UNIVERSITAT DE BARCELONA

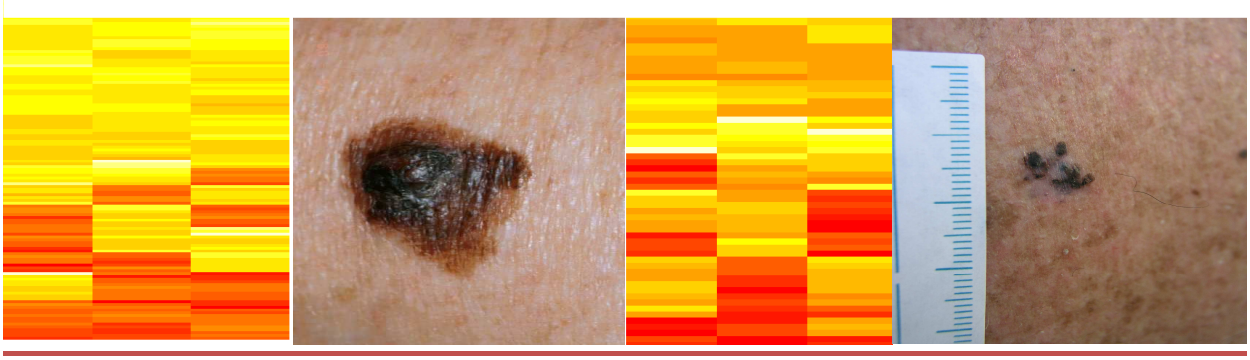
Genes y vías moleculares asociadas al riesgo y desarrollo del cáncer cutáneo

Joan Anton Puig Butillé

Tesis Doctoral I Joan Anton Puig Butillé

GENES Y VÍAS MOLECULARES ASOCIADAS AL RIESGO Y DESARROLLO DEL CÁNCER CUTANEO

Directora I Prof. Dra. Susana Puig Sardà



DOCTORAT EN MEDICINA.FACULTAT DE MEDICINA

TESIS DOCTORAL JOAN ANTON PUIG BUTILLÉ

GENES Y VÍAS MOLECULARES ASOCIADAS AL RIESGO Y DESARROLLO DEL CÀNCER CUTANEO

PRESENTACIÓN

Esta tesis doctoral refleja la trayectoria tanto de estudio como de investigación durante los años como investigador predoctoral contratado por el Centro de Investigación Biomédica en red de Enfermedades Raras (CIBERER). Fruto de la oportunidad de formación y trabajo en el grupo IDIBAPS "Melanoma: Imatge, genètica i immunologia" de la Unidad de Melanoma del Hopital Clínic, el cual forma parte del Grupo de Investigación en Genética de Enfermedades Raras (GICER), los resultados de esta tesis reflejan el enorme potencial de la genética molecular en la investigación del cáncer cutáneo, con un marcado perfil traslacional.

De forma global el cáncer cutáneo es la forma neoplásica más prevalente por lo que representa un problema de salud pública considerable. Durante los últimos años, se ha avanzado considerablemente en el conocimiento de la etiopatogénia del cáncer cutáneo gracias en parte, al uso de técnicas de genética molecular. Mientras que está bien establecido el papel de la radiación ultravioleta (RUV) en el desarrollo del cáncer cutáneo, los factores genéticos implicados en el riesgo o desarrollo de estas neoplasias así como su relación con la RUV son aún poco conocidos.

La tesis se presenta en formato de cuatro artículos científicos las cuales han sido publicados en revistas indexadas. El primer trabajo es el estudio de la desregulación del transcriptoma en células cutáneas debido a la presencia de mutaciones germinales en el gen *CDKN2A* o variantes no funcionales en el gen *MC1R* (ambos genes implicados en la susceptibilidad a cáncer cutáneo). Se identificaron perfiles de expresión y vías moleculares asociadas a las alteraciones germinales, por lo que dicha desregulación puede ser considerado un evento inicial en el desarrollo del cáncer cutáneo. De forma notable se demostró que los perfiles de expresión de las células fenotípicamente sanas se mantienen en las formas de cáncer cutáneo.

El segundo trabajo es un estudio retrospectivo observacional de pacientes de melanoma en el cual se analizó el papel del gen *MC1R* en el riesgo a desarrollar melanoma con características histopatológicas determinadas. Se identificó una variante sin repercusión fenotípica asociada al riesgo a desarrollar melanoma lentiginoso maligno.

En el tercer trabajo se presenta la caracterización molecular de un grupo de melanomas lentiginosos acrales. Se observaron alteraciones no conocidas hasta la fecha como son las ganancias de los genes *AURKA* y *TERT*. El trabajo sugiere la existencia de diferentes subgrupos moleculares en base a las alteraciones adquiridas y pone de manifiesto la importancia de otros genes, más allá del gen *CCND1* en este tipo de tumor cutáneo.

