas portan el gen silvestre de p53.

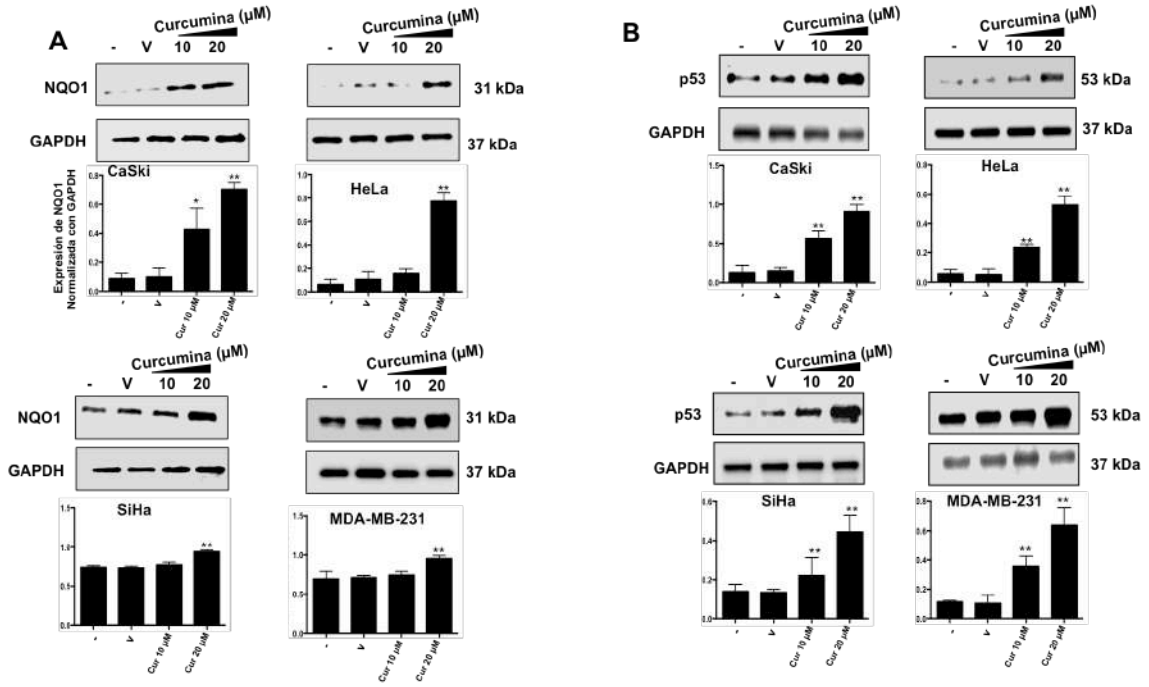

Figura 5. Efecto de la curcumina (cur) sobre los niveles de p53 y NQO1 en células CaSki, HeLa y SiHa y MDA-MB-231. Las células CaSki, HeLa y SiHa y MDA-MB-231 fueron tratadas con 10 μM y 20 μM curcumina (A) y NAD(P)H: quinona oxidorectusas 1 (NQO1, 31 KDa) y (B) p53 fueron detectados por Western blot. Las células fueron tratadas por 24 horas. Los valores son expresados como media ± DE de tres experimentos independientes. EL signo negativo (-) representa sin tratamiento, la V representa vehículo. Las diferencias estadísticas fueron determinadas usando ANOVA de una vía y el test de comparación multiple de Tukey; (*) $p < 0.005$, (**) $p < 0.001$ (-) vs V, (-) vs 10 μM de curcumina y (-) vs 20 μM de curcumina.

La curcumina incrementa los niveles de p21 y disminuye la viabilidad celular.

Para evaluar la funcionalidad de la vía p53 en respuesta la tratamiento con curcumina se evaluó la expresión de p21, se observa que la curcumina incrementa los niveles de p21 en las líneas celulares que tienen p53 *wild type*, (CaSki, HeLa, SiHa) sin embargo no fue observado este mismo efecto en MDA-MB-23, esta línea celular tiene una mutación en p53 que provoca pérdida de función (Figura 6A). Este resultado indica que la curcumina activa la vía Keap1/Nrf2 en nuestro modelo celular con p53 silvestre y además provoca la activación de la vía.

Para evaluar el efecto de la curcumina en la viabilidad celular se realizó en un ensayo de MTT. Las células fueron tratadas con 10 μM y 20 μM de curcumina; las células no tratadas (-) y células tratadas con vehículo (V) fueron usadas como control. El tratamiento con curcumina induce una disminución significativa en

células que son p53 silvestre (líneas celulares provenientes de cáncer cervicouterino), también se observa que a las mismas concentraciones las células MDA-MB-231 que tienen p53 mutado fueron resistentes al efecto citotóxico de la curcumina. Los resultados sugieren que el efecto citotóxico está relacionado con la activación de p53 silvestre (Figura 6B).

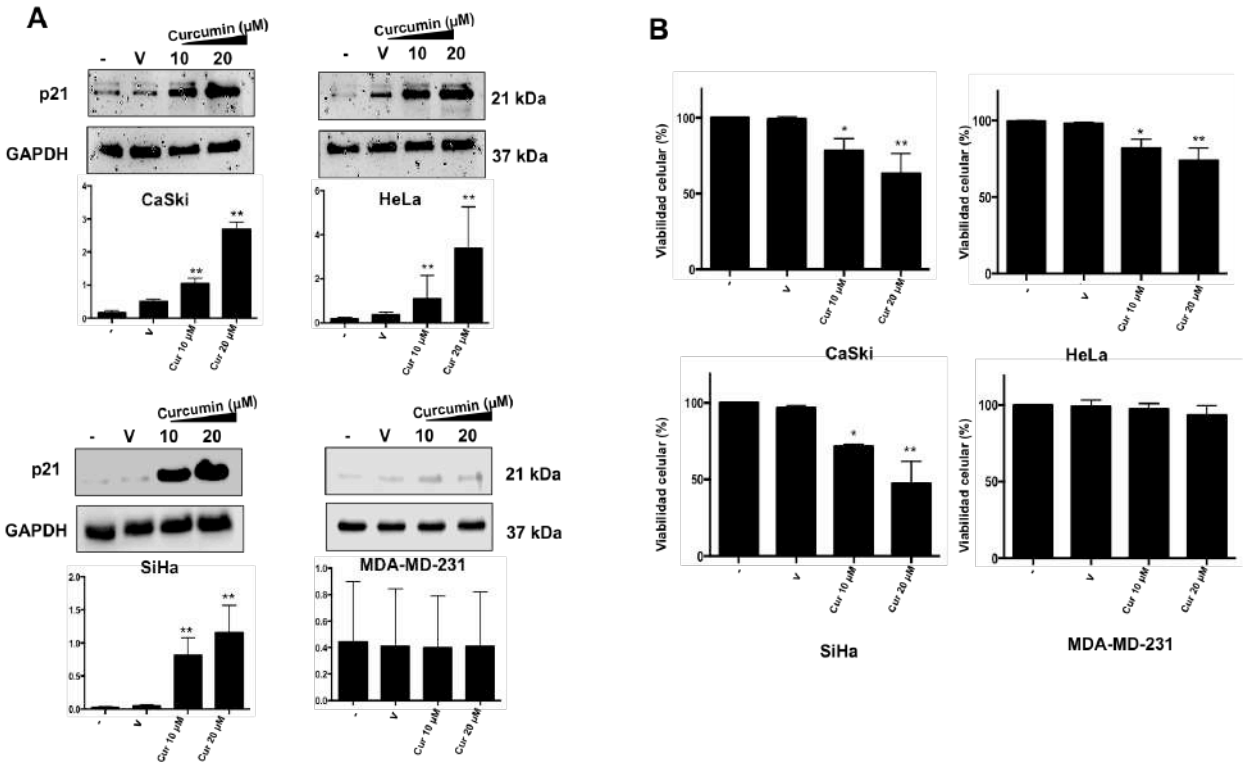

Figura 6: Efecto de curcumina (cur) sobre los niveles de p21 y la viabilidad en células CaSki, HeLa y SiHa y MDA-MB-231. Las células CaSki, HeLa, SiHa y MDA-MB-231 fueron tratadas por 24 horas con 10 μM y 20 μM de curcumina, los niveles de p21 fueron detectados por Western blot (A) y la viabilidad celular fue evaluada por MTT (B). Los valores están representados por media ± DE de tres experimentos independientes. El signo negativo (-) representa sin tratamiento, la V representa vehículo. Las diferencias estadísticas fueron determinadas usando ANOVA de una vía y el test de comparación multiple de Tukey; (*) $p < 0.005$, (**) $p < 0.001$ (-) vs V, (-) vs 10 μM de curcumina y (-) vs 20 μM of curcumina.

La curcumina incrementa la vida media de p53.

Es ampliamente conocido que la vida media de p53 es de 20 minutos en condiciones fisiológicas (Sullivan *et al*. 2018). Para evaluar el efecto que tiene el tratamiento con curcumina en la estabilidad de p53 se realizó un ensayo de pulso

y caza con cicloheximida, un inhibidor de la síntesis de proteínas (Buchanan *et al.* 2016). Se observó que la estabilidad de p53 en HeLa, SiHa Caski y MDA-MB-231 sin tratamiento con curcumina fue de 20 minutos (figura 7A).

Sin embargo cuando las células fueron tratadas con 20 μM de curcumina p53 fue detectado hasta los 60 minutos (figura 7B) estos resultados sugieren que la curcumina tiene la capacidad de prolongar la vida media de p53 es decir que la proteína se encuentre en células tumorales en un tiempo superior a 20 minutos.

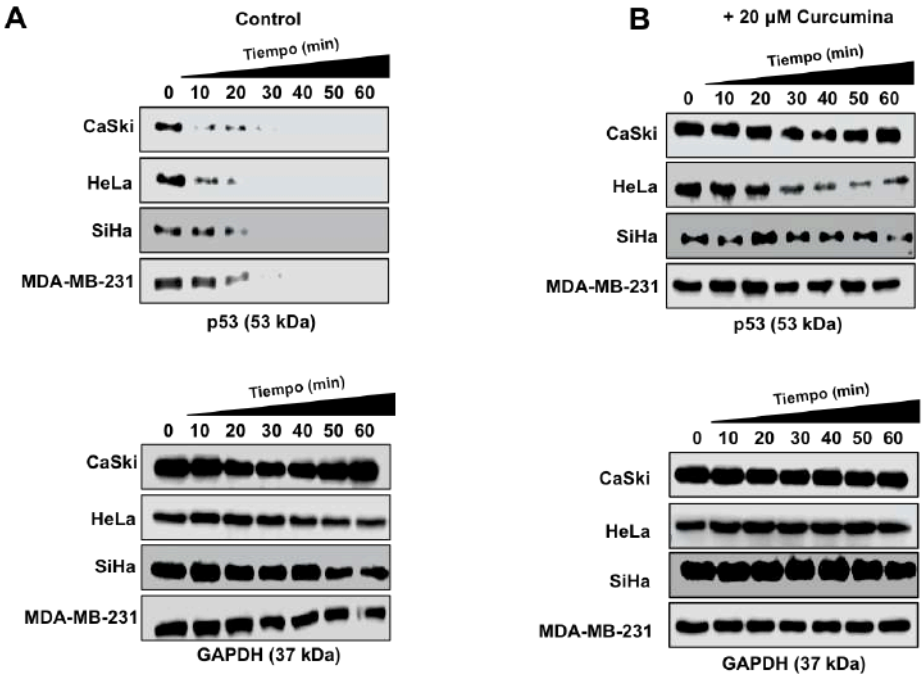

Figura 7. La curcumina estabiliza p53. Las células CaSki, HeLa, SiHa y MDA-MB-231 fueron tratadas por 24 horas con 20 μM de curcumina y la vida media de p53 (53 KDa) fue medida con un experimento de pulso y caza con cicloheximida (50 μg/ml), la extracción de proteina fue realizada cada 10 minutos (0-60) y el ensayo se reveló mediante Western blot, la estabilidad de p53 fue evaluada en (A) células control (sin curcumina) y en (B) células tratadas con curcumina.

La curcumina promueve la interacción entre NQO1-p53

Existen reportes de la interacción de NQO1 y p53 en condiciones de estrés (Das & Vinayak 2015, Kama et al. 2002), para evaluar si el tratamiento con curcumina promueve la interacción de p53 y NQO1 se realizó una inmunoprecipitación después del tratamiento con curcumina. En este ensayo se inmunoprecipitó p53 y se detectó NQO1 por un ensayo de *Western blot*, las células no tratadas se usaron

como control, los resultados muestran que existe interacción física entre NQO1 y p53 cuando las células HeLa, SiHa y CaSki fueron tratadas con curcumina (Figura 8A). Este resultado sugiere que el complejo NQO1-p53 podría estabilizar a p53 en respuesta al tratamiento. Para mostrar que el efecto del tratamiento con curcumina puede provocar la pérdidada de interacción entre p53 y su regulador negativo E6AP, se realizó una inmunoprecipitación, los resultados mostraron que el tratamiento con curcumina induce una pérdida de interacción entre p53 y E6AP (Figura 8B).

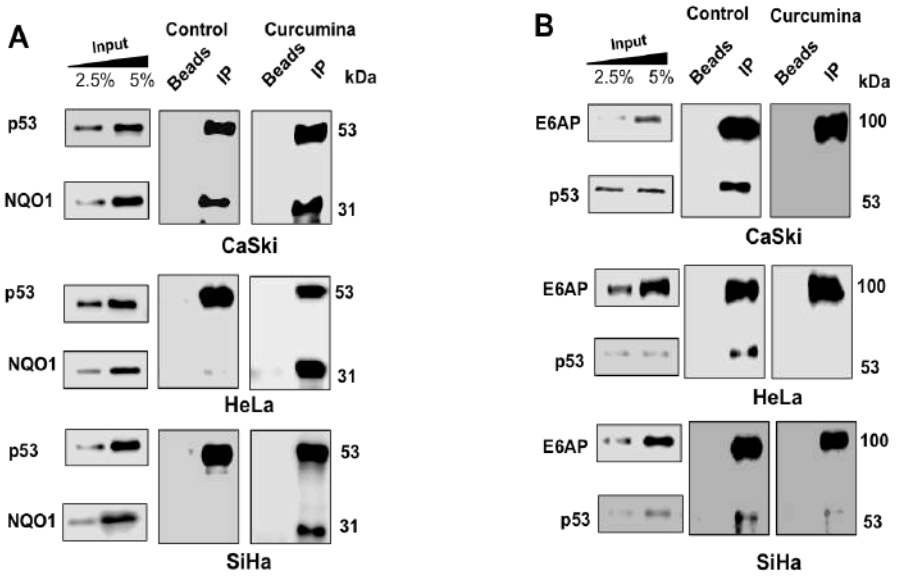

Figura 8. La curcumina promueve la interacción entre p53 y NQO1. Las células CaSki, HeLa, SiHa y MDA-MB-231 fueron tratadas por 24 horas con 20 μM de curcumina y NQO1 (31 KDa) fue detectado por *Western blot* después de realizer una inmunoprecipitación de p53 (53 KDa) de los extractos. En el *input* se detectaron p53 y NQO1 en concentraciones crecientes de proteína (2.5 and 5%). (A) La interacción de p53-NQO1 fue evaluada después del tratamiento con curcumina. Las lineas celulares fueron tratadas por 24 horas con 20 μM de curcumina y p53 (53 KDa) fue detectado por *Western blot* después de la inmunoprecipitaciónde E6AP (100 KDa). (B) En el *input* se detectaron p53 y E6AP en concentraciones crecientes de proteína (2.5 and 5%).

La curcumina es necesaria para la estabilización de p53 y la formación del complejo NQO1-p53.

Para determinar si la actividad enzimática de NQO1 es necesaria para la estabilidad de p53 cuando la curcumina está presente, las células se trataron con curcumina y posteriormente 100 μM de dicumarol (un inhibidor de la actividad de

NQO1) durante 4 horas. Para evaluar la estabilidad de p53 se realizó un ensayo de pulso y caza. Los resultados mostraron que la vida media de p53 fue inferior a 60 minutos cuando las células se trataron con dicumarol (Figura 9A). Esto sugiere que la estabilización de p53 por NQO1 depende de que la actividad enzimática de NQO1 esté intacta. Para demostrar si NQO1 es responsable de la estabilidad de p53, utilizamos células de carcinoma pancreático (PANC1), una línea celular nula para NQO1 (Shieh *et al*. 2010, Traver *et al*. 1997). Primero tratamos las células PANC1 *wild type* (no transfectadas) con curcumina y, curiosamente, no observamos estabilización de p53 (Figura 9B), lo que sugiere que la presencia de curcumina no es suficiente para aumentar la vida media de p53. La expresión de NQO1 se restableció mediante la expresión exógena del mensajero NQO1 clonado en un plásmido (PCDNA-NQO1). Después de 48 h de transfección, se realizó un ensayo de pulso y caza en células transfectadas con PANC1, pero sin tratamiento con curcumina, y nuevamente no se observó la estabilización de p53 (Figura 9C) incluso cuando se expresó la proteína NQO1, lo que sugiere que la presencia de NQO1 no es suficiente para aumentar la vida media de p53. Sorprendentemente, cuando las células transfectadas se trataron con curcumina a una concentración de 20 µM, se observó una estabilización de p53 (Figura 9C). Este resultado mostró claramente que la curcumina es necesaria para promover la estabilización de p53, a través de la interacción con la proteína NQO1.