El cuarto trabajo tiene como objetivo explorar los efectos moleculares de un compuesto que contiene filtros UV y fotoliasa en pacientes con campo de cancerización. Se identificó el papel del gen *CPI-17* en la recuperación de un fenotipo cutáneo sano, además de poner de manifiesto el papel de la inflamación en el campo de cancerización.

TABLA DE CONTENIDOS

1. Cáncer cutáneo: tipos y epidemiología

El cáncer cutáneo es la neoplasia maligna más frecuente en el ser humano, compuesta por el cáncer cutáneo no melanoma, el cual son tumores que derivan de la proliferación de los queratinocitos, y el cáncer cutáneo melanoma el cual se origina en los melanocitos.

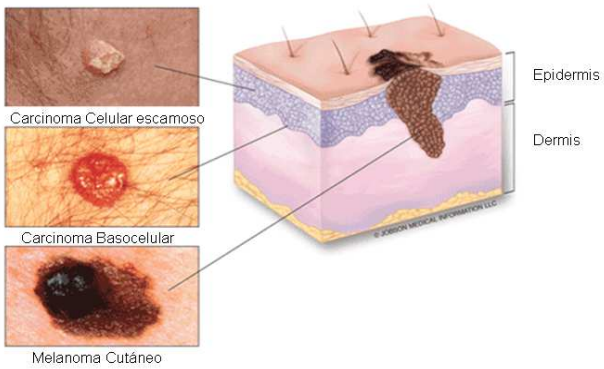


Figura 1. Tipos de cáncer cutáneo

CARCINOMA BASOCELULAR (BCC): Este tipo de tumor se desarrolla en los queratinocitos de la capa basal de la epidermis y de los folículos pilosebáceos y suelen desarrollarse en tejido cutáneo con daño solar de individuos con pieles claras y poca capacidad de bronceado [1]. Es un tipo de cáncer cutáneo poco agresivo y el porcentaje de casos que desarrolla metástasis se encuentra en 1 caso de cada 10.00 tumores [2-4]. La mayor incidencia de BCC se observa en personas de edad avanzada, siendo más frecuente en hombres. Al contrario, la mayoría de pacientes con una edad de debut menor de 40 años son mujeres, lo que podría deberse a determinados hábitos como el uso de camas de bronceado [5].

La mayoría de casos son esporádicos, aunque hay formas familiares (poco frecuentes) como los afectos del Síndrome de Gorlin.

CARCINOMA CELULAR ESCAMOSO (SCC): Se origina en los queratinocitos de la capa intermedia de la epidermis. Suele desarrollarse en zonas de la piel fotoexpuestas, aunque puede hacerlo en cualquier otra parte del cuerpo no expuesta al sol como en la lengua o en la mucosa bucal o genital. Normalmente se desarrollan en personas de avanzada edad, aunque pueden desarrollarse en pacientes más jóvenes, especialmente en aquellos con fototipos de piel claro y con poca capacidad de bronceado. Existen varios síndromes poco

prevalentes asociados a un mayor riesgo a desarrollar SCC como el Xeroderma Pigmentosum y la epidermodisplasia verruciformis.

Es un tipo de tumor más agresivo que el BCC, y el riesgo a desarrollar metástasis se encuentra relacionado con la zona de origen del tumor primario (los SSC originados en piel con daño solar son menos agresivos) y al grosor del tumor (20% de los tumores con un grosor > 5mm desarrollan metastasis)[6].

MELANOMA (MM): Se origina en los melanocítos de la capa basal de la epidermis. El fenotipo de riesgo asociado a desarrollar melanoma está definido por la presencia de múltiples nevus melanocíticos, y como en los tumores queratinocíticos, a fototipos claros con dificultad para broncearse y tendencia a quemarse [7], así como el color de ojos verdes o azules y/o cabello rubio o pelirrojo[8].

El 90% de casos de melanoma son esporádicos y un 5- 10% son casos de familias con agregación a la enfermedad (melanoma familiar). Se ha observado un aumento de la incidencia de MM, la cual se sitúa en un incremento anual del 6%. En los últimos 50 años se ha multiplicado en más del cuádruple, pasando de 3-4 casos a 10-15 casos por 100,000 habitantes y año, con especial repercusión entre mujeres jóvenes [9, 10]. Estudios a nivel mundial confirman que esta tendencia al alza se mantendrá al menos en las próximas dos décadas, y se espera que la incidencia se doble de nuevo[11].