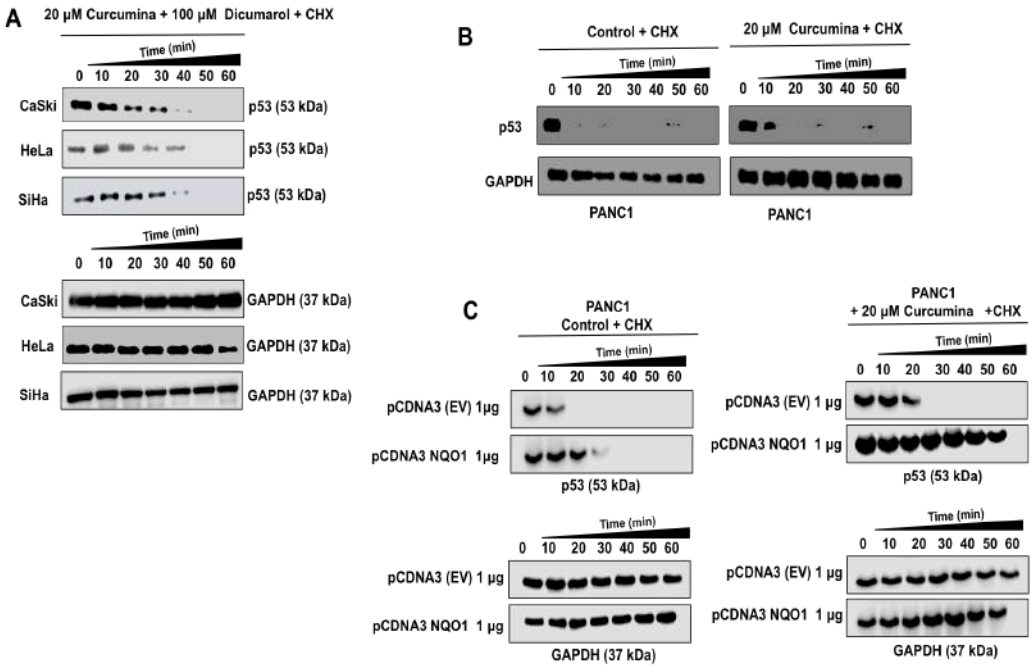

Figura 9. La curcumina incrementa la estabilidad de p53 en una manera dependiente de NQO1. Se realizó un análisis de pulso y caza en las líneas celulares tratadas por 24 horas con 20 μM curcumina y 4 horas con 100 μM de dicumarol (un inhibidor de la actividad de NQO1). (A) Se muestra el efecto de la inhibición de la actividad de NQO1 por dicumarol en la estabilidad de p53. Las células de carcinoma pancreático nulas a NQO1 (PANC1 cells) *wild type* y transfectadas con 1μg de pCDNA3 EV (empty vector) o con 1 μg de pCDNA3-NQO1 (para expresar la proteina completa NQO1) fueron tratadas con 20 μM de curcumina durante 24 h y posteriormente se realizó un ensayo de pulso y caza de p53. La caza de la proteina se realizó cada 10 minutos y se reveló por Western blot. Se muestra el efecto del tratamiento con curcumina en la estabilidad de p53 (B) en ausencia de NQO1 (PANC1 WT ó PANC1+ pCDNA3) y cuando los niveles de NQO1 son restaurados. CHX= cicloheximida

La curcumina tiene la capacidad de activar la vía Keap1/Nrf2, es decir la curcumina promueve la translocación de Nrf2 al núcleo, el cual se une a secuencias ARE e incrementa la expression de NQO1 (una enzima de fase II), NQO1 interacciona con p53, la formación de este complejo es favorecido por la presencia de curcumina, a su vez la interacción p53-NQO1 promueve la pérdida de interaccón entre p53 y su regulación negative E6AP.

En este trabajo se demostró que la curcumina incrementan los niveles y la vida media de p53, cuando p53 es *wild type* se puede activar la vía de p53 que incluye el increment en los niveles de p21 y la activación de la apoptosis (Figura 10).

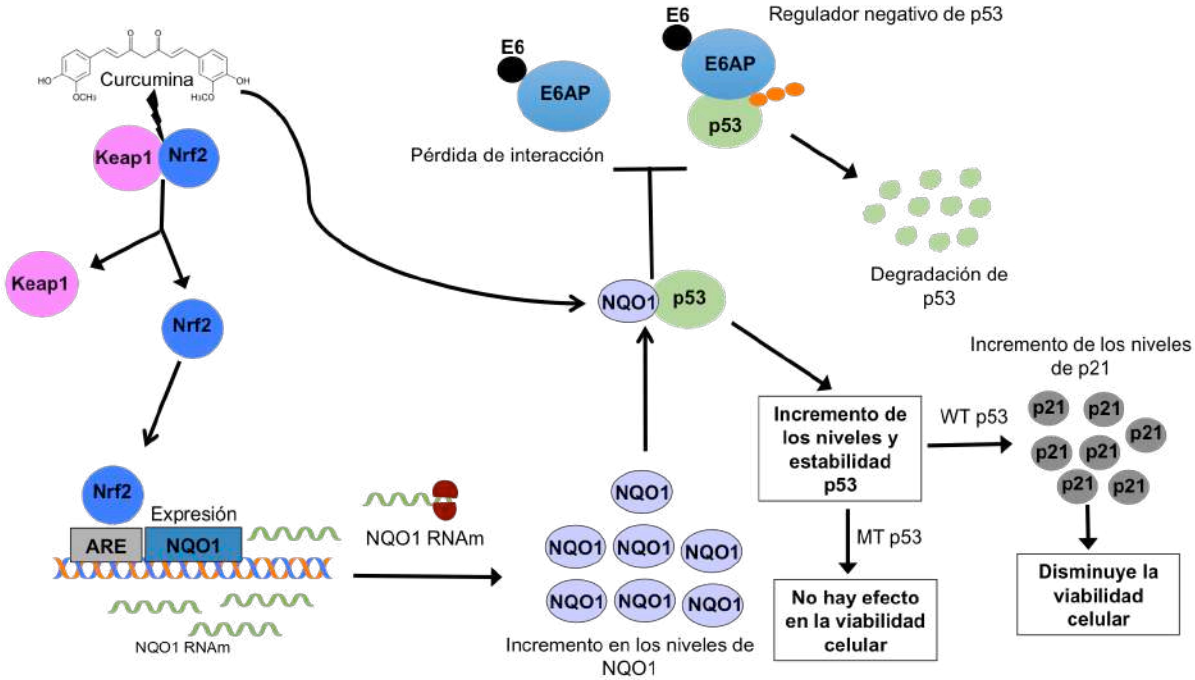

Figura 10. Modelo esquemático del efecto de la curcumina en la estabilización de p53. La curcumina activa la vía Keap1/Nrf2, Nrf2 es translocado al núcleo y se une a secuencias conocidas como elementos de respuesta a antioxidantes (ARE) incrementando la expression de NAD(P)H: quinona oxidoreductasa 1 (NQO1). NQO1 se une a p53 y promueve la pérdida de interacción entre p53 y su regulador negative E6AP. La curcumina también estabiliza la interacción NQO1-p53. En células con p53 wild type (WT) la vía de p53 es activada y tiene un efecto sobre la viabilida, sin embargo en las células con p53 mutado (MTp53) solo se observa su acumulación pero no hay efecto en la viabilidad celular.

10. Discusión:

p53 es un supresor de tumor que juega un papel central en la protección del genoma para prevenir la transformación celular (Lavin & Gueven 2006), por lo tanto las células deben mantener los niveles de p53 bajo control para evitar apoptosis anormal. Las mutaciones en p53 se relacionan con un estado patológico, por ejemplo en más del 50% de los cánceres se presenta una mutación en p53, pero las células utilizadas en este trabajo HeLa, SiHa y CaSki tienen p53 *wild type* (Hollstein et al. 1994, Srivastava et al. 1992) células como MDA-MB-231 tienen una mutación en TP53 que le confiere pérdida de función (Huovinen *et al.* 2011).

Las líneas celulares tumorales con p53 *wild type* puede reactivarse la vía de p53 y en consecuencia, promover la muerte celular y disminuir la viabilidad (Goodwin & DiMaio 2002). Por lo tanto, la restauración de p53 es una estrategia terapéutica prometedora, sin embargo, la estabilidad de p53 es un factor importante para la reactivación de la vía. El principal regulador negativo de p53 es MDM2, pero en líneas celulares de cáncer positivas a VPH como HeLa, SiHa y CaSki, la oncoproteína viral E6 forma un complejo con E6AP y su interacción física con p53 *wild type* promueve su degradación a través de la vía ubiquitina (Lane *et al.* 2010). La vida media de la proteína p53 es corta (20 min); durante los períodos de estrés celular, p53 se regula mediante un mecanismo de retroalimentación negativa. Aquí mostramos que la curcumina aumenta la vida media de p53 hasta 60 minutos y presentamos datos sobre el papel de NQO1 en la protección de p53 de su degradación que son consistentes con los datos reportados previamente (Park 2015). En este trabajo demostramos que la curcumina es necesaria para promover

la estabilización de p53 mediada por NQO1. NQO1 juega un papel importante en esta estabilización porque puede interactuar físicamente con p53. NQO1 se considera una enzima anticancerígena, ya que protege a las células del estrés oxidante (Mizumoto *et al.* 2019). Por lo tanto, el uso de compuestos naturales para inducir la expresión de NQO1 se ha convertido en una estrategia prometedora para la prevención del cáncer (Braicu *et al.* 2017). Los compuestos como la curcumina tienen la capacidad de translocar Nrf2 al núcleo y aumentar la expresión de proteínas antioxidantes de fase II como NQO1 (Ajaikumar B. Kunnumakkara et al. 2017, Taguchi & Yamamoto 2017). El aumento en los niveles de NQO1 es uno de los resultados de la translocación de Nrf2 al núcleo inducido por la curcumina. NQO1 es una proteína que puede formar complejos con otras proteínas para estabilizarlas, incluyendo a p53. En este trabajo, proponemos que la curcumina es necesaria para inducir NQO1 y promover su interacción con p53, lo que a su vez resulta en un aumento de la estabilidad de p53, al contrario de de lo que se ha publicado en algunos trabajos (Asher et al. 2005, Zeekpudsa et al. 2014). En 2005, Tsvetkov *et al*. demostraron que la curcumina y el dicoumarol desestabilizan p53 en células leucémicas mieloides de ratón M1 transfectadas con un plásmido que contiene un p53 sensible a la temperatura (M1-t-p53). Además, utilizaron células A31N-ts20 de ratón que tienen una ubiquitina E1 sensible a la temperatura, que se inactiva a 39°C y provoca la acumulación de p53, los mismos autores mostraron degradación de p53 *wild type* en la línea celular nula p53 HCT116 de células de cáncer colorrectal humano. En ese trabajo, las células se transfectaron con p53 *wild type* humano y se trataron con altas dosis de curcumina (60 μM) y dicumarol (300 μM) (Asher et al. 2005). En nuestro trabajo utilizamos líneas celulares derivadas de cáncer cervical humano; HeLa, SiHa y CaSki. Estas líneas celulares exhiben una baja expresión de p53 *wild type* basal debido a la presencia de la oncoproteína E6 del virus del papiloma humano (VPH), por lo tanto, en este caso el mecanismo para la degradación de P53 depende de la formación de un complejo entre E6 y E6AP. Finalmente, es importante enfatizar que utilizamos dosis bajas de curcumina (20 μM) y dicumarol (100 μM). Por otro lado, Zeekpudsa *et al*. en 2014 demostraron una mayor expresión de p53 cuando

la actividad NQO1 es inhibida por dicumarol. Los autores utilizaron dos líneas celulares de colangiocarcinoma relacionado con la infección de *Opisthorchis viverrini* (KKU-100 y KKU-M214) con niveles de expresión de NQO1 altos y bajos, respectivamente (Zeekpudsa et al. 2014). Para disminuir los niveles de NQO1, los autores utilizaron un ARN de interferencia específico para NQO1. Por el contrario, en nuestro trabajo utilizamos líneas celulares de cáncer de cuello uterino humano con p53 *wild type* y, lo que es más importante, usamos curcumina, que desempeña un papel central en promover la interacción interacción entre NQO1 y p53 y su estabilización. Vale la pena mencionar que en el trabajo de Zeekpudsa *et al*. la curcumina no se usó, nuestros resultados demuestran que la curcumina es necesaria para promover el mantenimiento de un entorno en el que p53 y NQO1 puedan interactuar y, por lo tanto, aumentar la vida media de p53.

Finalmente, nuestros datos también mostraron que la interacción física entre NQO1 y p53 promueve su pérdida de interacción con su regulador negativo E6AP. Además, NQO1 exhibe propiedades de enzimas catalíticas, primero informadas por Ernster y Navazio en 1950, que aquí mostramos que son necesarias para la estabilización de p53, ya que el dicumarol, un inhibidor de la actividad de NQO1, inhibe la estabilización de p53, consistente con lo anterior reportado (Asher et al. 2006, Levine & Oren 2010). Aquí proponemos un modelo en el que la estabilidad de p53 está determinada por dos factores que actúan juntos: la curcumina y el NQO1 (Figura 10). La curcumina activa la vía Keap1 / Nrf2 y promueve el aumento de los niveles de NQO1 y, en presencia de curcumina, NQO1 puede interactuar físicamente con p53 y promover su estabilidad. Además, la formación del complejo NQO1-p53 promueve la pérdida de interacción entre p53 y su regulador negativo E6AP. En las células tumorales positivas para VPH con p53 de *wild type*, como las usadas en este trabajo, la estabilización de p53 promueve una reactivación de la vía p53 y, por lo tanto, disminuye la viabilidad celular.

11. Conclusión:

Nuestros resultados demuestran que el tratamiento con curcumina participa en la regulación de la estabilidad de p53. Primeramente se demostró que el incremento de los niveles de p53 y su estabilidad no tiene una regulación transcripcional, ya que el sitio ARE encontrado en su promotor no responde a curcumina y la activación de la vía Keap1/Nrf2. Sin embargo pudimos demostrar que el tratamiento con curcumina aumenta los niveles de p53 y proporciona un entorno celular apropiado para que p53 y NQO1 interactúen. Al mismo tiempo, esta interacción promueve la pérdida de interacción de p53 con E6AP, el regulador negativo de p53 cuando está presente la oncoproteína E6 del virus del papiloma humano. En nuestro trabajo, las líneas celulares derivadas de tumores HeLa, SiHa y CaSki tienen intacta la vía de p53 debido a que p53 no tiene mutaciones, solo está en degradación constante por E6, por lo tanto el restablecimiento de p53 en este tipo de tumores conduce a la reactivación de la vía y la inducción de apoptosis en las células tumorales que lo conforman. La curcumina puede ser un potencial agente terapéutico para tumores con p53 *wild type*.

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13. Anexos

Anexo I

Redox Biology 28 (2020) 101320

Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

Curcumin stabilizes p53 by interaction with NAD(P)H:quinone oxidoreductase 1 in tumor-derived cell lines

Carlos César Patiño-Morales[a,b,c], Ernesto Soto-Reyes[b], Elena Arechaga-Ocampo[b], Elizabeth Ortiz-Sánchez[c], Verónica Antonio-Véjar[d], José Pedraza-Chaverri[e], Alejandro García-Carrancá[f,*]

[a] Posgrado en Ciencias Biomédicas, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Mexico City, Mexico
[b] Departamento de Ciencias Naturales, Universidad Autónoma Metropolitana, Unidad Cuajimalpa, Mexico City, 05300, Mexico
[c] División de Investigación Básica, Instituto Nacional de Cancerología, Mexico City, 14080, Mexico
[d] Laboratorio de Biomedicina Molecular, Universidad Autónoma de Guerrero, Chilpancingo Guerrero, 39080, Mexico
[e] Facultad de Química, Universidad Nacional Autónoma de México, Mexico City, 14080, Mexico
[f] Unidad de Investigación Biomédica en Cáncer, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México & Instituto Nacional de Cancerología, Secretaría de Salud, Mexico City, 14080, Mexico

ARTICLE INFO

Keywords:
P53
Curcumin
NQO1
E6AP
Tumour cell lines

ABSTRACT

Curcumin is a natural phytochemical with potent anti-neoplastic properties including modulation of p53. Targeting p53 activity has been suggested as an important strategy in cancer therapy. The purpose of this study was to describe a mechanism by which curcumin restores p53 levels in human cancer cell lines.

HeLa, SiHa, CaSki and MDA-MB-231 cells were exposed to curcumin and a pulse and chase and immunoprecipitation assays were performed. Here we showed that curcumin increases the half-life of p53 by a physical interaction between p53-NQO1 (p53 - NAD(P)H:quinone oxidoreductase 1) proteins after treatment with curcumin. Interestingly, the cell viability assay after treatment with curcumin showed that the cytotoxic activity was selectively higher in cervical cancer cells contained wild type p53 but not in breast cancer cells contained mutated p53. The cytotoxic effect of curcumin in cervical cancer cells was related to the complex p53-NQO1 that avoids the interaction between p53 and its negative regulator ubiquitin ligase E6-associated protein (E6AP). Finally, we demonstrated that in pancreatic epithelioid carcinoma cells (PANC1) that are knockout for NQO1, the reestablishment of NQO1 expression can stabilize p53 in presence of curcumin. Collectively, our findings showed that curcumin is necessary to promote the protein interaction of NQO1 with p53, therefore, it increases the half-life of p53, and permits the cytotoxic effect of curcumin in cancer cells containing wild type p53. Our findings suggest that the use of curcumin may reactivate the p53 pathway in cancer cells with p53 wild-type.

1. Introduction

Cancer is a diverse group of diseases characterized by abnormal cells growth and represents an important worldwide problem. Therefore, the search of therapeutic alternatives against tumoral cells has been a major challenge for scientific and commercial interest in the discovery of potent, safe and selective anti-cancer drugs [1]. Among them, antioxidants are molecules with therapeutic potential since they have the ability to neutralize reactive oxygen species but also have been attributed antitumor properties [2,3]. An example of these natural compounds with therapeutic potential is curcumin, a natural phenolic compound obtained from the roots of *Curcuma longa*. Structurally curcumin is a molecule with polar central and flanking regions separated by a lipophilic methionine segment. Also, it has distinct chemical properties, among these the presence of α,β–unsaturated compounds (Michael acceptor) that facilitate intermolecular interactions with another molecules [4,5]. The anti-tumor activities of curcumin have been demonstrated by some research groups [6,7]. Particularly, it has been shown that it can induce cyclin-dependent kinase inhibitors, therefore this promotes p53 restoration in wild-type and mutant p53 cancer cell lines and represses the growth of numerous cancer cell lines by the phosphatidylinositol 3-kinase pathway (PI3K, AKT), Ras, and β-catenin

* Corresponding author. Laboratory of Virus and Cancer, Instituto Nacional de Cancerología, Av. San Fernando No. 22, Sección XVI, Tlalpan, CP 14080, Mexico City, Mexico.
E-mail address: carranca@biomedicas.unam.mx (A. García-Carrancá).

https://doi.org/10.1016/j.redox.2019.101320
Received 22 May 2019; Received in revised form 27 August 2019; Accepted 3 September 2019
Available online 09 September 2019

pathways [8]. Curcumin is also a powerful antioxidant because it activates the Kelch-like ECH-associated protein 1-nuclear factor (erythroid-derived 2)-like 2 pathway (Keap1/Nrf2) under oxidative microenvironment [9]. The Nrf2 pathway is regulated by Keap1 by mediating its degradation, but when cells are exposed to electrophiles or oxidants, Nrf2 is stabilized and translocate into the nucleus, binding to the antioxidant response element (ARE) located in the promoter region of antioxidant genes and upregulates their transcription. Nrf2 functions as a transcriptional factor with multiple targets as NAD(P)H: quinone oxidoreductase 1 (NQO1) [10,11]. This protein has multiple functions including neutralization of reactive oxygen species, detoxification of quinones and stabilization proteins for example; there is a report showing that NQO1 can interact with p53 causing its stabilization [12,13]. p53 is a tumour suppressor gene product that can block cell progression through the cell cycle when deoxyribonucleic acid (DNA) is damaged [14]. It is localized into nucleus, where it functions as transcription factor of DNA sequence-specific in genes as p21. The activation of p53 can activate different gene pathways, resulting in apoptosis, cell-cycle arrest, DNA repair or senescence, among others [15]. Because of its biological importance, mutations in the *TP53* tumour suppressor gene are observed in greater than 50% of all human cancers. The vast majority of p53 mutations that are associated with human cancer occur at the region of DNA binding recognition [16]. Moreover, mutant p53 in human cancer is commonly expressed at high levels and is more stable than wild-type p53 [17].