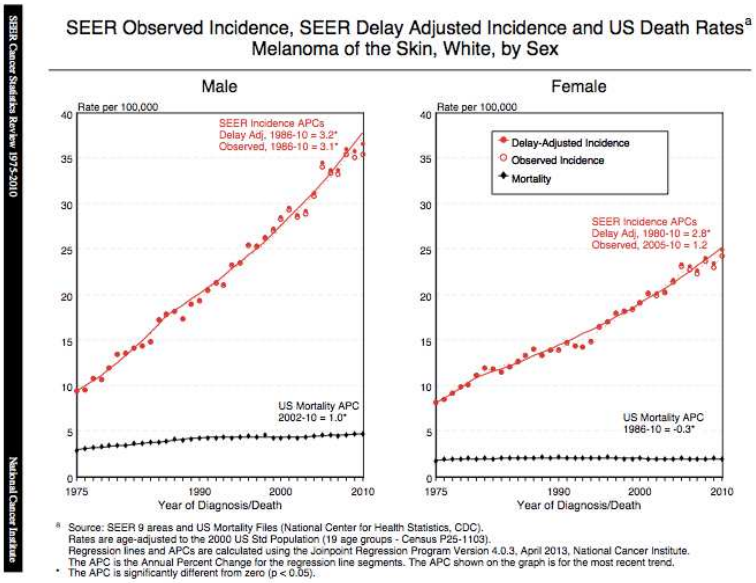


Figura 2. Curvas de evolución de incidencia y mortalidad (nuevos casos/1000.000 hab) de melanoma desde 1975 a 2010 según la SEER National Cancer Statistics de EEUU

La incidencia y morbilidad asociada al cáncer cutáneo difiere considerablemente según el tipo. Aproximadamente, el 90% de los casos de cáncer cutáneo corresponden a tumores queratinocíticos, de los cuales aproximadamente un 80% son BCCs y un 20% son SCC [12].

Desafortunadamente, una estimación precisa de su incidencia es difícil de obtener puesto que estos tipos de cáncer frecuentemente no se reportan en los registros de cáncer. Mientras que estos tipos de cáncer en poblaciones caucásicas son un problema de salud pública a causa de su elevada incidencia, suponen una baja morbilidad en dichas poblaciones. Se estima que se diagnostican cada año 3,5 millones de cánceres de piel BCC o SCC (presentándose en alrededor de 2.2 millones de estadounidenses, ya que algunas personas tienen más de uno) y solamente 2.000 personas mueren al año a causa de este tipo de neoplasias, siendo la mayoría de ellos personas de edad avanzada o personas con el sistema inmunológico suprimido [13]. Por otro lado, aunque la incidencia de MM es notablemente menor (<10% de casos de cáncer cutáneo), es el subtipo que presenta el mayor índice de mortalidad; siendo el responsable del 75% de las muertes por cáncer de piel.

A nivel epidemiológico, el aumento de la incidencia del cáncer cutáneo observado en poblaciones caucásicas, se debe en parte, al papel crítico que tiene la radiación ultravioleta (RUV) como factor ambiental asociado a estas neoplasias; y por consiguiente, la capacidad de respuesta a la radiación y los hábitos de fotoexposición del individuo. Múltiples estudios, han demostrado el incremento de riesgo a desarrollar melanoma [14, 15] o cáncer cutáneo no melanoma [1, 16] en base a la radiación solar. El estudio caso-control multinacional más extenso hasta la fecha (1732 pacientes europeos con cáncer cutáneo y 1550 controles) confirma la importancia de la exposición solar como factor ambiental, y la pigmentación como factor genético en la etiopatogénia del cáncer cutáneo [17]. Además, ilustra las diferencias entre los diferentes tipos de cáncer, ya que mientras que el tipo SCC está muy relacionado con la exposición solar crónica o acumulativa, la relación entre RUV y MM es más compleja (Tabla 1). Tal y como muestra el estudio, el MM está más asociado a la exposición intermitente e incluso no se observa un aumento del riesgo relacionado con los trabajos realizados al aire libre.

Green y col. ya en 1992 establecieron las primeras hipótesis sobre la influencia de la localización anatómica, y por tanto el tipo de exposición solar, en la diferente respuesta de los melanocitos, y por ello, pudiendo variar el riesgo y tipo de MM desarrollado (Figura 3). Así se empezó a diferenciar entre el papel de la exposición solar intermitente intensa, implicada en el MM de tronco y extremidades, de la exposición crónica continua, implicada en el MM de cabeza y cuello[18].

TABLA 1: Factores de riesgo a desarrollar cáncer cutáneo en población Europea.

Se muestran los valores de Odd Ratio (OR) del análisis multivariable ajustado por edad, sexo, fototípo y país. * número bajo de individuos en esta categoría (datos adaptados del estudio de deVries E. et al [17])