Here, we investigated the mechanism of the activation of p53 mediated by curcumin. We showed that curcumin promotes the complex formation of NQO1-p53 leading to p53 stabilization [18]. High levels of NQO1 are not enough for the p53 stabilization; we demonstrated that the presence of curcumin is necessary to stabilize the p53-NQO1 interaction. Also, this interaction can promote the loss interactions between p53 and its negative regulators. The effect of curcumin on p53 levels is differential between the cancer cell lines because it only has effect on cell viability of HeLa, SiHa and CaSki but not in MDA-MB-231. So curcumin is a molecule with an important therapeutic potential in cancer cells with p53 wild type.

2. Materials and methods

2.1. Chemicals and reagents

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dicumarol, cycloheximide (CHX), dimethyl sulfoxide (DMSO), curcumin (C1386), protease inhibitor cocktail tablets EDTA-free (S8830), protein G sepharose (GE28), Trizma base (T1503), sodium chloride (NaCl S9888) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pierce BCA Protein Assay Kit (23225) and lipofectamine plus transfection reagent (15338100) were from ThermoFisher (Waltham, MA, USA) Nonidet P-40 (CAS 68412-54-4), anti-p53 mouse monoclonal antibody (DO-1), anti-NQO1 mouse monoclonal antibody (H9), anti-E6AP (E4) mouse monoclonal antibody, anti-lamin A/C (2A1) mouse monoclonal antibody, anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, L8) goat polyclonal antibody, donkey anti-goat IgG-HRP (sc-2020), and goat anti-mouse IgG-horse radish peroxidase (HRP, sc-2005) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Dulbecco's Modified, Eagle Medium high glucose (DMEM 11965–084) and fetal bovine serum (10500056) were from GIBCO.

2.2. Cell lines and culture

Cell lines HeLa, SiHa and CaSki were cultured in DMEM supplemented with 10% fetal bovine serum. MDAMB-231 cells were cultured in Dulbecco's Modified Eagle Medium Nutrient Mixture (DMEM, GIBCO, 11320–033) supplemented with 10% fetal bovine serum. All cell lines were cultured at 37°C in a 5% CO_2 incubator.

2.3. Western blot

The cells samples lysates were extracted with lysis buffer composed of 50 mM Tris, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 10 mM sodium phosphate, and a complete tablet protease Inhibitor Cocktail per 100 ml of buffer, and the protein concentration in the lysates was quantified using an enhanced bicinchoninic acid protein assay kit with bovine serum albumin as a standard. The total protein extract will be used for western blot analysis. Equal amounts of total protein were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred into a nitrocellulose membrane, followed by incubation overnight to 4°C using the following dilution of primary antibodies: anti-p53 (1:100), anti NQO1 (1:1000), anti-MDM2 (1:500), anti-E6AP (1:1000), anti-lamin A/C (1:500), anti-GAPDH (1:1000) and following by incubation with secondary antibody in blocking solution 1 h room temperature; anti-mouse (1:10000), anti-goat (1:20000) finally protein expression levels were visualized with Li-COR C-DiGit chemiluminescence western blot scanner and UVP Imaging system.

2.4. Pulse and chase assays

The cells were seeds in p35 plates at density of 1.5×10^5 cells/plate and treated with curcumin at concentration of 20 μM for 24 h, the treatment with curcumin was removed and the cells were washed with PBS, continuing with the treatment with CHX with a concentration of 50 μg/ml as previously reported [19,20], the CHX treatment is a standard protein synthesis inhibitor and aliquots of cells were collected every then minutes starting on 0 min, 10 min, 20 min so on until 60 min immediately following addition of the compound cells were lysed with lysis buffer composed of 50 mM Tris, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 10 mM sodium phosphate, and a complete tablet protease Inhibitor Cocktail per 100 ml of buffer, p53 protein abundance at each time point was analyzed, by western blot as described.

2.5. Immunoprecipitation assays

The cells were seeded in p60 plates at density of 2.5×10^5 cells/plate and treated with 20 μM curcumin for 24 h and then lysed, after centrifugation, the clear cell lysate was separated from the pellet of cell and then incubated 2 h at 4°C with 50 μl Protein G sepharose, 1 μg of antibodies and 0.5 mg/ml RNAse A, after incubation the sepharose beads coupled to protein G were washed 20 times with lysis buffer. For western blot, the resulting immunoprecipitates were resolved by SDS-PAGE, then the gel contents were transferred to a nitrocellulose membrane and probed with specific antibodies, finally protein expression levels were visualized with Li-COR C-DiGit chemiluminescence western blot scanner (LI-COR Biosciences, Lincoln, Nebraska USA).

2.6. Cell viability assays

Cell viability was determined by the MTT (M2128) assay [21]. HeLa, SiHa, CaSki and MDA-MB-231 cells (5×10^4 cells/well) were seeded in a 96-well plate and treated with 10 μM and 20 μM curcumin (C1386) for 24 h. After addition 10 μl per well MTT (5 mg/ml) solution the cells were incubated at 37°C for 4 h, the formazan crystals were dissolved using 50 μl DMSO [41]. The cell viability was determined by measuring the absorbance at 570 nm on a Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA).

2.7. Transfection of PANC1 cells with NQO1 plasmids

PANC1 cells (Null to NQO1 were obtained from American Type Culture Collection (ATCC CRL-1469) were transient transfected using Lipofectamine plus transfection reagent with 1 μg pCDNA3 NQO1 or 1 μg pCDNA3 (Empty Vector) the plasmids were obtained from addgene nonprofit plasmid repository. The results were obtained from three

separate biological replicates. The cells transfected and PANC1 wild type (non transfected) were treated with curcumin, pulse and chase assay was performed. The expression levels of p53 protein were analyzed by western blot as described above.

2.8. Statistical analysis

Results are expressed as mean ± SD. Statistical tests were performed using GraphPad PRISM version 6.0c. The ANOVA test with Tukey was applied to compare the means of groups, a $p < 0.05$ was significant.

3. Results

3.1. Curcumin treatment increases the levels of NQO1, p53 and p21

Previous studies have shown that curcumin is capable to activate the Keap1/Nrf2 pathway in order to increase the expression of phase II enzymes including NQO1 [22]. To determine the participation of curcumin on the Keap1/Nrf2 in our model, we evaluate the activation of the Keap1/Nrf2 pathway through the translocation of Nrf2 to the nucleus in Hela, CasKi and SiHa cervical cancer cells lines and MDA-MB-231 breast cancer cells in response to curcumin treatment. We confirmed the immunodetection of Nrf2 in the nuclear extract after 20 μM of curcumin treatment in all cell lines (data not shown) and observed the overexpression of NQO1 (Fig. 1A). Therefore, we determine that curcumin induces the nuclear translocation of Nrf2, an event that indicates the activation of the Keap1/Nrf2 pathway and the increased levels of NQO1 that is consistent with the Nrf2 nuclear translocation. Then, we observed that curcumin treatment also increases the p53 levels (Fig. 1B). All together, these results indicate that curcumin may have an important role in the activating the p53 pathway. To evaluate the functionality of p53 pathway in response to curcumin treatment the expression of its target p21 was evaluated. We showed that curcumin increases the expression of p21 in cervical cancer cell lines with p53 wild-type genotype (CaSki, HeLa and SiHa) meanwhile, this effect was not observed in MDA-MB-231 cell line that has mutated p53 (Fig. 2A) which has decreased p53 activity. These results indicate that curcumin activates the Keap1/Nrf2 pathway in our cellular model with wild-type p53 and leads to the activation of p53 pathway.

In order to evaluate the effect of curcumin on the cell viability a MTT assay was performed. Cells were treated with 10 μM and 20 μM of curcumin; non-treated cells (−) and cells treated with vehicle (V) were used as control. Curcumin treatment induces a significant decrease on the cell viability was observed at the p53 wild type cervical cancer cell lines. Interestingly, at the same concentrations, MDA-MB-231 cells with p53 mutated were resistant to the cytotoxic effect of curcumin. These results suggest that the cytotoxic effect is related with the activation of the p53 wild type (Fig. 2B).

3.2. Curcumin increases the half life time of p53

It is widely reported that the half-life time of p53 is 20 min in physiological conditions [23]. In order to evaluate the effect of curcumin on the half-life of p53 we analyzed its stability after treatments with curcumin or cycloheximide, a well-known inhibitor of protein synthesis [24]. It was found that p53 stability of HeLa, SiHa, CaSki and MDA-MB-231 cells without treatment with curcumin was 20 min (Fig. 3A), however when cells were treated with 20 μM curcumin, p53 protein was detected until 60 min (Fig. 3B). These results suggest that curcumin is able to enhance the p53 half-life of tumor cells.

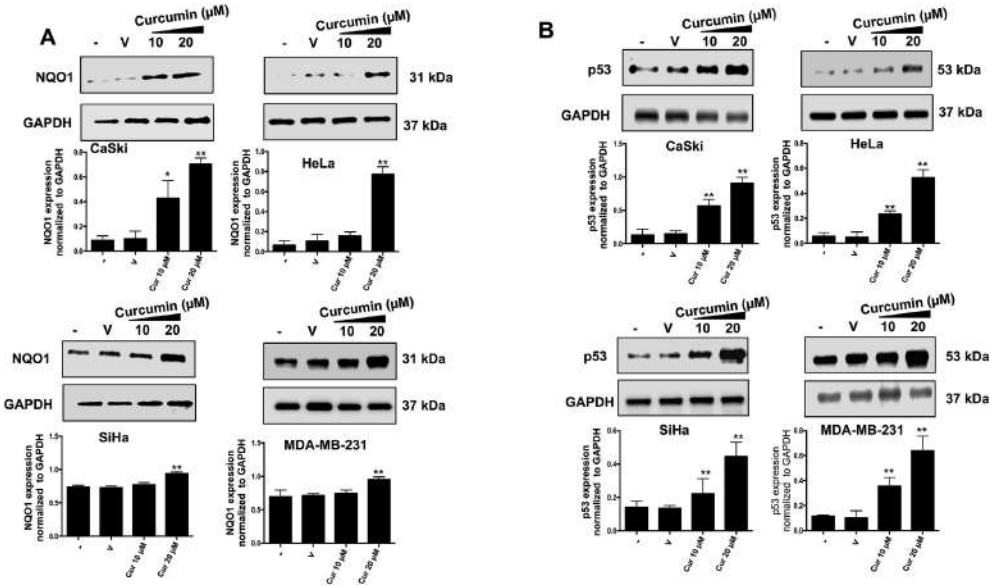

Fig. 1. Effect of curcumin (Cur) on NQO1, p53 levels in CaSki, HeLa, SiHa and MDA-MB-231 cells. CaSki, HeLa, SiHa and MDA-MB-231 cells were treated with 10 μM and 20 μM of curcumin and (A) NAD(P)H: quinone oxidoreductase 1 (NQO1, 31 kDa) and (B) p53 were detected by immunoblotting. The cells were treated for 24 h. The values are means ± SD of three independent experiments. The negative sign (−) represents without treatment, the V represents Vehicle. Statistical differences in A and B were determined using one-way ANOVA and Tukey's multiple comparison test; (*) $p < 0.005$, (**) $p < 0.001$ (−) vs V, (−) vs 10 μM of curcumin and (−) vs 20 μM of curcumin.

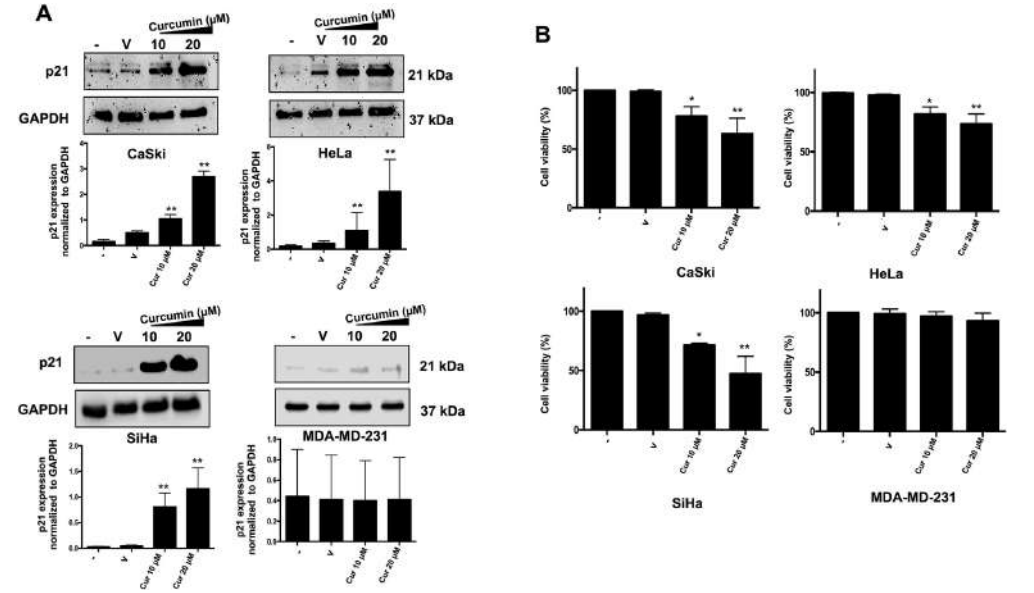

Fig. 2. Effect of curcumin (Cur) on p21 levels and cell viability in CaSki, HeLa, SiHa and MDA-MB-231 cells. CaSki, HeLa, SiHa and MDA-MB-231 cells were treated for 24 h with 10 μM and 20 μM of curcumin and (A) p21 (21 kDa) levels were detected by immunoblotting and (B) Cell viability was evaluated using MTT. The values are means ± SD of three independent experiments. The negative sign (−) represents without treatment, the V represents Vehicle. Statistical differences in A and B were determined using one-way ANOVA and Tukey's multiple comparison test; (*) $p < 0.005$, (**) $p < 0.001$ (−) vs V, (−) vs 10 μM of curcumin and (−) vs 20 μM of curcumin.

3.3. Curcumin promotes the interaction between p53 and NQO1

The NQO1 interaction with p53 under stress oxidative conditions has also been observed [25,26]. In order to evaluate whether curcumin promotes the physical interaction of p53 with NQO1, immunoprecipitation assays were performed. In these assays, p53 was inmunoprecipitated and then NQO1 was revealed by western blot assay. Non-treated cells were used as control. The input material was

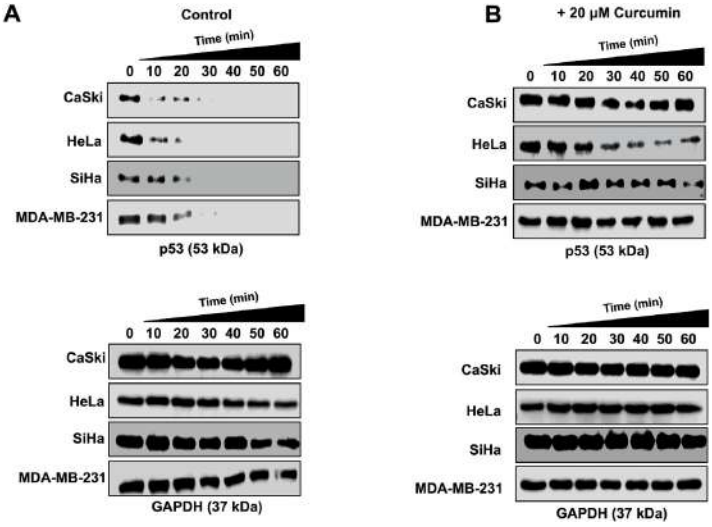

Fig. 3. Curcumin stabilizes p53. CaSki, HeLa, SiHa and MDA-MB-231 cells were treated with 20 μM of curcumin for 24 h and the half-life of p53 (53 kDa) was measured with a pulse and chase experiment with cycloheximide (50 μg/ml), the protein extraction was performed every 10 min (0–60) and revealed by immunoblot, the stability of p53 was evaluated in (A) control cells (without curcumin) and in (B) cells treated with curcumin.

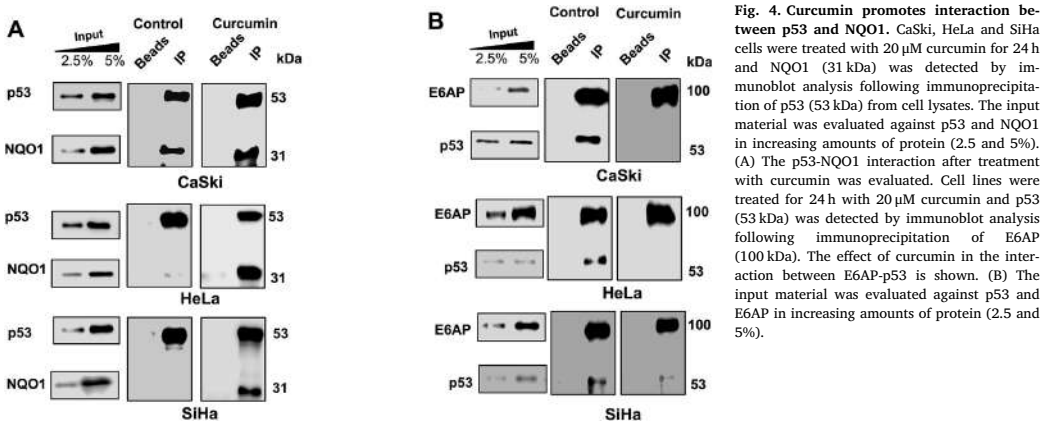

Fig. 4. Curcumin promotes interaction between p53 and NQO1. CaSki, HeLa and SiHa cells were treated with 20 μM curcumin for 24 h and NQO1 (31 kDa) was detected by immunoblot analysis following immunoprecipitation of p53 (53 kDa) from cell lysates. The input material was evaluated against p53 and NQO1 in increasing amounts of protein (2.5 and 5%). (A) The p53-NQO1 interaction after treatment with curcumin was evaluated. Cell lines were treated for 24 h with 20 μM curcumin and p53 (53 kDa) was detected by immunoblot analysis following immunoprecipitation of E6AP (100 kDa). The effect of curcumin in the interaction between E6AP-p53 is shown. (B) The input material was evaluated against p53 and E6AP in increasing amounts of protein (2.5 and 5%).

evaluated against p53 and NQO1 in increasing amounts of protein (2.5 and 5%). It was found that NQO1 physically interact with p53 when HeLa, SiHa and CaSki cells were treated with curcumin (Fig. 4A). These results suggested that NQO1-p53 complex could be stabilizing p53 in response to curcumin treatment for altered cell viability.