	BCC (N=620)	SCC (N=409)	MM (N=360)
Fototipo de piel (clasificación Fitzpatrick	**OR (IC95%)**	**OR (IC95%)**	**OR (IC95%)**
I	2,01 (1,31-3,08)	3,72 (2,19-6,33)	2,36 (1,38-4,02)
II	1,51 (1,11-2,04)	2,59 (1,73-3,88)	2,27 (1,51-3,43)
Presencia de nevus comunes	1,42 (1,11-1,83)	no significativo	2,11 (1,47-3,04)
Presencia de nevus atípicos	no significativo	no significativo	6,08 (4,24-8,71)
Signos de fotoenvejecimiento	1,87 (1,45-2,42)	4,23 (2,90-6,17)	1,51 (1,09-2,09)
Presencia de efélides en la cara			
Pocas	1,50 (1,18-1,91)	2,37 (1,72-3,27)	1,67(1,25-2,22)
Algunas	2,04 (1,44-2,90)	3,69 (2,40-5,68)	2,22 (1,48-3,34)
Muchas	3,01 (1,63-5,58)	3,99 (1,90-8,37)	no significativo*
Quemaduras solares en la infancia			
1-2 veces	no significativo	no significativo	1,43 (1,04-1,95)
3-5 veces	1,55 (1,09-2,21)	1, 55(1,98-2,45)	1,63 (1,09-2,44)
6 -10 veces	2,33 (1,62-3,36)	2,32 (1,46-3,70)	2,65 (1,76-4,00)
Tener/haber tenido trabajos al aire libre	1,55 (1,24-1,94)	1,91 (1,43-2,55)	no sign
Practicar/haber practicado aficiones al aire libre	1,61 (1,28-2,03)	1,47 (1,09-1,97)	1,51 (1,14-2,00)
Vivir en paises tropicales			
5-10 años	4,49 (1,56-12,89)	no significativo	no significativo

De igual manera, la clasificación clínico-patológica clásica de Clark apoya esta teoría, puesto que el MM tipo de extensión superficial, el 60% en nuestro entorno, se asocia al tronco y extremidades, debuta en edades más jóvenes, y se asocia más frecuentemente a la presencia de nevus melanocíticos y exposición solar intermitente. El MM tipo lentigo maligno, se presenta en áreas de daño actínico marcado, en pacientes de edad más avanzada e independientemente de la presencia de nevus.

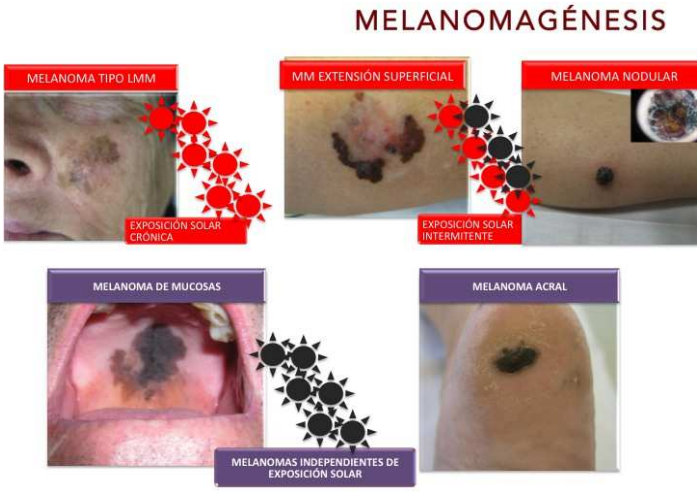


Figura 3: Melanomagénesis: Los diferentes subtipos de melanoma y su relación con la radiación UV.

El MM tipo lentiginoso acral, es la forma menos común de melanoma. Generalmente ocurre en las plantas de los pies, las palmas de las manos o en la matriz ungueal por lo que no encajaría en el modelo de la foto inducción. La no asociación a la radiación solar se observa también en el hecho de que su prevalencia es igual en diferentes zonas geográficas, indepedientemente de la raza y el fototipo cutáneo. Es el más el frecuente en razas más pigmentadas puesto que en estas poblaciones, la frecuencia de tipos marcadamente relacionados con la radiación solar es muy baja, debido al efecto protector del propio fenotípo [19].

2. Queratosis actínicas y campo de cancerización

La queratosis actínica o queratosis solar, es una neoplasia intra-epidérmica común caracterizada por la atípia de los queratinocítos y originada en áreas de la piel con marcado daño solar. Se presenta, habitualmente, en pacientes de edad avanzada y el riesgo es mayor en hombres, en individuos con fototipos claros y que no se broncean fácilmente, con color de pelo rojo o claro y/o color de ojos azul [20]. También se observa un mayor riesgo en pacientes con el Síndrome de Rothmund-Thompson, de Cockayne, o Xeroderma Pigmentosum [21].

Las queratosis actínicas son consideradas los estadios iniciales de un continuo biológico desde que se identificaron como lesiones pre-neoplásicas relacionadas con el cáncer cutáneo. Aunque las lesiones puede mantenerse estables por muchos años (el ratio de transformación maligna en un paciente es menor del 0.1% por año [22]), las no tratadas pueden progresar hacia SCC en 8-20% de pacientes [23] y son un factor de riesgo a