In order to show that the effect of curcumin could avoid the interaction between p53 and its negative regulators ubiquitin ligase E6-associated protein (E6AP) a immunoprecipitation assay was performed (Fig. 4B). In order to address this question cells with wild type p53 were used for this purpose because we did not observe any effect on cell viability in MDA-MB-231. To evaluate the change in the direct interaction between E6AP and p53 in response to curcumin treatment, E6AP protein was inmunoprecipitated and then p53 was revealed by western blot assay. Non-treated cells were used as control. The input material was evaluated against p53 and E6AP in increasing amounts of protein (2.5 and 5%). Results showed that curcumin treatment induces loss of interaction between p53 and E6AP (Fig. 4B). All together, our results showed that curcumin induces loss in the E6AP-p53 interaction and favors interaction of NQO1-p53 protein complex to promote cytotoxicity on the cervical cancer cells with p53 wild type.

3.4. Curcumin is necessary for complex NQO1-p53 and p53 stabilization

To determine whether the enzymatic activity of NQO1 is required for p53 stability when curcumin is present, the cells were treated with curcumin and after they were treated 4 h with 100 μM of dicumarol (a NQO1 activity inhibitor). To evaluate p53 stability a pulse and chase assay was performed. Results showed that p53 half-life time was less than 60 min when the cells were treated with dicumarol (Fig. 5A). This suggests that stabilization of p53 by NQO1 depends on an intact enzymatic activity of NQO1. In order to demonstrate if NQO1 is responsible for p53 stability, we used pancreatic epithelioid carcinoma cells (PANC1), a cell line null to *NQO1* gene [27,28]. We first treated wild type cells PANC1 (non-transfected) with curcumin and interestingly, observed no stabilization of p53 (Fig. 5B), suggesting that the presence of curcumin is not sufficient to increase the half-life of p53. NQO1 expression was reestablished by exogenous expression of NQO1 messenger cloned in a plasmid (PCDNA-NQO1). After 48 h of transfection, pulse and chase assay was performed in PANC1 transfected cells, but without curcumin treatment, and again the stabilization of p53 was not observed (Fig. 5C) even when NQO1 protein was expressed, suggesting that the presence of NQO1 is not enough to increase the half-life of p53. Surprisingly, when transfected cells were then treated with 20 μM curcumin, stabilization of p53 was indeed observed (Fig. 5C). This result clearly showed that curcumin is necessary to promote the stabilization of p53, via interaction with the NQO1 protein.

4. Discussion

Tumor suppressor p53 plays a central role in protecting the genome and preventing cell transformation [29]. However, normal cells must maintain p53 levels under tight control to prevent death. Despite mutations in the p53 gene occurring in 50% of all cancers, cervical cancer cells as HeLa, SiHa and CaSki cells retain functional wild-type p53 [30,31] while, MDA-MB-231 cells has a loss-of-function mutation in TP53 gene [32]. Therefore, cancer cell lines with wild type p53 can reactivate downstream pathways and consequently promote cell death and decrease cell viability [33]. Hence, p53 restoration may be a promising therapeutic strategy, however, stability of p53 is an important factor for the reactivation pathway. The main negative regulator of p53 is MDM2, however in HPV-positive cancer cell lines like HeLa, SiHa and CaSki, viral oncoprotein E6 forms a complex with E6AP and their physical interaction with wild-type p53 promotes its degradation via the ubiquitin pathway [34]. The half-life of the p53 protein is short (20 min); during periods of cellular stress, the p53 protein is regulated by a negative feedback mechanism. Here we show that curcumin increases the half-life of p53 up to 60 min and present data on the role of NQO1 in protecting p53 from degradation which are consistent with data previously reported [35]. However, in this work we demonstrated that curcumin is necessary to promote NQO1-mediated stabilization of p53. NQO1 plays an important role in this stabilization because it can physically interact with p53. NQO1 is even considered an anticancer enzyme since it protects cells from oxidative stresses [36]. Thus, the use of dietary compounds to induce the expression of NQO1 has emerged as a promising strategy for cancer prevention [37]. Compounds as curcumin had the ability to translocate Nrf2 which in turn bind to ARE in the promoter region of antioxidant enzymes and increase the expression of phase II antioxidant proteins such as NQO1 [38,39]. The increase in NQO1 levels is one of the results of the translocation of Nrf2 to the nucleus induced by curcumin. NQO1 is a protein that can form complexes with other proteins to stabilize them, including p53.

In this report, we propose that curcumin is necessary to induce NQO1 and promote its interaction with p53, which in turn results in an increase of the p53 stability, contrary to previous reports [40,41]. In 2005, Tsvetkov et al. showed that curcumin and dicoumarol destabilized p53 in M1 mouse myeloid leukemic cells transfected with a plasmid containing a temperature-sensitive p53 [135 Ala → Val] (M1-t-

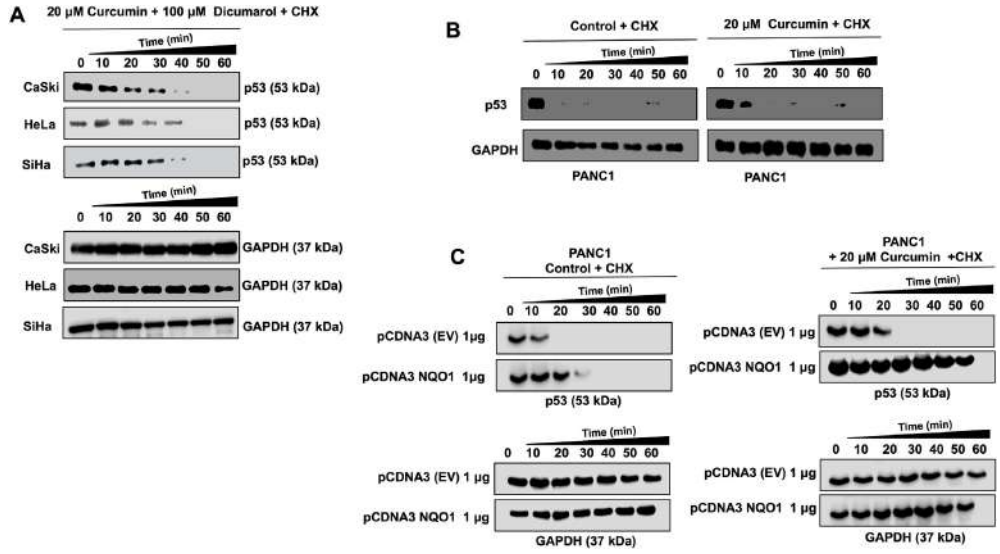

Fig. 5. Curcumin increases p53 stability in a NQO1 dependent manner. Pulse and chase analysis were performed in cell lines treated for 4 h with 20 μM curcumin 24 h and 100 μM dicumarol (NQO1 Inhibitor). (A) the effect of the inhibition of NQO1 activity by dicumarol on p53 stability is shown. Pancreatic carcinoma cells null to NQO1 (PANC1 cells) wild type and transfected with 1 μg pCDNA3 EV (empty vector) or with 1 μg of pCDNA3-NQO1 (to express the complete NQO1 protein) were treated with 20 μM for 24 h and after pulse and chase of p53 (53 kDa) was performed. The chase of proteins was performed every 10 min and revealed by immunoblot, the effect of treatment with curcumin on p53 stability in (B) absence of NQO1 (PANC1 WT or PANC1 + pCDNA3) and when (C) NQO1 levels are restored is shown. CHX = cycloheximide.

p53). M1-t-p53 protein has a wild-type conformation and activity at 32°C, but not at 37°C. In addition, they used mouse A31N-ts20 cells which had a temperature-sensitive E1 ubiquitin activating enzyme, that is inactivated at 39°C and causes accumulation of p53. They observed degradation of M1-t-p53 by low doses of curcumin (20 μM) and dicumarol (100 μM) in mouse cells growth at two different temperature conditions (39 °C or 32 °C). However, the same authors showed degradation of WT p53 in the p53-null cell line HCT116 from human colorectal cancer cells [40], In that report, cells were transfected with human WT p53 and treated with high doses of curcumin (60 μM) and dicumarol (300 μM) [40]. In our report we used cell lines derived from human cervical cancer; HeLa, SiHa, and CaSki. These cell lines exhibit a low basal wild-type p53 expression due to the presence of the Human Papillomavirus (HPV) E6 oncoprotein, therefore, in this case the mechanism for degradation of P53 is dependent on the formation of a complex between E6 and the E6AP protein. Finally, it is important to emphasize that we used low doses of curcumin (20 μM) and dicumarol (100 μM).

On the other hand, Zeekpudsa et al. in 2014 reported an increased expression of p53 when NQO1 activity is inhibited by dicoumarol. The authors used two *Opisthorchis viverrini*-related cholangiocarcinoma cell lines (KKU-100 and KKU-M214) with high and low NQO1 expression levels, respectively [41]. To decrease NQO1 levels, the authors used a specific NQO1 siRNA. On the contrary, in our work we used human cervical cancer cell lines with wild-type p53 and importantly, we use curcumin, which plays a central role in directing the interaction between NQO1 and p53 to promote its stabilization. It is worth to mention that in the report of Zeekpudsa et al., curcumin was not used. Our results indeed demonstrate that curcumin is necessary to promote the maintenance of an environment in which p53 and NQO1 can interact, and therefore increase the half-life of p53.

Finally, our data also showed that the physical interaction between NQO1 and p53 promote its loss of interaction with its negative regulator E6AP. Moreover, NQO1 exhibits catalytic enzyme properties, first reported by Ernster and Navazio in 1958 [42], that we here shown necessary for the stabilization of p53, since dicumarol, an inhibitor of NQO1 activity, inhibits the stabilization of p53, consistent with the previously reported [43,44].

We here propose a model in which p53 stability is determined by two effectors acting together: curcumin and NQO1 (Fig. 6). Curcumin activates the Keap1/Nrf2 pathway and promote the increase of NQO1 levels and, in the presence of curcumin, NQO1 can interact physically with p53 and promote its stability. Also, the formation of the NQO1-p53 complex promote the loss of interaction between p53 and its negative regulator E6AP. In cancer cells with wild-type p53, like those positive for HPV, the stabilization of p53 promotes a reactivation of the p53 pathway, and therefore decrease cell viability.

5. Conclusions

Our results demonstrate the importance of curcumin in the regulation of p53 stability. In this work we demonstrate that curcumin treatment increases p53 levels and provides an appropriate cellular environment for p53 and NQO1 to interact. At the same time this interaction promotes the loss of interaction of p53 with its negative regulator E6AP, the negative regulator of p53 when HPV E6 oncoproteins are present. In our work, HeLa, SiHa and CaSki tumor-derived cell lines that have wild-type p53 and downstream pathways intact, therefore curcumin may be a potential therapeutic agent for tumors with wild type p53.

Author's contributions

"CCPM, ESR, and AGC conceived the study, designed the

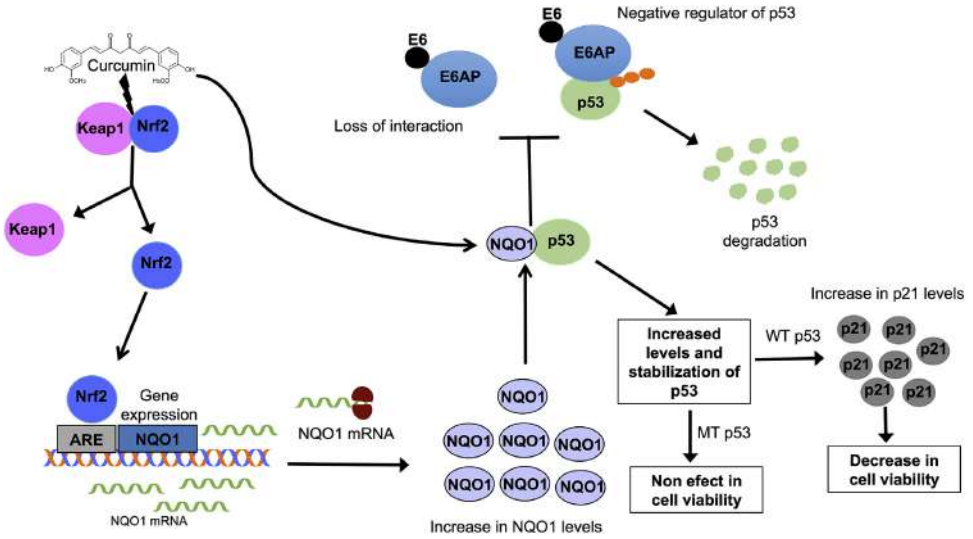

Fig. 6. Schematic model of effects of curcumin in the p53 stabilization. Curcumin activates the Kelch-like ECH-associated protein 1-nuclear factor (erythroid-derived 2)-like 2 pathway (Keap1/Nrf2) pathway; Nrf2 is translocated into the nucleus and binds to the antioxidant response element (ARE) sequences increasing the expression of NAD(P)H: quinone oxidoreductase 1 (NQO1). NQO1 is translated and binds to p53 promoting the loss of interaction between p53 and its negative regulators. Curcumin also stabilizes the interaction between NQO1-p53. In cells with wild-type p53 (WT p53) the p53 pathway is activated and has an effect on cell viability whereas in cells with mutated p53 (MT p53) there is only accumulation of p53 without effect on cell viability.

experimental strategy, analyzed the results, and drafted the manuscript. CCPM. EAO, and VAV carried out molecular biology studies and performed statistical analysis. EOS and JPC participated in drafting the discussion and helped to revise the manuscript. All authors read and approved the final manuscript."

Funding

This work was supported by Consejo Nacional de Ciencia y Tecnología (CONACyT No. 253804, to AG-C) and by Fundación Miguel Alemán, A.C. (to AG-C).

Conflicts of interest

The authors declare no conflicts of interests.

Acknowledgments

Carlos César Patiño Morales is a doctoral student from Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México (UNAM) and received fellowship from CONACYT. CVU 416000. We thank to the Unidad de Investigación Biomédica en Cáncer, Universidad Nacional Autónoma de México-Instituto Nacional de Cancerología (Mexico City) and to the Departamento de Ciencias Naturales, Universidad Autónoma Metropolitana, Unidad Cuajimalpa (Mexico City).

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Anexo II

www.oncotarget.com Oncotarget, 2018, Vol. 9, (No. 24), pp: 17028-17042

Research Paper

CTCF-KDM4A complex correlates with histone modifications that negatively regulate *CHD5* gene expression in cancer cell lines

Lissania Guerra-Calderas[1], Rodrigo González-Barrios[1], Carlos César Patiño[1], Nicolás Alcaraz[4], Marisol Salgado-Albarrán[1], David Cantú de León[3], Clementina Castro Hernández[1,2], Yesennia Sánchez-Pérez[1], Héctor Aquiles Maldonado-Martínez[5], Inti A. De la Rosa-Velazquez[6], Fernanda Vargas-Romero[7], Luis A. Herrera[1,2], Alejandro García-Carrancá[1,2] and Ernesto Soto-Reyes[1]

[1]Cancer Biomedical Research Unit, Instituto Nacional de Cancerología (INCan), Mexico City, Mexico

[2]Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México (UNAM), Mexico City, Mexico

[3]Clinical Research, Instituto Nacional de Cancerología (INCan), Mexico City, Mexico

[4]The Bioinformatics Centre, Section for RNA and Computational Biology, Department of Biology, University of Copenhagen, Copenhagen, Denmark

[5]Department of Surgical Pathology, Instituto Nacional de Cancerología, Mexico City, Mexico

[6]Genomics Lab, Universidad Nacional Autónoma de México, Red de Apoyo a la Investigación-CIC and Instituto Nacional de Ciencias Médicas y Nutrición "Salvador Zubirán", Mexico City, Mexico

[7]Instituto de Fisiologia Celular-Neurociencias, Universidad Nacional Autonoma de Mexico (UNAM), Mexico City, Mexico

***Correspondence to**: Ernesto Soto-Reyes, **email:** ctcf@ciencias.unam.mx*

***Keywords:** KDM4A; CTCF; histone demethylation; H3K36me; CHD5*

***Received:** August 10, 2017* ***Accepted:** February 26, 2018* ***Published**: March 30, 2018*

ABSTRACT

Histone demethylase KDM4A is involved in H3K9me3 and H3K36me3 demethylation, which are epigenetic modifications associated with gene silencing and RNA Polymerase II elongation, respectively. *KDM4A* is abnormally expressed in cancer, affecting the expression of multiple targets, such as the *CHD5* gene. This enzyme localizes at the first intron of *CHD5*, and the dissociation of KDM4A increases gene expression. *In vitro* assays showed that KDM4A-mediated demethylation is enhanced in the presence of CTCF, suggesting that CTCF could increase its enzymatic activity *in vivo,* however the specific mechanism by which *CTCF* and *KDM4A* might be involved in the *CHD5* gene repression is poorly understood. Here, we show that CTCF and KDM4A form a protein complex, which is recruited into the first intron of *CHD5*. This is related to a decrease in H3K36me3/2 histone marks and is associated with its transcriptional downregulation. Depletion of *CTCF* or KDM4A by siRNA, triggered the reactivation of *CHD5* expression, suggesting that both proteins are involved in the negative regulation of this gene. Furthermore, the knockout of *KDM4A* restored the *CHD5* expression and H3K36me3 and H3K36me2 histone marks. Such mechanism acts independently of *CHD5* promoter DNA methylation. Our findings support a novel mechanism of epigenetic repression at the gene body that does not involve promoter silencing.

INTRODUCTION

Gene regulation in eukaryotes is driven in part by chromatin architecture, where histone post-translational modifications play a major role in this process [1]. In particular, the methylation of lysine residues in histones is involved in transcriptional activation and repression, depending on specific lysines and the degree of methylation. For example, H3K4me3 and H3K36me3 are associated with transcriptional activation, while H3K9me3 and H3K27me3 are related with transcriptional repression [2].