desarrollar BCC y MM [24]. Diversos estudios indican que el 60% de SCCs invasivos se originan en queratosis actínicas y que el 82-97% de SCCs presentan queratosis actínicas contiguas al tumor [22, 25, 26]. Dado que pacientes con SCC, en la misma área fotoexpuesta donde se origina el tumor, presentan otras lesiones, como las queratosis actínicas en distintos estadios evolutivos; se ha postulado que los primeros eventos genéticos en el campo de un tumor, podrían llevarse a cabo por la expansión clonal de células pre-neoplásicas afectadas por cambios genómicos en un área de tejido cutáneo dañado (incluso antes de ser valorables por la histopatología), las cuales conducirían hacia la adquisición del fenotipo maligno. Este hecho ha sido denominado campo de cancerización. Este concepto sugiere que la piel clínicamente normal alrededor del área de queratosis actínicas ya revela cambios moleculares similares a un carcinoma por lo que los tratamientos actuales van dirigidos a tratar todo el campo de cancerización, para eliminar otras lesiones que pueden ser prácticamente inapreciables pero que podrían continuar su evolución en un futuro.

Actualmente, varios autores incluso clasifican las queratosis actínicas como un tipo de SSC más que una lesión precursora [21, 27]. Aunque no existe un consenso en este aspecto, la hipótesis que estas lesiones necesitan adquirir determinadas alteraciones antes de una expresión clínica con carácter maligno es plausible [28].

3. Radiación Ultravioleta (RUV): Factor ambiental asociado al cáncer cutáneo

1. Efectos biológicos de la RUV

Los estudios epidemiológicos ponen de manifiesto que la radiación ultravioleta (RUV) es la principal causa del cáncer cutáneo. Aunque la RUV es únicamente el 5% de la radiación emitida por el Sol, es la responsable de los principales efectos dañinos de la radiación solar, y desde el año 2003 ya está incluida dentro de la lista oficial del *National Institute of Environmental Health Sciences* [29] de EEUU como cancerígeno conocido y demostrado.

Tanto de forma aguda como crónica o acumulativa, provoca quemadura solar, inmunosupresión, fotoenvejecimiento cutáneo y ocular, melanoma y cáncer cutáneo no melanoma.

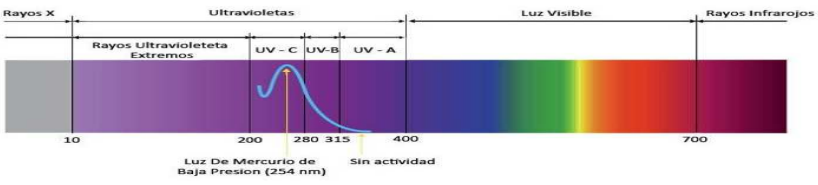


Figura 4: Espectro electromagnético de la luz solar

Debido a la mayor esperanza de vida, y a cambios en los hábitos lúdico-deportivos y estéticos a partir del siglo XX, la carcinogénesis inducida por la RUV se ha convertido en un auténtico problema socio-sanitario, sin olvidar que cerca de 3 millones de personas al año, pierden la visión a causa del daño actínico ocular y las cataratas fotoinducidas [30, 31].

Por otro lado, la RUV es necesaria para la vida, y ejerce también efectos beneficiosos mayoritariamente a través de la síntesis de la vitamina D en la piel, necesaria para el metabolismo músculo-esquelético, fosfo-cálcico, y también interviene en diversas funciones de homeostasis hormonal e incluso anticancerígenas. Estudios epidemiológicos han demostrado un posible efector protector frente al desarrollo de diferentes tipos de neoplasias, y a su vez, un también un papel pronóstico de la evolución del cáncer [32]. Existen datos contradictorios sobre la necesidad de fotoprotección extrema en los pacientes con cáncer cutáneo como es el caso de los afectos de melanoma[33]. Como ejemplo, el estudio caso-control de Berwick y col. en el cual la supervivencia de los pacientes de melanoma era mejor en los casos de mayor exposición solar, una vez ajustado el análisis por el resto de factores pronóstico [34].

2. Fotocarcinogénesis

La RUV es absorbida por diferentes cromóforos en la piel, y el ADN es su principal diana y donde provoca sus mayores efectos dañinos, por lo que la RUV es una pieza fundamental en la fotocarcinogénesis melanocítica y queratinocítica[35]. La RUV ejerce estas funciones deletéreas a través de acciones directas e indirectas, agudas y acumulativas, de inmunosupresión, daño directo del ADN celular, y daño indirecto a través del estrés oxidativo y formación de radicales superóxido.

La RUVB supone un 5% de la RUV que atraviesa la capa de ozono, es la de mayor energía y menor longitud de onda (λ 280-320nm), por lo que alcanza la epidermis hasta la unión dermoepidérmica. La RUVA, de menor energía pero mayor longitud de onda, alcanza estructuras hasta la hipodermis (λ 320-400nm) y supone más del 95% de la RUV que recibimos en la superficie terrestre, dependiendo de las condiciones meteorológicas y geográficas puede variar [36, 37].

La RUVB se ha considerado la responsable del mayor daño biológico, principalmente a largo-medio plazo de la fotocarcinogénesis, y de forma aguda de la quemadura o eritema solar. Molecularmente se conoce bien su capacidad en generar dímeros de ciclobutano de pirimidina (DCP), y sus fotoproductos (6-4PPs), los cuales son los principales causantes del daño en el ADN (Figura 6). Los DCP son específicos del daño por RUV, pueden cuantificarse y se sub-clasifican según qué dímero de bases nitrogenadas, y fotoproducto se forme; siendo más característicos de UVB los DCP TT, TC y CT, y los 6-4PP TC y TT.

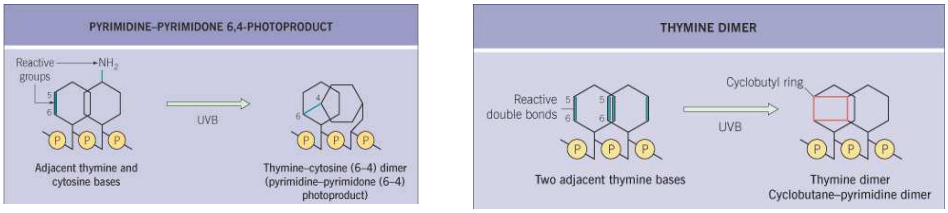


Figura 5: Fotoproductos originados por la radiación ultravioleta

La RUVA, clásicamente, se había considerado de menor capacidad carcinogénica, más responsable de la inducción de melanina y de la fotoinmunosupresión [38], principalmente a través de la isomerización de la forma trans- a la cis- del ácido urocánico, otro cromófero cutáneo. Causa principalmente roturas y entrecruzamientos entre proteínas y cadenas simples del ADN, y genera radicales superóxido, responsables del daño oxidativo en la célula. Sin embargo, actualmente, se ha demostrado que la RUVA por sí sola tiene capacidad de producir daño directo genético, generando también DCP (principalmente dímeros TT), por una vía independiente del daño oxidativo [39].

El cáncer cutáneo se incluiría en el modelo de carcinogénesis consistente en iniciación, promoción y progresión tumoral, donde el acúmulo de alteraciones genéticas, sumado a la ineficacia en la eliminación de células anormales, y a factores ambientales favorecedores, explicaría la génesis tumoral. Probablemente la fotocarcinogénesis de los tumores queratinocíticos es diferente a la que induce los melanomas. Se sabe que melanomas y carcinomas exhiben diferentes mutaciones y activación de oncogenes, lo que probablemente traduce diferente tipo de daño del ADN.

3. Fotoadaptación

La sensibilidad a la RUV en el hombre presenta una variabilidad de hasta 1000 veces entre individuos, es decir que ante un mismo tipo y cantidad de RUV los efectos dañinos provocados varían enormemente. Los principales determinantes de esta variabilidad están relacionadas con la pigmentación y por tanto con la capacidad de síntesis de melanina; y con los diferentes procesos de inflamación y reparación del daño del ADN, es la llamada fotoadaptación [40, 41]. Según las capacidades de cada persona, se alcanza una fotoadaptación y un resultado final del daño fotoinducido, agudo y acumulativo o crónico. Por lo que genes implicados en los procesos relacionados con la adapatación a la RUV pueden modular la respuesta al daño y por consiguiente estar implicados en el riesgo a desarrollar cáncer cutáneo.

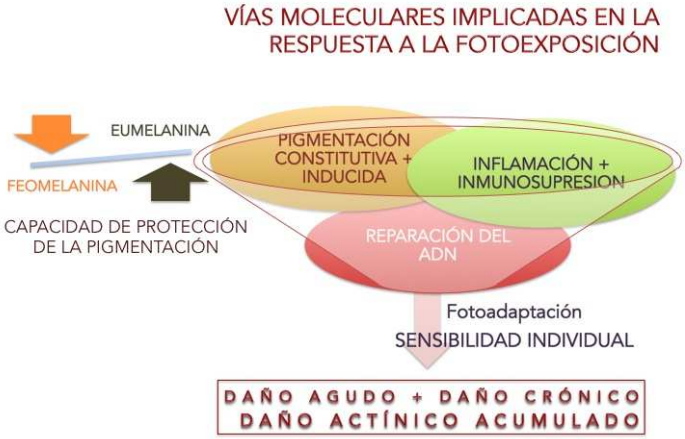


Figura 6: Respuesta a la fotoadaptación

4 . REPARACIÓN DEL DAÑO DEL ADN

1. Vías moleculares de reparación en humanos

Las mutaciones adquiridas en el ADN y provocadas a menudo por agentes exógenos, son generalmente dañinas. Se estima que a diario cada célula humana sufre una media de 25.000 cambios espontáneos de bases del global de $3x10^9$ que posee su genoma, como consecuencia de las agresiones que recibe. La RUV es la fuente principal de daño que recibe el ADN de las células de la piel.