Although, it was long thought that lysine methylation was a stable and irreversible process, recent reports have found approximately 25 enzymes capable of removing the methyl groups of lysines in histones. These enzymes are grouped into two families depending on their chemical mechanism of demethylation, the oxidases and the oxygenases [3]. The majority of histone demethylases belong to the second family, including lysine (K)-specific demethylase 4A (KDM4A). KDM4A actively removes the methyl groups from H3K36me3 to produce H3K36me2 [3]. In particular, H3K36me3 is enriched in genes that are transcriptionally active and is associated with recruitment of RNA polymerase II and transcriptional elongation, loss of H3K36me3 leads to transcriptional repression [4].

KDM4A is overexpressed in several types of cancer, including breast cancer [5]. One of the target genes of KDM4A is chromodomain helicase DNA binding protein 5 gene (*CHD5*). *CHD5* was identified as a tumor suppressor gene, and it has been reported deregulated in glioma, colon, lung, ovarian, prostate and breast cancers. Thus, based on its likely involvement as a tumor suppressor gene (TSG) in neuroblastomas, gliomas, and many common adult neoplasms, CHD5 may play an important developmental role in many other tissues besides the nervous system and testis [6]. Particularly, this gene is involved in cell proliferation, apoptosis and senescence by regulating $p19^{Arf}$, modulating p53 activity [6]. KDM4A has been reported to negatively regulate *CHD5* by its recruitment to the first intron [7]. Neither the mechanism by which KDM4A negatively regulates *CHD5* nor the mechanism by which KDM4A is recruited to this target site are known. Furthermore, *in vitro* assays have shown that the demethylation frequency of KDM4A increases up to 80% in the presence of the architectural protein CTCF [8], suggesting that CTCF may play a major role in the activity of KDM4A which has not been addressed until now. Hence, the aim of this study was to elucidate the mechanism underlying the role of CTCF and KDM4A on histone modifications and in the downregulation of *CHD5*.

RESULTS

KDM4A is highly expressed in MCF7, MDA-MB-231 and HeLa cell lines

As a first approach, we evaluated the expression of *KDM4A* in four different cell lines using RT-qPCR. We observed that *KDM4A* was highly expressed in MCF7 and MDA-MB-231 cell lines compared to the expression levels of the non-tumorigenic epithelial breast cell line MCF 10A (Figure 1A). Previously, *KDM4A* has been reported to be highly expressed in HeLa cells [9], hence we used this cell line as a positive control. Immunofluorescence assays show that KDM4A is located mainly at the nucleus in the neoplastic cell lines (Figure 1B), but it is not detected in the non-tumorigenic breast cell line MCF 10A (Figure 1B). We also observed *CHD5*, which has been reported to be regulated by KDM4A and highly expressed in the MCF 10A cells compared with MCF7, MDA-MB-231 and HeLa cells (Figure 1C) [7]. Additionally, *CHD5* is only detected in the MCF 10A cell line, where *KDM4A* is not present (Figure 1B and 1D). When looking into breast cancer cell line expression data available at the Cancer Cell Line Encyclopedia we found that 83.34% (50/60) of these cell lines show high expression of *KDM4A,* while not expressing *CHD5.* In this regard, MCF7 and MDA-MB-231 cell lines exhibit the same behavior that we observed previously in our results (Figure 1 and Supplementary Figure 1A) [10]. In contrast to what is observed in cell lines, we did not find a significant correlation between *KDM4A* and *CHD5* expression in breast cancer patients (Supplementary Figure 1B) from The Cancer Genome Atlas (TCGA). We argue that this could be due to the heterogeneity of the tumor tissue or tumor subtypes.

DNA methylation at the *CHD5* gene promoter is not the main mechanism of epigenetic silencing in the neoplastic cell lines

Some authors have reported that DNA methylation at *CHD5* gene promoter can alter the expression of this gene in several cancers and neoplastic cell lines [11, 12]. Thus, we analyzed the methylation status along the *CHD5* gene locus of 743 breast cancer patients and 98 normal samples obtained from TCGA (Ilumina Human Methylation 450 K) through the TCGA wanderer web service [13]. This panel measures the methylation levels of 485,000 CpG sites distributed along the genome, of which 63 CpGs fall within the *CHD5* gene region (Figure 2A); of these sites, 8 CpGs are located within the gene promoter, the remaining 55 sites are distributed along the gene body. At the gene body, 34 CpGs are found to be methylated (having Beta-value ≥0.6, which is considered as a methylated region) in 50% of the patients, and 20 of these 34 sites that are present at the gene body are methylated

in 80% of the patients. Nevertheless, when evaluating the mean methylation levels of the 8 CpG sites located within the gene promoter region (Highlighted part of the figure with a rectangle in Figure 2A) (Ensembl version 75), we observed that only 1 out of the 743 patients shows promoter methylation, where the CpG methylation Beta value is less than 0.6, indicating that *CHD5* gene promoter is considered as not methylated (Figure 2B). In order to determine if the absence of methylation in the *CHD5* promoter was restricted only to breast cancer, we also looked into the methylation status in other neoplasms such as Low-Grade Gliomas or Glioblastomas where we also did not find methylation at the promoter region (Supplementary Figure 1C). Hence, these datasets point out that DNA methylation at the promoter region is not related with *CHD5* gene silencing, suggesting that there may be other mechanisms related to its repression in breast cancer (Figure 2A).

Given the methylation status of *CHD5* gene in breast cancer patients found in TCGA, we aimed to characterize DNA methylation status at the *CHD5* promoter. We carried out a methylation sensitive-PCR assay (MS-PCR) at the CpG island which we observed to be unmethylated in 742 patients (Figure 2B and 2C). We found DNA methylation at the *CHD5* promoter to be absent in most of the cell lines, with the exception of MDA-MB-231 (Figure 2D); a similar finding was previously reported by Mulero-Navarro and Esteller [12]. As a positive methylation control of the assay we used an *in vitro*-methylated DNA (IVD) (Figure 2D).

Results from the MS-PCR reinforce the observation of the methylation status in the TCGA patients, where DNA methylation at the *CHD5* gene promoter is not a common mechanism involved in repression of *CHD5*. Therefore, we focused on another epigenetic mechanism that is independent of DNA methylation, such as the histone demethylase KDM4A.

The localization of KDM4A at the *CHD5* first intron correlates with the decrease of H3K36me3 and H3K36me2 in neoplastic cell lines

In 2012, Mallette and colleagues demonstrated by chromatin immunoprecipitation (ChIP) assays that KDM4A is located at *CHD5* first intron in the U2OS cell line, and that the depletion of KDM4A increased *CHD5* mRNA and protein levels [7]. Nevertheless, the mechanism by which KDM4A negatively regulates transcription of the *CHD5* gene remained unclear.

One epigenetic mark relevant to transcriptional elongation is H3K36me3. This histone mark is mainly enriched in gene bodies, where a decrease in its

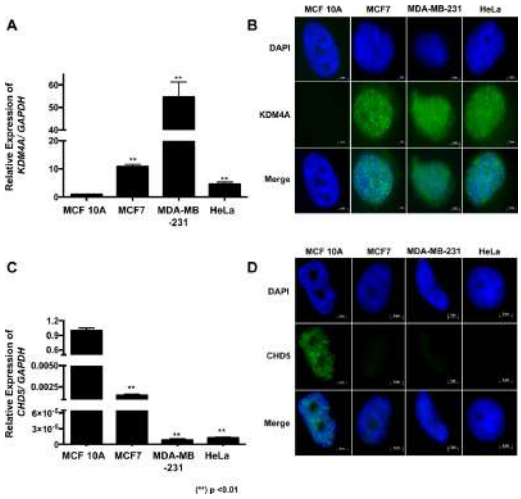

Figure 1: KDM4A overexpression correlates with CHD5 decrease in neoplastic cell lines. (**A**) Expression profile of the human *KDM4A* gene in MCF 10A, MCF7, MDA-MB-231 and HeLa cell lines obtained by RT–qPCR. The data were normalized against GAPDH expression in three independent experiments. (**B**) The presence and localization of KDM4A in MCF 10A, MCF7, MDA-MB-231 and HeLa cells were assessed by immunofluorescence assay. (**C**) Expression profile of *CHD5* gene in the MCF 10A, MCF7, MDA-MB-231 and HeLa cell lines obtained by RT–qPCR. The data were normalized against GAPDH expression in three independent experiments. (**D**) The presence and localization of *CHD5* in MCF 10A, MCF7, MDA-MB-231 and HeLa cells were assessed by immunofluorescence assay. The DNA was stained with DAPI. (**) $p < 0.01$ compared with the MCF 10A cell line. Statistical differences were determined using Student's *t* test.

trimethylated form is associated with gene silencing. In some genes, such silencing is not related to inactivation of the gene's promoter. Since KDM4A is capable of removing this histone mark, we speculated that demethylation of H3K36me3 could play a role in the downregulation of *CHD5* gene expression. To assess our hypothesis, we performed a ChIP assay to determine whether KDM4A could be found at the *CHD5* first intron in our cell lines. KDM4A was present at this region in the MCF7, MDA-MB-231 and HeLa cell lines; and was not detected in the non-neoplastic cell line MCF 10A (Figure 2E). One of the best-characterized gene targets of KDM4A is the region located -1922 bp upstream from the TSS of *ASCL2* [14]. Therefore, we used this region as a positive control of ChIP assay to confirm the presence of KDM4A in all the cell lines, and the 27th exon of the *RB* gene as negative control (Supplementary Figure 2A). To determine the impact of the presence of KDM4A on histone marks related to transcriptional elongation, we analyzed the abundance of H3K36me3 and H3K36me2 at the *CHD5* first intron by ChIP assay (Figure 2F). As a positive control for the H3K36me3 histone modification, we used the ENCODE database to identify a region that is enriched with this histone mark in different cell lines; based on the

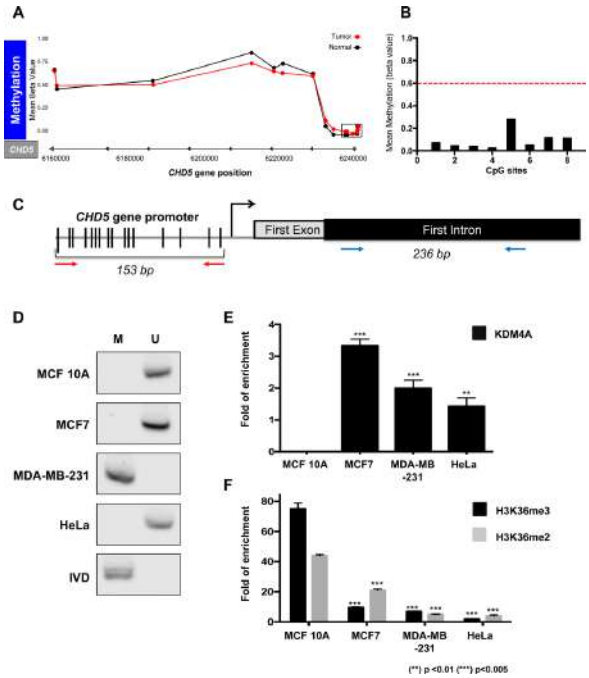

Figure 2: CHD5 repression is associated to histone demethylation by KDM4A at the first intron and not to promoter DNA methylation. (**A**) TCGA DNA methylation levels (Illumina 450 K data) in 743 breast cancer patients (tumor) and 98 non-neoplastic samples (normal) in CHD5 gene locus. 63 CpG sites were analyzed along the gene. Cutoff ≥0.6 Beta-values represents methylated status. The locus marked by a rectangle represents the methylation status of the promoter region; X axes represents the *CHD5* gene position (GRCh 37/hg19) (**B**) the graphic represents the 8 CpGs sites analyzed of the promoter region of 743 patients. The dot line is the threshold of DNA methylation (>0.6 = Methylated). (**C**) Schematic representation of the *CHD5* gene that includes the promoter region and the *CHD5* first intron region analyzed by MS-PCR (153 bp PCR product) and ChIP assays (236 bp PCR product) respectively. The red arrows represent the primers employed for MS-PCR and blue arrows for ChIP qPCR (**D**) Promoter DNA methylation status was assessed by MS-PCR in MCF 10A, MCF7, MDA-MB-231 and HeLa cells. DNA from lymphocytes was methylated *in vitro* by SssI methyltransferase and used as a methylated DNA positive control (IVD). M represents methylated, and U represents non-methylated. (**E** and **F**) qPCR evaluation of the products obtained from the ChIP assay of the *CHD5* first intron, precipitated with anti-KDM4A (D), anti-H3K36me3 and anti-H3K36me2 (E) antibodies in MCF 10A, MCF7, MDA-MB-231 and HeLa cells. As a negative control, we used the IgG antibody included in the OneDay ChIP kit (Diagenode, NJ, USA, Kch-onedIP-180). (**) $p < 0.01$ and (***) $p < 0.005$ compared with the MCF 10A cell line. Statistical differences were determined using Student's *t* test.

results of this analysis, we decided to use the second intron of the *GAPDH* gene. As a negative control, we employed the third exon of the silenced gene *MYOG* (Supplementary Figure 2B). When we compared the enrichment of the methyl marks present in the intron 1 region to the non-neoplastic cell line MCF10A we found that the presence of KDM4A was associated with a decrease in these epigenetic marks in the tumor cell lines (Figure 2F). These results suggest that the presence of KDM4A could alter epigenetic marks related to transcriptional elongation and thus affect gene transcription (Figure 2E and 2F).

CTCF and KDM4A coexist at the *CHD5* first intron in neoplastic cell lines

Given that CTCF plays a major role in the demethylation function of KDM4A [8], we decided to characterize its expression in our cellular model. By RT-qPCR, we observed that CTCF was overexpressed in the MCF7, MDA-MB-231 and HeLa cell lines when compared to the MCF 10A cells (Figure 3A). In addition, CTCF was located in the nucleus of all the cell lines evaluated (Figure 3B).

To determine if CTCF could participate in KDM4A's demethylation activity, we decided to evaluate by ChIP assay the presence of CTCF in the first intron of *CHD5*. As a negative and positive controls, we used the 27th exon of *RB* gene and *WRAP53* promoter region, respectively (Supplementary Figure 2C). Our results show that CTCF is found in this region in all cell lines evaluated (Figure 3C and 3D), including MCF 10A. This is the same region where KDM4A was shown to be present in the neoplastic cell lines (Figure 2C). Since MDA-MB-231 exhibits promoter methylation, we decided to focus only in MCF7 and HeLa, where *CHD5* is repressed even though its promoter region is not methylated. Thus, to determine the coexistence of CTCF and KDM4A at the same genomic region, we performed a ChIP/re-ChIP experiment in the MCF7 and HeLa cell lines (Figure 4A). A first immunoprecipitation was performed with each of the antibodies (KDM4A or CTCF), and a subsequent immunoprecipitation was performed with a second antibody (KDM4A-CTCF or CTCF-KDM4A). As a negative control assay, we used the antibody of interest followed by IgGs or the IgGs followed by the antibody of interest. As a positive control for KDM4A recruitment, we analyzed the region -1922 bp from the *ASCL2* TSS (Supplementary Figure 3A). For CTCF, we employed the *WRAP53* promoter region as a positive control (Supplementary Figure 3B). As a negative control for KDM4A and CTCF, we evaluated the 27th exon of the *RB* gene (Supplementary Figure 3C). The ChIP/ReChIP results showed a co-existance of CTCF and KDM4A at the first intron of *CHD5* both in MCF7 and HeLa cells (Figure 4A). Using the ChIP and ChIP/ReChIP results we evaluated the percentage of co-occupancy in MCF7 and HeLa cell lines (Figure 4B). These results suggest that the higher co-occupancy of KDM4A and CTCF is associated with an increase in *CHD5* repression. Also, these results imply a possible interaction between CTCF and KDM4A.

CTCF and KDM4A form a protein complex in neoplastic cell lines

In order to demonstrate the physical interaction between CTCF and KDM4A a co-immunoprecipitation assay in HeLa cells was performed. This was carried out by an immunoprecipitation against CTCF and revealed with a CTCF antibody (Figure 4C). Subsequently, the proteins obtained from the CTCF Immunoprecipitation (IP) were used in an independent experiment and were revealed against KDM4A (Figure 4D). Our data shows a detectable interaction between endogenous CTCF and endogenous KDM4A in HeLa cells (Figure 4C and 4D). Our results demonstrate a novel protein complex formed by CTCF and KDM4A, which may be localized at the first intron of the *CHD5* gene (Figure 4).

KDM4A and *CTCF* siRNA knock down is associated with the reactivation of *CHD5* expression in neoplastic cell lines

To determine the participation of KDM4A in the repression of *CHD5*, HeLa and MCF7 cells were transfected with siRNAs against *KDM4A*. At 72 hours, post-transfection with the siRNA, expression analyses of the *KDM4A* and *CHD5* genes were performed by RT-qPCR. The results revealed that *KDM4A* mRNA decreased after transfection (Figure 5A). The decrease of KDM4A in the MCF7 and HeLa cell lines induced the reactivation of *CHD5* mRNA, even above the basal expression of MCF 10A (Figure 5B). Because CTCF and KDM4A can potentially form a protein complex, we further investigated which was the participation of CTCF in the repression of *CHD5*. Therefore, we transient transfected a small hairpin RNA expression vector against CTCF (pCT1) in MCF7 and HeLa cells (Figure 5C). Our results show that diminishing of CTCF leads to a reactivation of *CHD5* expression similar to MCF 10A (Figure 5D). Taken together, our results suggest that the presence of KDM4A and CTCF at the first intron of *CHD5* acts as repressors of *CHD5* expression in neoplastic cells.

KDM4A knockout (KO) in MCF7 reestablish the H3K36me3 histone mark at the first intron of *CHD5* and reactivates gene expression

In order to further validate that KDM4A is negatively regulating *CHD5* we establish a Knockout model ($KDM4A^{KO}$) using CRISPR/Cas9 KO system (Santa Cruz, sc-404599 and sc-404599-HDR). This system employed three gRNAs that target exon 3 and

8 of the *KDM4A* gene (Supplementary Figure 4A). We selected cells by puromycin treatment and further enrich our KDM4A^{KO} by FACS cell sorting selecting the highest fluorescent cells (Supplementary Figure 4B). As control, we employed a non-targeting gRNA plasmid (Mock) (Santa Cruz, sc-418922).