Sin embargo el organismo tiene diferentes vías intracelulares específicas, y eficazmente dirigidas a prevenir la carcinogénesis inducida por carcinógenos, son las llamadas vías de reparación del ADN (Figura 7.a) [42]. El grado de daño determina el proceso utilizado por la célula, ya sea inicialmente mediante la detención del ciclo celular, identificación y reparación del ADN dañado, o si el daño es muy extenso o la reparación ha sido ineficaz mediante la inducción de la senescencia o muerte celular (apoptosis) (Figura 7.b)

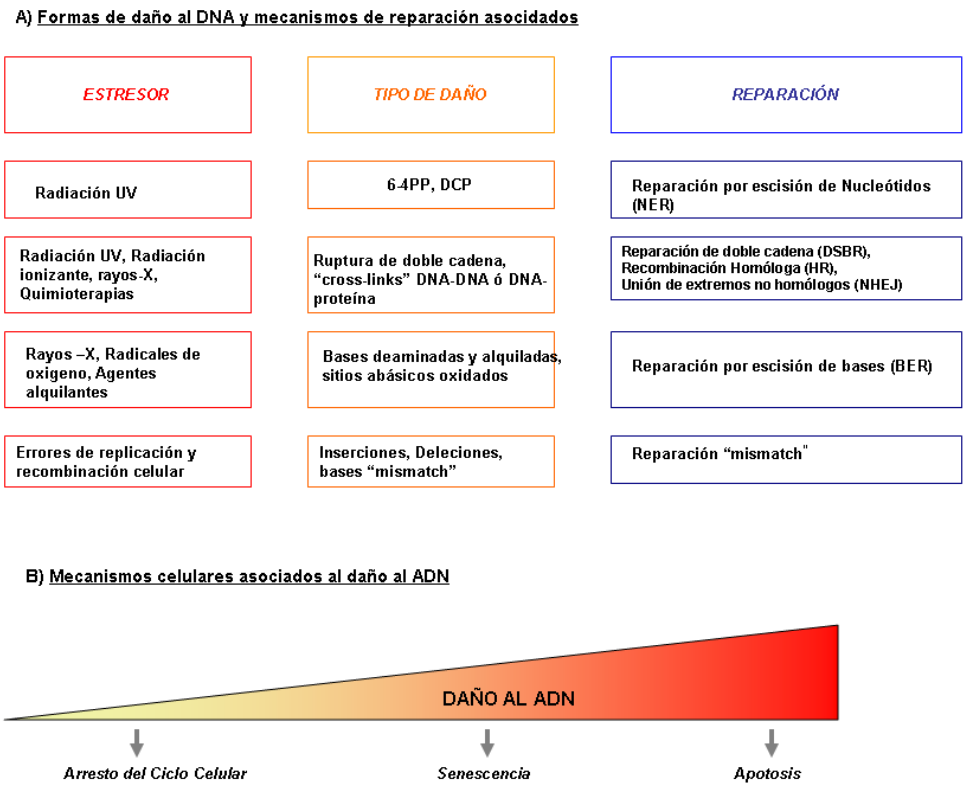


Figura 7: Daño al DNA: La figura 7.a muestra los diferentes formas de daño y los mecanismos de reparación. La figura 7.b muestra los procesos de respuesta celular según el grado de daño celular.

Una de las vías de regulación celular ante el fotodaño es la activación de los *"checkpoints"* del ciclo celular los cuales promueven el arresto del ciclo, tanto en la transición de fase G1 a la S, en la de G2 a la de mitosis, o en la fase de replicación del ADN. Según el tipo de daño del ADN inducido por un carcinógeno, se activará o detendrá el ciclo celular mediado por una vía diferente[43].

Diversas proteínas están implicadas en la regulación del ciclo celular cuya función es inducir un estacionamiento o inhibición del ciclo, de forma que haya tiempo suficiente para la reparación de dicho daño, como por ejemplo: p53 (gen *TP53*), p21 (gen *CDKN1A*) y p14 y p16 (ambas codificadas por el locus *CDKN2A*). En caso contrario, se induce la apoptosis celular, evitando que la célula se replique y aparezca un clon aberrante.

El caso más evidente de alteración en los mecanismo de identificación y reparación del daño del ADN son los pacientes afectos de Xeroderma Pigmentoso (XP). Estos pacientes son portadores de diferentes tipos de mutaciones germinales en genes implicadaos en la reparación del DNA, lo que provoca un mayor riesgo de neoplasias ante cualquier agresión carcinogénica, en especial a la luz solar. Así la mayoría de enzimas implicadas en las vías de reparación se deenominan con las iniciales XP.

Se conocen distintos mecanismos: 1) Escisión de bases (gen *XRCC1*); 2) Escisión de nucleótidos (genes *XPA-G, y XPV*) [42] y 3) Reparación de la doble cadena homóloga (gen *XRCC3*) [44]. Según qué polimorfismos y/o mutaciones presenten dichos genes la reparación del daño inducido por RUV será más o menos eficiente [45, 46].