We evaluated by Western Blot the protein expression of KDM4A in MCF7 Mock and KDM4A^{KO} cells, where a 63.6% reduction of KDM4A in KDM4A^{KO} cells is observed (Figure 6A). We also performed RT-qPCR analysis of *CHD5* expression in MCF10A, MCF7 Mock and KDM4A^{KO} cells. Here we observe a significant reactivation of the *CHD5* expression in KDM4A^{KO} cells, with levels similar to the observed in MCF10A (Figure 6B). In order to evaluate if such reactivation is related to the loss of KDM4A of the *CHD5* first intron, we performed a ChIP analysis of KDM4A. Our results show a significant loss of KDM4A in KDM4A^{KO} compared to Mock cells (Figure 6C). Regarding our previous results that suggest that KDM4A-CTCF complex regulates

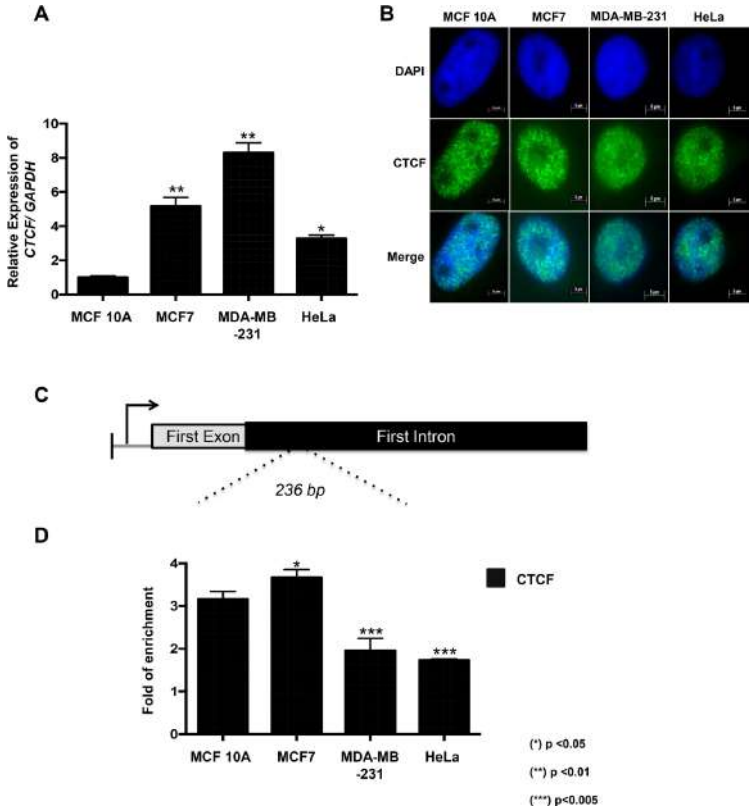

Figure 3: CTCF is overexpressed in neoplastic cell lines and is recruited to CHD5 first intron. (**A**) Expression profile of the human *CTCF* gene in the MCF 10A, MCF7, MDA-MB-231 and HeLa cell lines was obtained with RT–qPCR. The data were normalized against GAPDH expression in three independent experiments. (*) $p < 0.05$ and (**) $p < 0.01$ compared to the MCF 10A cell line. Statistical differences were determined using Student's *t* test. (**B**) The presence and localization of CTCF in MCF 10A, MCF7, MDA-MB-231 and HeLa cells were assessed by immunofluorescence assay. (**C**) Schematic representation of the *CHD5* gene that includes the promoter region and the CHD5 first intron region (236 bp PCR product). (**D**) qPCR analysis of the *CHD5* first intron was performed on the DNA obtained from anti-CTCF ChIP assays in MCF 10A, MCF7, MDA-MB-231 and HeLa cells. As a negative control, we used the IgG antibody included in the OneDay ChIP kit (Diagenode, NJ, USA, Kch-onedIP-180). The data was evaluated by qPCR at *CHD5* first intron and the data is expressed in fold of enrichment over IgG immunoprecitpitation. Statistical differences were determined using Student's *t* test. (*) $p < 0.05$ and (**) $p < 0.01$ compared to the MCF 10A cell line.

CHD5, we evaluated if CTCF could be affected by the loss of KDM4A at the *CHD5* first intron. Our results show that CTCF binding is independent of KDM4A presence, suggesting that CTCF may acts as repressor when it is in a complex with KDM4A (Figure 6D). Because of the obtained results, we attempted to establish a CTCF^{KO} model, however these cells were not viable so the experimental approach was not possible. The generation of a CTCF^{KO} model has been an experimental challenge for different research groups. Particularly, alteration in the abundance of CTCF affects cell proliferation and can even be causal of a lethal phenotype in murine models [15–17].

One of the central questions we wanted to address is whether the loss of KDM4A could restore the H3K36me3 pattern at the *CHD5* first intron. Therefore, we performed a ChIP analysis of H3K36me2/3 in MCF10A, MCF7

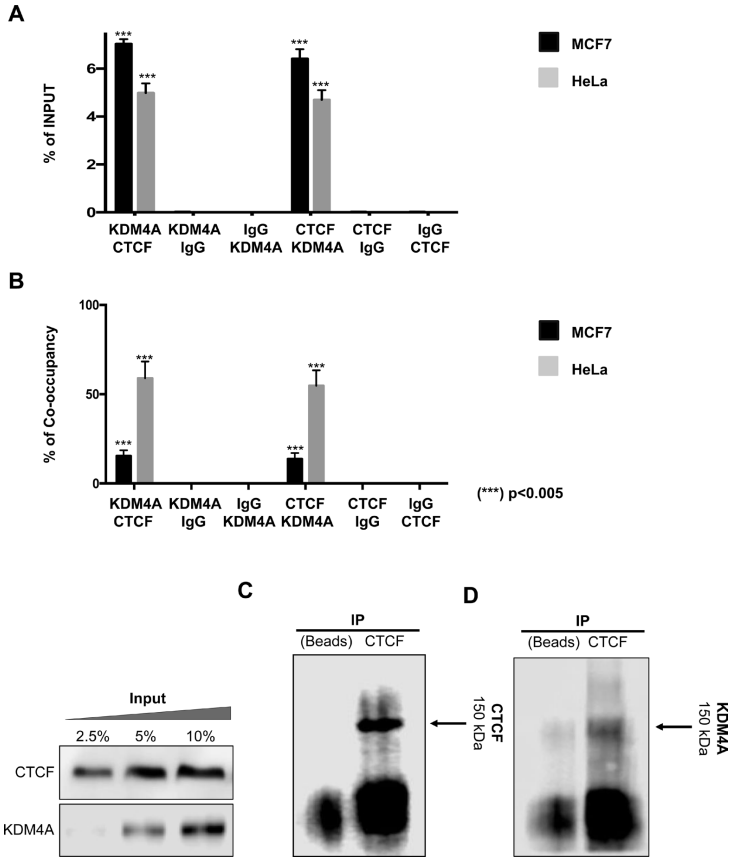

Figure 4: CTCF-KDM4A complex is located at the CHD5 first intron in MCF7 and HeLa cell lines. (**A**) ChIP/re-ChIP assays were performed using the antibodies shown in the first row and subsequentially immunoprecipitated by the antibodies described at the second row in the MCF7 and HeLa cells. The data was evaluated by qPCR at *CHD5* first intron and the results are represented as % of input. Statistical differences were determined using Student's *t* test, (***) $p < 0.005$ compared with IgGs. (**B**) Co-occupancy analysis was performed in accordance to Geisberg and Struhl [38]. For occupancy analysis, the ChIP-Re-ChIP data from both experiment data (CTCF-KDM4A or KDM4A-CTCF) were used. Also, the co-occupancy of IgG experiments was evaluated. The results are represented in % of co-occupancy. Statistical differences were determined using Student's *t* test, (***) $p < 0.005$ compared with IgGs. (**C–D**) A Co-immunoprecipitation assay was performed against CTCF and revealed with CTCF (150 kDa) (C). Using the proteins obtained from the CTCF IP we revealed employing a KDM4A antibody (150 kDa) (D). To the left, the input material was evaluated against CTCF and KDM4A in increasing amounts of protein (2.5, 5 and 10 %).

Mock and KDM4A^{KO} cells. We found a H3K36me3 recovery in KDM4A^{KO} cells, which does not affect the H3K36me2 (Figure 6E). This suggests that the loss of KDM4A demethylase allows the reincorporation of H3K36me3 at the first intron of *CHD5*, favoring the reactivation of the gene expression.

DISCUSSION

Epigenetic alterations are a common feature of cancer processes [18, 19]. Mainly, key epigenetic components, which include methylases and demethylases such as KDM4A as well as architectural proteins like CTCF, are deregulated [5]. Several studies have reported that KDM4A is highly expressed in breast cancer tissues. This demethylase removes the methyl group of H3K9me3 and H3K36me3, with the former related to heterochromatin and the repression of transcription [20], while the latter is enriched in the bodies of genes that are transcriptionally active and is associated with the recruitment of RNA polymerase II and the process of transcriptional elongation [4]. Hence, H3K36me3 alteration could affect gene transcription without disturbing the gene promoters, suggesting a novel mechanism of gene dysregulation not associated with regulatory regions.

CHD5 is a gene that encodes an enzyme which belongs to the helicase family (chromodomain helicase DNA-binding protein 5) [21]. The CHD5 protein can function as a tumor suppressor by regulating apoptosis

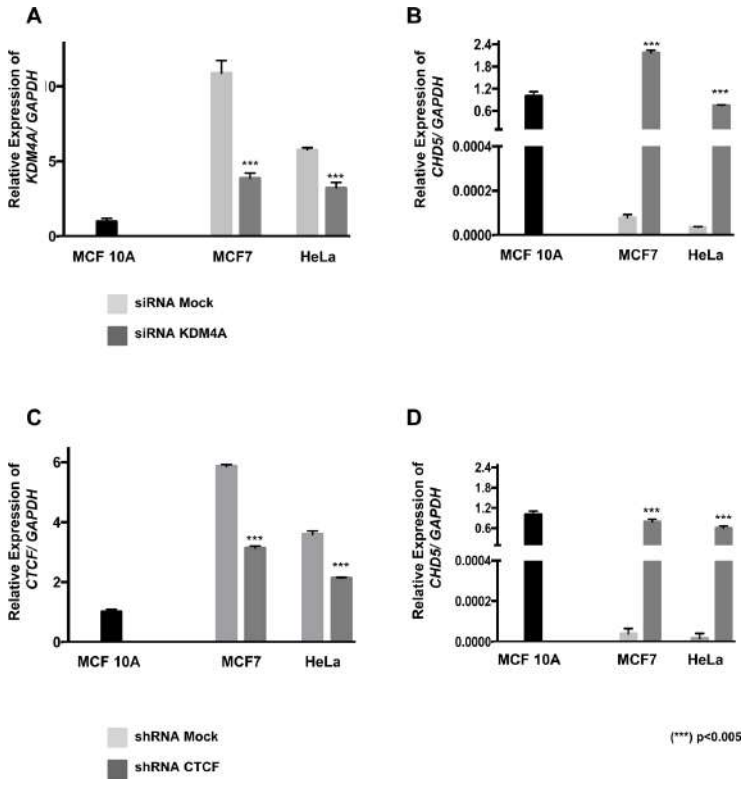

Figure 5: The *CHD5* expression is reactivated by CTCF and KDM4A knockdown in MCF7 and HeLa cells. Analysis of *KDM4A* (**A**) and *CHD5* (**B**) expression in MCF7 and HeLa cell lines following *KDM4A* siRNA transfection. Analysis of *CTCF* (**C**) and *CHD5* (**D**) expression in MCF7 and HeLa cell lines following *CTCF* shRNA transfection. Data were normalized against *GAPDH* expression in three independent experiments using MCF 10A cells as the normal expression control. siRNA mock and shRNA mock transfected cells were used as negative controls. Statistical differences were determined using Student's *t* test compared with mock-transfected cells. (***) $p < 0.005$.

and cellular senescence, and is involved in the p19^{Arf}/p53 pathway by interacting with MDM2 [22, 23]. Because this interaction leads to the attenuation of MDM2-mediated p53 degradation [24], CHD5 and p19^{ARF} help to stabilize p53. In addition, CHD5 inhibits clonogenic growth *in vitro* as well as tumor xenograft growth, suggesting that its inactivation may be involved in cancer development [11]. Some studies have suggested that *CHD5* can be inactivated by genetic [25] or epigenetic processes, but these reports focused mainly on its repression by DNA promoter methylation [11, 12, 26–28]. Analysis of TCGA datasets show that the *CHD5* promoter region is not methylated in breast cancer patients, which suggests that another epigenetic mechanism could be involved in gene repression. In this regard there is evidence that suggests that alteration at the *CHD5* promoter is not the major mechanism of repression of this gene [12].

Previously, it was reported that KDM4A localizes to the *CHD5* first intron and the reduction in KDM4A leads to an increase of *CHD5* expression in U2O2 cells; this

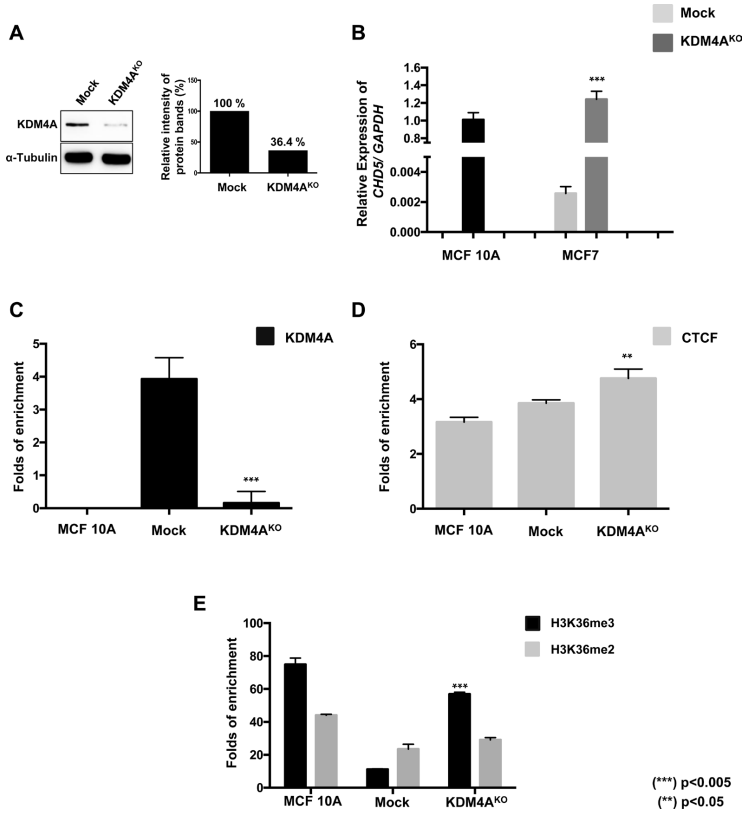

Figure 6: The KDM4A knockout promotes the reestablishment of the H3K36me3 histone mark at the first intron and the reactivation of the expression of CHD5 gene. (**A**) Characterization of KDM4A protein abundance by immunoblots in MCF7 cells transfected with a non-targeting gRNA plasmid (Mock) or KDM4A^{KO} CRISPR/Cas9 and HDR plasmids. The quantitation of the relative intensity of the protein bands showed a decrease of 63.6% of KDM4A in KO cells. (**B**) *CHD5* expression analysis in Mock and KDM4A^{KO} cells. Data were normalized against *GAPDH* expression in two independent experiments using MCF 10A cells as the normal expression control. (**C, D, E**) qPCR evaluation of the *CHD5* first intron from DNA obtained from the ChIP assay using anti-KDM4A (C), anti-CTCF (D) and anti-H3K36me3 and anti-H3K36me2 (E) antibodies in MCF 10A cells, Mock and KDM4A^{KO} cells. As a negative control, we used the IgG antibody included in the OneDay ChIP kit (Diagenode, NJ, USA, Kch-onedIP-180). (**) $p < 0.01$ and (***) $p < 0.005$ compared with the MCF7 Mock cells.

indicates that KDM4A could be associated with *CHD5* repression [7]. However, since KDM4A was not found at the promoter of *CHD5*, the mechanism of how KDM4A downregulates *CHD5* remained unclear.

The overexpression of *KDM4A* is known to be associated with cell proliferation and poor prognosis in several cancers [29, 30]. Identifying how KDM4A inhibits gene expression has a therapeutic impact on cancer in the future; therefore, understanding the molecular mechanisms underlying the effects of KDM4A and their implications in cancer are an important topic for future clinical research [31]. Our findings show that KDM4A functions as a repressor of the *CHD5* TSG by affecting epigenetic marks associated with elongation and not by regulating the gene promoter. This phenomenon has been reported in other cellular models, where KDM4A/C specifically alters H3K36me3 [32]. The phenomenon is also associated with the loss of RNA polymerase II recruitment in transcribed regions of the *GFAP* gene [32]. Our results suggest a novel mechanism of *CHD5* gene repression, where the decrease of H3K36me3/2 at the gene body could lead to transcriptional repression. One hypothesis is that this phenomenon occurs due to lack of phosphorylation of the second serine in the carboxy terminal domain of RNA polymerase II, which results in the enrichment of H3K36me2 and a decrease of transcriptional elongation, or due to an increase in repressive histone marks.

In vitro assays have reported that the presence of CTCF increases the demethylation frequency of KDM4A by up to 80%, suggesting that CTCF has a role in the

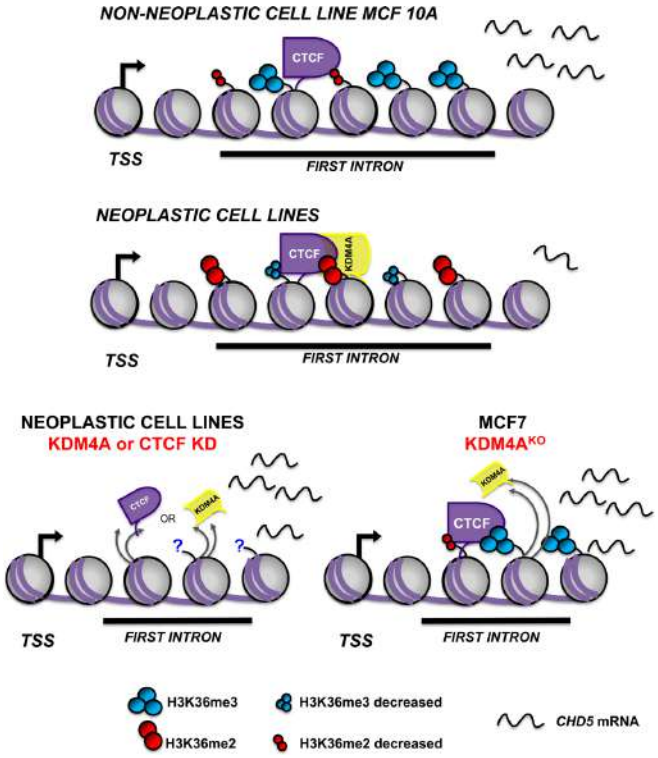

Figure 7: Schematic model of *CHD5* transcriptional repression mediated by CTCF-KDM4A protein complex. CTCF-KDM4A protein complex is recruited to the first intron of the *CHD5* gene and promotes demethylation of histone H3K36me. In non-neoplastic cells, CTCF is located at the first intron of *CHD5*, and H3K36me3/2 are enriched. These events correlate with *CHD5* expression. In contrast, in the neoplastic cells, CTCF-KDM4A protein complex promotes the demethylation of H3K36me3/2 and leads to gene repression. CTCF or KDM4A knockdown (KD) reactivates *CHD5* gene expression. The loss of KDM4A in KDM4A^{KO} cells leads to the reestablishment of the H3K36me3 histone mark at the first intron and the reactivation of *CHD5* gene expression.

demethylation function of KDM4A [8]. Additional support for these datasets was provided by another study that demonstrated that CTCF can interact with the KDM5B histone demethylase and increase its demethylation activity in breast cancer cell lines [33]. CTCF has been reported to act occasionally as a transcriptional repressor of genes, such as *c-MYC*, *Bax*, *Xist* and *hTERT*, by interacting with SIN3A and recruiting HDACs or by preventing the binding of transcription factors that affect expression [34–38]. Interestingly, we observed a protein complex formed by CTCF-KDM4A, which is found at the first intron of *CHD5*. When we evaluated the co-occupancy of KDM4A and CTCF, we showed that the HeLa cell line exhibits a higher percentage of co-occupancy in comparison with MCF7 cell line. Our results suggest that KDMA4 acts as a transcriptional repressor when it is in complex with CTCF. The loss of KDM4A at *CHD5* first intron restores H3K36me3 histone mark and recovers *CHD5* gene expression. Therefore, we propose a novel mechanism of transcriptional repression mediated by KDM4A and CTCF (Figure 7). To date it is unknown if this complex is related with the repression of other genes, and what could be the implications of this complex in diseases such as cancer. Further studies are needed to understand the biological meaning of this new regulatory mechanism.

MATERIALS AND METHODS

Cell culture

MCF 10A cells were cultured in 1 part DMEM-Dulbecco's Modified Eagle Medium (GIBCO, 11965-084) to 1 part of Dulbecco's Modified Eagle Medium/Ham's F-12 Nutrient Mixture (DMEM/F-12, GIBCO, 11320-033) supplemented with 10% fetal bovine serum (GIBCO, 10500056), 2 mM L-Glutamine (GIBCO, 25030081), 10 ng/Ml EGFRh (Invitrogen), 120 mU/mL insulin and 1 µg/mL hydrocortisone (SIGMA). MCF7 and MDA-MB-231 cells were cultured in Dulbecco's Modified Eagle Medium/Ham's F-12 Nutrient Mixture (DMEM/F-12, GIBCO, 11320-033) supplemented with 10% fetal bovine serum. HeLa cells were cultured in DMEM high glucose (GIBCO, 11965-084) supplemented with 10% fetal bovine serum. All cell lines were cultured at 37° C in a 5% CO_2 incubator.

Expression analysis

Total RNA was extracted using TRIzol (Invitrogen, 15596018) according to the manufacturer's specifications. 2 µg of total RNA were reverse transcribed in a final volume of 40 µL using the Kit GeneAmp® RNA PCR KIT (Applied Biosystems, N8080143) as described by the manufacturer's protocol. Gene expression levels for *KDM4A, CTCF*, and *CHD5* were determined using the primers listed in Supplementary Table 1; *GAPDH* was used as an internal control. The qPCRs were performed using Thermo Maxima SYBR Green/ROX 1 PCR Master Mix (Thermo Scientific, K0222) with a StepOnePlus Real–Time PCR System (Applied Biosystems). All reactions were run in triplicate, and the average C_t values were used for quantification. The plots show the mean of three biological replicates. The analysis of the relative quantification of target genes was performed using the $\Delta\Delta C_t$ method as described by Livak [39].

Immunofluorescence assays

Cells were cultured in 22 × 22 mm coverslips at least 18 h before the immunofluorescence staining was performed. The cells were fixed in 2% formaldehyde for 10 min and then washed three times with 1x PBS for 5 min each. Subsequently, cells were permeated with 2% Triton X-100 in 1× PBS for 20 min and then washed three times with 1× PBS for 5 min. Non-specific antigens were blocked by incubating the cells with 1% fetal bovine serum in 1× PBS for 40 min at room temperature. Then, the cells were incubated with the primary antibodies diluted in blocking solution for 60 min at 37° C. The coverslips were washed three times with 2% Triton X-100 in 1× PBS for 3 min; in between these washes, the cells were quickly rinsed with 1× PBS. Afterwards, the coverslips were incubated with the secondary antibodies diluted in blocking solution for 60 min at room temperature in the dark. The cells were washed three times with 2% Triton X-100 in 1× PBS for 3 min; in between these washes, the cells were quickly rinsed with 1× PBS. Finally, the coverslips were mounted on a previously cleaned slide with 10 µL-15 µL mounting medium with DAPI (Vector Labs, H-1200). To prevent drying and movement under the microscope, the coverslips were sealed with nail polish and then stored in the dark at 4° C. For all experiments, at least 100 cells from three coverslips were analyzed. The antibodies used are listed in Supplementary Table 2. The cells were observed using a Zeiss Axio Imager A2 epifluorescence microscope (Carl Zeiss), and the images were analyzed using AxioVision 4.8 software (Carl Zeiss). The concentrations and quantities of antibodies were chosen based on the manufacturer's specifications.

CHD5 Promoter methylation analysis by MS-PCR

DNA was obtained from the cell lines by phenol/chloroform extraction. 500 ng of genomic DNA were modified using the EZ DNA methylation Gold kit (ZYMO, D5006). The MS-PCR assay was performed with DNA treated with sodium bisulfite. The primers for MS-PCR were designed using Methyl Primer Express software and are listed in Supplementary Table 3. As a positive control, 1 µg of DNA from lymphocytes of a

healthy donor was methylated *in vitro* (IVD) for 8 h using SssI methyltransferase (NEB, M226S).

Chromatin immunoprecipitation (ChIP) and ChIP/re-ChIP assays

Cells were cultured until 80% confluence, and then, chromatin was extracted in accordance with the protocol of the OneDay ChIP kit (Diagenode, Kch-onedIP-180). ChIP assays were performed following the manufacturer's instructions. For all experiments, at least two chromatin preparations were analyzed. As a negative control, we used an IgG antibody included in the kit. The antibodies used are listed in Supplementary Table 4.

The ChIP/re-ChIP assays were performed following the method previously described by [40]. In brief, cells were treated according to the first steps in the ChIP assay and then incubated at 37° C in 10 mM DTT in 1X ChIP buffer for 30 min. Eluents were then diluted at 1.5 mL with ChIP buffer and incubated with the indicated second antibody overnight. The following day, protein A agarose beads were added to the solution, which was then incubated for 3 h at 4° C. The DNA-protein-antibody complexes were washed three times with 1X ChIP buffer. Finally, the DNA-protein complexes were treated with proteinase K overnight, and to break the crosslinked complexes, the samples were boiled for 10 min. The DNA was extracted as suggested by the OneDay ChIP kit protocol, and qPCR was performed with the specific primers listed in Supplementary Table 5.

The obtained results represent experiments from four separate amplifications that were used to calculate the standard deviation. qPCRs were done in triplicate using fast optical 96-well qPCR plates. Then, the oligonucleotides were amplified in triplicate by a fast optical 96-well qPCR plate (Applied Biosystems). The qPCR was performed using Thermo Maxima SYBR Green/ROX 1 PCR Master Mix (Thermo Scientific, K0222) with a StepOnePlus Real–Time PCR System (Applied Biosystems). We used the concentration of antibodies indicated by the manufacturer's specifications.

ChIP and ChIP-Re-ChIP data analysis

The oligonucleotides were validated with a standard curve performed with Input serial dilutions. The amplification efficiency (AE) value was calculated as AE = 10^(−1/slope). The percentage of the input was calculated as % input = AE^(Ctinput – CtChIP) × Fd (Fd = factor Dilution) × 100, using 10% of the input value as reference. Afterwards, to calculate the fold of enrichment of the immunoprecipitated proteins we used the following equation fold of enrichment = % input (ip)/% input (IgG) as described in the OneDay ChIP (Diagenode) manufacturer's manual. For the ChIP/ Re-ChIP analysis, we calculated the % of the input using the 10% of the input as reference and compared the data obtained from IgG.

Co-occupancy data analysis

To determine the co-occupancy of CTCF and KDM4A at the first intron of *CHD5* gene, we used the fold of enrichment over the background for each individual ChIP. The percentage of co-occupancy, was calculated according to Geisberg and Struhl [41]: % co-occupancy = 100 (AB–A)/(A × B–A), where A and B represent the IP of each experiment, and AB the ChIP-Re-ChIP assay. The occupancy was determined in the ChIP-Re-ChIP data for both experiments (CTCF-KDM4A or KDM4A-CTCF), as well as the co-occupancy in the IgG experiments, with negative results plotted with a value of 0.

CTCF and KDM4A knockdown

HeLa and MCF7 cells were transient transfected using Xfect transfection reagent (Clontech, 631317) following the manufacturer's specifications, using 2.5 µg of an small hairpin RNA expression vector against CTCF (pCT1) kindly provided by Ko Ishihara (Institute of Molecular Embriology and Genetics, Kumamoto University, Japan) [42]. As a mock control, we employed the empty vector from pSilencer-3.1-H1 puro (Ambion).

For KDM4A knockdown, siRNA transfections were performed using KDM4A SMART pool siRNAs (Dharmacon, E-004292-00-0010) and non-targeting siRNA (Dharmacon, D-001910-01-05). HeLa and MCF7 cells were seeded at 3×10^4 cells/well and 6×10^4 cells/well, respectively, in 12-well plates. 24 h later, the cells were transfected with ACCELL siRNA Delivery Media (Dharmacon, B-005000-500) over 72 h according to the manufacturer's protocol. The results were obtained from three separate biological replicates. RNA and cDNA were obtained as previously described.

Co-immunoprecipitation of CTCF and KDM4A (Co-IP)

Extracts from HeLa cells were prepared with IP lysis buffer containing 50 mM Tris-HCl (pH 8.0), NaCl 150 mM and 1% of NP40 supplemented with 2× complete protease inhibitor cocktail (Sigma-Aldrich). The cell lysate was cleared by centrifugation at 13,000 rpm for 10 minutes at 4° C. The proteins were incubated with 2 µg of anti-CTCF (Santa Cruz Biotech, sc-5916) or without antibody (using beads) and the complex were precipitated employing 25 µL of Protein A/G magnetic beads (Pierce, 88802) and incubated at 4° C approximately 16 h. The beads were recovered with a magnetic stand, and washed five times for 20 minutes with IP lysis buffer. Finally, proteins were eluted by boiling in 1× Laemmli buffer and evaluated by Western Blot using antibodies against CTCF (Santa Cruz Biotech, sc-5916) and KDM4A (Cell Signaling, JMJD2A #5328) as two independent experiments. At least three independent biological replicates were evaluated.

CRISPR/Cas9 KO and HDR plasmids transfection

We used the X-fect Transfection Reagent (PT5003-2) to transfect 1 µg of the CRISPR/Cas9 KDM4A KO Plasmid (sc-404599) and 1 µg of the HDR Plasmid (sc-404599-HDR). In brief, 3×10^5 cells were seeded in a 6-well chamber, 24 h before plasmid transfection. We diluted 1 µg of the KDM4A KO and HDR plasmids onto 100 µL of Xfect Reaction Buffer. Afterwards, we added 2 µL of the Xfect Polymer and incubated for 15 min. Finally, we distributed the entire 100 µL of nanoparticle complex solution dropwise to the cell culture medium. 48 h after transfection we evaluate the GFP and RFP expression by epifluorescence microscopy (Carl Zeiss, AXIO Imager D2). We selected the transfected cells with media supplemented with Puromycin (3 µg/mL), changing the media every 24 h for at least 5 days.

Flow cytometry and cell sorting

$KDM4A^{KO}$ or Mock cells were resuspended at a concentration of 1×10^6 cells/mL in DMEM/F-12, containing 10% FBS and 1X antibiotic-antimycotic. First, cells were filtered through a 70 µm cell strainer and subsequently through a 40 µm cell strainer and sorted on a FACSAria III Cell Sorting Flow Cytometer (BD Biosciences, San Jose, CA). Prior to sorting, MCF7 WT was used for cell size and autoflorescence measures. It was determined that the Mock cells did not show RFP florescence, while the KO population that was positive for RFP, only cells with the highest fluorescence were sorted (Supplementary Figure 4B). 2.61×10^5 cells were sorted into DMEM/F12 medium containing 10% FBS, and 2X antibiotic-antimycotic and were seeded in a p60 cell culture plate. The $KDM4A^{KO}$ population, that exhibit RFP+ high expression, and Mock sorted cells were used for the subsequent experiments.

Abbreviations

KDM4A: Lysine specific demethylase 4; CTCF: CCCTC-Binding Factor; CHD5: chromodomain helicase DNA binding protein 5; qPCR: quantitative PCR; ChIP: Chromatin immunoprecipitation; Co-IP: co-immunoprecipitation; MS-PCR; Methylation-specific PCR; H3K36me3: trimethylation of histone H3 at lysine 36; H3K36me2: demethylation of histone H3 at lysine 36; TSG: tumor suppressor gene.

Author contributions

LGC, RGB and ESR designed experimental strategy, analyzed the results, and drafted the manuscript; CCP, performed the Co-IP and Western Blot assays, MSA carried out the siRNA assays and drafted the manuscript; NA performed the bioinformatics analysis; FVR and CCH participated in the DNA methylation analysis; DCL, HAMM, LAH and AGC analyzed and discussed the results; YSP and IARV, participated in the manuscript discussion. All authors have contributed to seen and approved the manuscript.

ACKNOWLEDGMENTS

L. Guerra-Calderas is a doctoral student in the Programa de Doctorado en Ciencias Bioquímicas, UNAM, and received a fellowship from CONACyT (378681). We thank Dr. Guillermo Juárez Vega for his technical assistance with cell sorting.

CONFLICTS OF INTEREST

The authors declare no conflicts of interests.

FUNDING

This work was supported by the Consejo Nacional de Ciencia y Tecnología (CONACyT) by the Fondo Sectorial de Investigación en Salud y Seguridad Social (FOSISS, grant number 0261181), CONACyT 182997 and CB-CONACyT 284748. This research project had support from the National Cancer Institute of Mexico (INCan).

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ORIGINAL ARTICLE

Antiproliferative Activity of *Haematoxylum brasiletto* H. Karst

Bello-Martínez J[1], Jiménez-Estrada M[2], Rosas-Acevedo JL[3], Avila-Caballero LP[1], Vidal-Gutierrez M[4], Patiño-Morales C[5], Ortiz-Sánchez E[5], Robles-Zepeda RE[4]

[1]Laboratory of Chemistry of Natural Products, School of Chemical and Biological Sciences, Guerrero State University, Chilpancingo, Guerrero, [2]Department of Natural Products, Institute of Chemistry, Mexico National Autonomous University (UNAM), Ciudad de México, [3]School of Regional Development Sciences, Guerrero State University, Acapulco, Guerrero, [4]Department of Chemical and Biological Sciences, Sonora State University, Blvd. Luis Donaldo Colosio esq. Rosales s/n Col. Centro, Hermosillo, Sonora, [5]National Institute of Cancerology, Tlalpan, Ciudad de México, Mexico

Submitted: 19-10-2016 Revised: 17-11-2016 Published: 11-07-2017

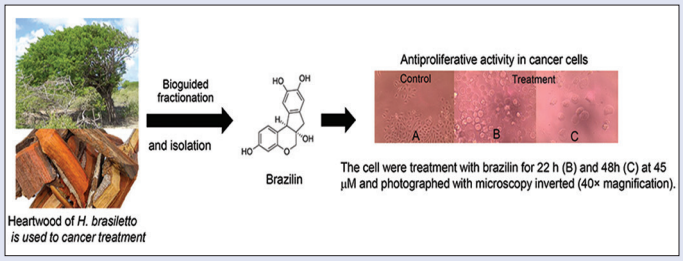
ABSTRACT

Background: *Haematoxylum brasiletto* is a tree that grows in Central America, commonly known as "Palo de Brasil," which is used in the traditional medicine for the treatment of cancer and gastric ulcers. **Objective:** The aim of this study was to isolate the compounds responsible for antiproliferative activity of *H. brasiletto*. **Materials and Methods:** A bioassay-guided fractionation of ethanol extract of *H. brasiletto* was performed using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide cell proliferation assay to measure the antiproliferative activity on six human cancer cell lines (A549, LS180, HeLa, SiHa, MDA-MB-231, and NCI-H1299) and one human noncancer cell line (ARPE-19). The ethanol extract was partitioned with hexane, dichloromethane, and ethyl acetate. The active dichloromethane fraction was fractioned by silica-column chromatography, and active subfractions were separated using preparative-thin layer chromatography. The chemical structure of an isolated compound was elucidated with different chemical and spectroscopic methods. **Results:** The flavonoid brazilin (1) was isolated from the heartwood of *H. brasiletto*. The measurement of antiproliferative activity showed that brazilin can inhibit the growth of SiHa, MDA-MB-231, A549, and NCI-H1299 cell lines by 50% at doses of 44.3, 48.7, 45.4, and 48.7 μM, respectively. Furthermore, the flavonoid showed a high antiproliferative activity on LS 180 and HeLa with IC50 values of 62.2 and 71.9 μM, respectively. Brazilin also exhibited a high antiproliferative activity on the human noncancer cell line ARPE-19 with an IC50 value of 37.9 μM. **Conclusions:** Brazilin: (6a*S*,11b*R*)-7,11b-Dihidro-6*H*-indeno[2, 1-*c*] cromeno-3,6a, 9,10-tetrol was isolated; this compound demonstrated antiproliferative activity against several human cancer cell lines. This work demonstrated that brazilin, a flavonoid isolated and characterized of *H. brasiletto*, has antiproliferative activity against cancer cell lines.

Key words: Antiproliferative activity, flavonoids, *Haematoxylum brasiletto*

SUMMARY

- The flavonoid brazilin was isolated from the heartwood of *H. brasiletto*
- Brazilin is able to inhibit the growth of SiHa, MDA-MB-231, A549 and NCI-H1299 cancerous cell lines
- Brazilin exhibited a moderate antiproliferative activity on the human non-cancer cell line ARPE-19
- Brazilin demonstrated to have antiproliferative activity against human cancer cell lines and could be a potential source of anticancer agents.



Abbreviations used: MTT: [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium]; FBS: Fetal bovine serum; TLC: Thin layer chromatography.

Correspondence:

Dr. Robles-Zepeda RE,
Department of Chemical and Biological Sciences, Sonora State University, Blvd. Luis Donaldo Colosio esq. Rosales s/n Col. Centro, Hermosillo, Sonora, Mexico.
E-mail: rrobles@guayacan.uson.mx

DOI: 10.4103/pm.pm_466_16

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INTRODUCTION

Cancer is a group of diseases that represent a serious public health problem.[1] Cancer is the second highest cause of morbidity and mortality worldwide, with approximately 14.1 million new cases and 8.2 million cancer-related deaths. In addition, 32.6 million people are currently living with some kind of cancer.[2] Thus, cancer is one of the main causes of death in developed countries.[3] Some of the most common cancer types, such as breast, cervical, oral, and colorectal cancers have high cure rates when detected early and treated according to effective means, including surgery, radiation therapy, and chemotherapy. However, all these treatments are also accompanied of severe side effects such as tingling, burning, weakness or numbness in the hands, feet, or both, weak, sore, tired, or achy muscles, loss of balance, and shaking or trembling.[4]

The use of natural products derived from plants, animals, or microorganisms for medicinal purposes has a long history.[5] Medicinal plants constitute an important natural source of bioactive compounds with multiple applications that can be used for therapeutic purposes. Their chemical components, with possibly novel mechanisms of action, provide the basis for the synthesis of pharmaceutical products.[6] Plants are the major source of anticancer drugs.[7] In this way, ethnobotanical

Cite this article as: Bello-Martinez J, Jiménez-Estrada M, Rosas-Acevedo JL, Avila-Caballero LP, Vidal-Gutierrez M, Patiño-Morales C, *et al*. Antiproliferative activity of *Haematoxylum brasiletto* H. Karst. Phcog Mag 2017;13:S289-93.

knowledge of medicinal plants represents an alternative for the identification of secondary metabolites with antiproliferative activity that could be a promising source of future anticancer drugs.[8,9]

Haematoxylum brasiletto H. Karst (Fabaceae), native from Mexico and extending into Central America, commonly known as "Palo de Brasil," is used by rural communities in the State of Guerrero, Mexico, as traditional treatment for hypertension, stomach upsets, mouth infections, diarrhea, gastric ulcers, and cancer.[10] An ethanolic extract of the stem bark of *H. brasiletto* was found to inhibit the growth of *Escherichia coli* O157:H7 (EHEC), verotoxin production, and adhesion of *E. coli* O157:H7 to HeLa cells.[11] *H. brasiletto* has shown antimicrobial activities against *Staphylococcus aureus* 375, *S. aureus* ATCC 25923, and *Enterococcus faecium* 379.[12]

To provide scientific validation of traditional medicinal use of *H. brasiletto* for the treatment of cancer, in the present study, We evaluated the bioguided antiproliferative activity of *H. brasiletto*.

MATERIALS AND METHODS

Chemicals and reagents

All solvents used were of analytical grade. Methanol (PubChem CID: 887), ethanol (PubChem CID: 702), n-hexane (PubChem CID: 8058), dichloromethane (PubChem CID: 6344), ethyl acetate (PubChem CID: 8857), and sulfuric acid (PubChem CID 1118) were purchased from Fermont chemicals (Monterrey, NL, Mexico). CD_3OD was purchased from Cambridge Isotopes Laboratories, Inc., (Tewksbury, MA, USA). Water was purified by Milli-Q instrument (Millipore, Bedford, MA, USA). Dulbecco's Modified Eagle's Medium (DMEM) high-glucose, L-glutamine solution 200 mM (PubChem CID: 24895310), L-arginine monohydrochloride (PubChem CID: 87640969), L-asparagine (PubChem CID: 24890831), sodium pyruvate solution 100 mM (PubChem CID: 24899804), penicillin–streptomycin solution (PubChem CID: 86591708), doxorubicin hydrochloride (PubChem CID: 31703), dimethyl sulfoxide (DMSO) (PubChem CID: 679), trypsin–EDTA solution 0.25%, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (PubChem CID: 64965), phosphomolybdic acid (PubChem CID 24845315), and ceric sulfate (PubChem CID 159684) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum was obtained from Gibco Life Technologies (Grand Island, NY, USA). Silica gel 60 (70–230) mesh and silica gel 200–400 mesh were purchased from Sigma-Aldrich (St. Louis, MO, USA). Preparative-thin layer chromatography (TLC) glass-backed 20 cm × 20 cm silica gel plates (2.0 mm thickness) and TLC aluminum-backed silica gel plates (0.2 mm thickness) were obtained from E.M. Merck (Germany).

Plant material

The heartwood of *H. brasiletto* was collected at Mochitlán, Guerrero, Mexico. 99°21'19.03" W; 17°29'03.27" N to 1042 msnm in March 2015. The specimens were taxonomically identified by Professor María de los Angeles Venalonzo Martínez, a voucher of classification was assigned (UAGROHBH15) and was deposited in the Herbarium of Universidad Autónoma de Guerrero. All plant materials were air-dried in the shade at room temperature. The dried samples were powdered and stored at 4°C.

Preparation of ethanolic extract and solvent fractions

The plant extract was obtained based on the methodology described by González-Salvatierra *et al*.[13] In brief, the ethanolic extract of the powder of heartwood of *H. brasiletto* (1 kg) was obtained by maceration with 96% ethanol (EtOH) at room temperature for 10 days with regular manual stirring twice daily. The combined EtOH extracts were evaporated under reduced pressure in a rotatory evaporator to yield the crude extract (50 g). The crude extract (HBM-1) was suspended in 250 mL of an aqueous (3:2 water [H_2O]/methanol [MeOH]) mixture and the resulting suspension was fractioned by successive liquid–liquid partition with *n*-hexane (Hx), dichloromethane (CH_2Cl_2), and ethyl acetate (EtOAc) to produce the corresponding low (HBM-2A [500 mg]), low-medium (HBM-2B [5 g]), and medium (HBM-2C [20 g]) polarity fractions, respectively. The ethanol crude extracts and their fractions were analyzed by TLC. A sample of 100 µg is dissolved in 100 µL of CH_2Cl_2 and is applied to the plate using a capillary tube, and then placed in a chromatographic chamber and eluted with a suitable system. The plate is observed under ultraviolet (UV) light and the bands of interest were evaluated for their retention factor (Rf). Chromatographic analyses were performed on 5.5 cm^2, 0.2 mm thick silica gel plates (E.M. Merck DC Alufolien). All extracts were stored at −4°C in amber glass vials until use.

Isolation of bioactive metabolites of *Haematoxylum brasiletto*

Column chromatography (CC) was performed using silica gel 60 (70–230 mesh, Sigma). While silica gel 200–400 mesh (Sigma) was used for flash CC, vacuum liquid chromatography (VLC) purifications were carried out using TLC-grade silica gel (Merck). Gel-preparative TLC purifications were performed using glass-backed 20 cm × 20 cm silica gel plates (2.0 mm thickness, Merck). For analytical TLC analyses, aluminum-backed silica gel plates (E.M. Merck, 0.2 mm thickness) were used. Chromatograms were examined under UV light and then visualized by dipping the plates in a solution of phosphomolybdic acid (20 g) and ceric sulfate (2.5 g) in 500 mL of sulfuric acid (5%), followed by drying and heating to 100°C.[14]

VLC purification of the bioactive dichloromethane fraction (5 g), using a gradient elution with mixtures of CH_2Cl_2:EtOAc: MeOH, produced 11 major fractions (3A-3K). Fraction 3B (1.45 g) was purified by CC eluting with *n*-hexane: EtOAc: MeOH (45:50:5) to produce 8 new fractions (4A-4H). Final purification of fraction 4C (152.5 mg) using a multiple elution (3×) preparative-TLC eluting with EtOAc: $CHCl_3$:MeOH (70:30:10 + 50 µL of formic acid/10 mL of solution) resulted in the isolation of brazilin (1) (54 mg), which was identified by comparing its spectroscopic and spectrometric data with those in literature.[15]

Structure elucidation

The structure of isolated compound was determined by different spectroscopic analyses such as IR, NMR spectra (^{1}H NMR, ^{13}C DEPT 135 and DEPT 90), and two-dimensional experiments, such as hydrogen–hydrogen correlation (H–H COSY), heteronuclear multiple bond coherence (HMBC), heteronuclear single quantum coherence (HSQC), mass spectrometry, and also by comparison with literature data. For obtaining IR spectrum, we used a Bruker Tensor 27 spectrometer coupled with ATR. ^{1}H NMR and ^{13}C NMR were acquired on a Bruker Avance III, operating in 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR. Samples were dissolved in CD_3OD. Chemical shifts were given in δ (ppm), and coupling constants (J) are expressed in Hertz (Hz). Residual not deuterated solvent peak (δ_H 4.87, 3.31 and δ_C 49.15) was set as reference, and tetramethylsilane (TMS) was used as an internal standard. DART-MS (Direct Analysis in Real Time Mass Spectrometry) was measured using a Joel AccuTOF JMS-T100 LC Mass Spectrometer (Japan) and positive ion [M^+ H^+] was identified. Fourier-transform infrared (FT-IR) spectra were taken on a Bruker Tensor 27 spectrometer with photodiode detector using KBr pellets method for sample preparation.

Cell lines and cell culture

Cell lines such as ARPE-19 (human retinal pigmented epithelium), HeLa (human cervix carcinoma), SiHa (human cérvix squamous cell carcinoma), MDA-MB-231 (human mammary gland epithelial adenocarcinoma), NCI-H1299 (human lung carcinoma; nonsmall cancer cell), A549 (human alveolar adenocarcinoma), and LS 180 (human colorectal adenocarcinoma) were purchased from the American Type Culture Collection (ATCC, Rockville, MD).[16] All cell cultures were maintained in DMEM supplemented with 5% heat-inactivated fetal calf serum (D5F) and grown at 37°C at an atmosphere of 5% CO_2.

Cell proliferation assay

To evaluate the effect of plant extracts on the proliferation of seven cell lines, cell proliferation was determined using the standard MTT assay.[17] In brief, 10,000 cells (50 µL) were added into each well of a flat 96-well plate. After 12 h incubation at 37°C at an atmosphere of 5% CO_2 to allow cell attachment, the cell cultures were incubated with 50 µL of medium containing different concentrations of either crude extract or fractions, and the cell cultures were incubated for 48 h. The crude extract or fraction was first dissolved in DMSO and then diluted in D5F. Control cell cultures were incubated with DMSO (final concentrations of DMSO: 0.06%–0.5%). The antitumor drug doxorubicin was used as a positive control due to its wide use in the clinic for the treatment of a broad spectrum of cancers.[18]

In the last 4 h of the cell culture, 10 µL of MTT stock solution (5 mg/mL) was added to each well. Formazan crystals were dissolved with acidic isopropanol, and the plates were read in an ELISA plate reader, using a test wavelength of 570 nm and a reference wavelength of 630 nm. The absorbance of the wells was read within 15 min of adding isopropanol. The antiproliferative activity of extracts was reported as IC_{50} values (IC_{50} was defined as the concentration of extract evaluated which inhibits cell proliferation by 50%).[19] Values of proliferation of at least three experiments, in triplicate, were log transformed, normalized, and nonlinear regression analysis was used to generate a dose-response curve to calculate IC_{50} values. The differences in means were analyzed using one-way analysis of variance (one-way ANOVA) followed by Tukey's test GraphPad Prism 5 (GraphPad Software, Inc., CA, USA).

RESULTS

Flavonoid isolated from ethanol extract of *Haematoxylum brasiletto*

In this study, the *in vitro* antiproliferative activity of the ethanol extract and isolated compound from *H. brasiletto* against seven cell lines was evaluated. Purification of the dichloromethane partition of ethanol extract of the heartwood of *H. brasiletto* led to the isolation of one known compound: brazilin, ($C_{16}H_{14}O_5$) (6a*S*,11b*R*)-7,11b-Dihidro-6*H*-indeno[2,1-*c*] cromeno-3,6a, 9,10-tetrol [Figure 1]. brazilin was characterized and identified by its spectroscopic data (^{1}H NMR, ^{13}C NMR, DEPT, COSY HSQC, HMBC, and IR) and by comparison with published values [Table 1], this compound was previously described.[15] The flavonoid brazilin has been previously isolated from *H. brasiletto*[20,21] and *Caesalpinia sappan*.[22]

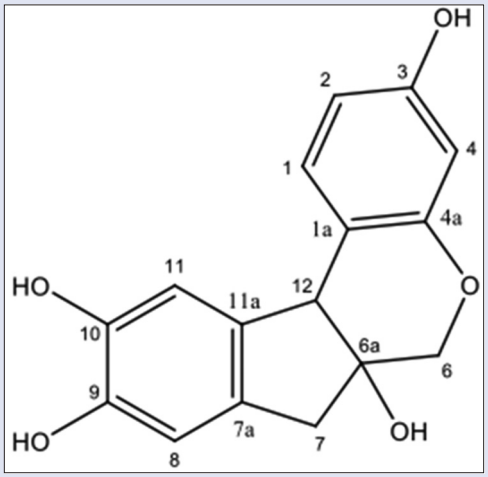

Figure 1: Chemical structure of the flavonoid brazilin

Table 1: Spectroscopic data analysis of Brazilin from *Haematoxylum brasiletto*

Carbon	$^{13}C^a$	$^1H^b$ (m, J in Hz)
1	131.35	7.17 (dd, 8.3, 0.8)
1a	115.58	
2	112.45	6.70 (s)
3	157.86	
4	109.96	6.46 (dd, 8.3, 2.5)
4a	155.72	
6	78.09	3.68 (d, 11.3)
		3.92 (d, 11.3)
6a	70.87	
7	42.92	2.76 (d, 15.7)
		3.01 (d, 15.5)
7a	132.21	
8	112.86	6.59 (s)
9	137.46	
10	145.34	
11	104.28	6.28 (d, 2.5)
11a	145.65	
12	51.09	3.95 (s)

[a]The chemical shifts are expressed in δ values (ppm). Spectra recorded in CD_3OD at 100.6 MHz. [b]The chemical shifts are expressed in δ values (ppm), m, and coupling constants are in Hz. Spectra recorded in CD_3OD at 400 MHz. m: Multiplicity; Hz: Hertz

Antiproliferative activity

Recently, the antiproliferative activity of the extracts of *H. brasiletto* against A549, RAW 264, and L-929 cells was evaluated using the MTT assay, which demonstrates mitochondrial activity of cells and is commonly used to measure the cell viability. These previous results prompted us to perform the present study in which the aim was to isolate the compounds responsible for antiproliferative activity of the ethanol extract of *H. brasiletto.*

The flavonoid isolated of chromatographic fraction of dichloromethane fraction of *H. brasiletto* was evaluated for its effects on proliferation of a panel of six human cancer cell lines (A549, LS180, H1299, HeLa, SiHa, and MDA-MB-231) and a normal (noncancer) human cell line (ARPE-19). The broad-spectrum chemotherapeutic agent, doxorubicin, was included as a positive control and for comparison purposes as it can induce apoptosis for intercalation into DNA and disruption of topoisomerase-II DNA repair.[23] The results are presented in Table 2. The antiproliferative activity of brazilin was evaluated [Table 2], demonstrating that it causes a moderate inhibitory effect on the growth in the human SiHa, MDA, A549, and H1299 cell lines at IC_{50} values of 44.3, 48.7, 45.4, and 48.7 µM, respectively. In LS180 and HeLa, a low effect at IC_{50} 62.2 and 71.9 µM, respectively, was observed.

Brazilin showed a moderate antiproliferative effect on noncancer ARPE-19 cell line with IC_{50} value of 37.9 µM, suggesting that the antiproliferative activity of brazilin is nonselective.

Table 2: *In vitro* antiproliferative activity of flavonoid brazilin from *Haematoxylum brasiletto* on seven cell lines measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

	Cell lines IC_{50} (μM)						
	A549	LS 180	H1299	HeLa	SiHa	MDA	ARPE 19
Brazilin[a]	45.4±2.5	62.2±2.9	48.7±1.9	71.9±2.3	44.3±0.7	48.7±1.9	37.9±0.7
Doxorubicin[a]	8.3±0.2	4.7±0.3	6.5±0.8	5.6±0.2	4.1±0.13	4.6±0.4	7.77±0.4

IC_{50} values represent a mean±SD (n=3) of three independent experiments. SD: Standard deviation. [a]Doxorubicin was used as a control positive

DISCUSSION

In this work, we demonstrated that brazilin, a flavonoid isolated and characterized of *H. brasiletto*, has antiproliferative activity against cancer cell lines. Rivero-Cruz[12] reported that brazilin inhibited the growth of *S. aureus* 375, *S. aureus* ATCC 25923, and *E. faecium* 379. Moreover, brazilin was described as an inhibitor of NO synthase,[24] xanthine oxidase,[22] protein kinase C,[25] and aldose reductase enzymes.[26] However, there are no reports of research of antiproliferative activity induced by brazilin on cell lines.

Flavonoids with antiproliferative effect, such as kaempferitrin and curcumin, have previously been described.[27] Several studies indicate that curcumin possesses reactive oxygen species (ROS)-inducing or pro-oxidant activity.[28] It is known that ROS, including the superoxide anion, hydrogen peroxide, and hydroxyl radical, are known to mediate apoptosis induced by some cancer chemopreventive and therapeutic agents.[29] Moreover, curcumin induces hypomethylation of the miR-203 promoter and subsequent upregulation of miR-203 expression. This leads to downregulation of miR-203 target genes Akt2 and Src that culminates in decreased proliferation and increased apoptosis of bladder cancer cells.[30] Kaempferitrin may induce both transcription-independent and transcription-dependent pathways of p53 as it upregulates pro-apoptotic proteins and downregulates antiapoptotic proteins. Moreover, it has been described that an increase in p53 levels leads to cell cycle arrest at G1 phase.[31]

However, brazilin is more active than other compounds, such as a 5-fluorouracil, usually used in anti-tumoral clinical therapy which has IC_{50} values >100 μM in HeLa. Likewise, other natural products isolated from Sonora propolis in Mexico, such as CAPE, galangin, and xanthomicrol, exhibit an IC_{50} ≥60 μM in HeLa and A549 cell lines.[19] In the same way, kaempferitrin, isolated from *Justicia spicigera*, shows IC_{50} >45 μM in HeLa.[32]

CONCLUSIONS

In this study, using a bioassay-guided method, we isolated one flavonoid with antiproliferative activity from *H. brasiletto*: brazilin (6a*S*,11b*R*)-7,11b-Dihidro-6*H*-indeno[2,1-*c*] cromeno-3,6a, 9,10-tetrol; this compound demonstrated antiproliferative activity against selected human cancer cells.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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