También se ha relacionado la expresión de la proteína p16 (inducida por exposición a RUV como mecanismo reparador del daño de ADN) y del gen *MC1R* con la reparación. De forma que se demuestra una estrecha relación entre síntesis de melanina y reparación del ADN. El gen *MC1R* juega un papel importante en fotocarcinogénesis a través de la estimulación de producción de p53 y de p16 ante un daño fotoinducido [47] o a través de la vía de MITF la cual está implicada en la proliferación celular tumoral, apoptosis y vías de reparación del ADN [48].

Por otra parte se ha sugerido que la RUVA podría tener incluso un mayor papel en la melanomagénesis por la combinación de daño directo del ADN e indirecto del daño oxidativo, que se potenciaría más aún a través de una mayor síntesis de melanina. En un estudio reciente se pudo demostrar que tanto la RUVB como la RUVA inducen DCP de igual manera en melanocitos como queratinocitos. En cambio, el daño oxidativo inducido por UVA (a través de 8-oxo-7,8-dihydroguanine) fue el doble en melanocitos (2.2 veces superior), lo que apoya también las hipótesis de que a mayor síntesis de melanina en el melanocito, mayor estrés oxidativo sufre la célula [49].

2. Fotoreactivación y fotoliasa

La vía de reparación por escisión de nucleótidos (NER) reconoce y remueve eficazmente los 6-4PP, sin embargo el reconocimiento y eliminación de los DCPs mediante esta vía es limitado [50, 51]. Muchos organismos (bacterias, algas, crustáceos o peces) excepto los mamíferos placentarios presentan un mecanismo de reparación adicional llamado fotoreactivación; llevado a cabo por unas enzimas monoméricas llamadas fotoliasas, las cuales reconocen y reparan eficazmente ambos fotoproductos[52].

En el proceso de fotoreactivación, la fotoliasa se une a los DCPs en una reacción independiente de la luz. Luego se requiere un fotón cuya longitud de onda esté comprendida entre 300 y 500nm para que la enzima se active y pueda donar un electrón al dímero. Esto último inicia una reorganización electrónica que finalmente restablece las dos pirimidinas intactas.

El potencial de las fotoliasas en la prevención del cáncer cutáneo ha sido reconocido en base a los resultados obtenidos en varios estudios. Jans J et al. han demostrado los beneficios de la expresión de la fotoliasa en la piel de ratones transgénicos [53, 54]. La expresión transgénica de la enzima en ratones irradiados, reducía drásticamente los niveles de 6-4PP en epidermis y dermis; y la capacidad de reparación de las lesiones DCPs aumentaba un 40%. Además, observaron un aumento de la resistencia contra los efectos inducidos por la RUV y una frecuencia de mutaciones en el gen *TP53* similar a la observada en ratones control no irradiados. Además, se ha observado que la aplicación tópica en la piel humana de fotoliasa encapsulada en lisosomas proporciona una protección contra el daño inducido por la RUVB [55]. En la piel irradiada con RUVB, la

aplicación tópica del compuesto seguida de una exposición a luz fotoreactivadora reducía un 40-45% el número de dimeros inducidos por la RUV.

5. Susceptiblidad al cáncer cutáneo

En los últimos años, se han identificado múltiples genes que incrementan el riesgo a desarrollar cáncer cutáneo. Por un lado, se han identificado variantes comunes en genes implicados en pigmentación, reparación del ADN o en el metabolismo de carcinógenos. Por otro lado, se han identificado genes que se encuentran mutados de forma germinal en formas etiopatológicas hereditarias poco frecuentes, y que confieren un elevado riesgo a desarrollar cáncer queratinocítico (genes de alto riesgo) como son las alteraciones en el gen *PTCH1* en afectos del Síndrome de Gorlin, el gen *RECQL4* en afectos del Síndrome Rothmund–Thomson, el gen *RECQL3* en afectos del Síndrome de Bloom o los genes *XP* en pacientes de Xeroderma Pigmentosum [56]. También se ha identificado mutaciones germinales en individuos con elevado riesgo a desarrollar MMs en los genes *CDKN2A* y *CDK4 y* más recientemente en el gen *BAP1* [57].

1. Papel del gen *MC1R* en la pigmentación y riesgo a cáncer cutáneo

La melanina es el pigmento que determina el color de cabello, ojos y piel en humanos. El proceso de síntesis de la melanina se lleva a cabo en el melanocito, y tiene lugar en el melanosoma, el compartimento subcelular especializado en este proceso. La síntesis de melanina es un proceso gradual que tiene lugar durante la maduración del melanosoma. Los melanosomas maduros son transportados, mediante las proyecciones dendríticas del melanocito, hacia los queratinocitos circundantes de la piel o del folículo (Figura 8).

Existen dos tipos de melanosomas que difieren en tamaño, forma, estructura y composición química. Los eumelanosomas son los compartimentos donde se sintetiza el pigmento eumelanina (pigmento marrón-negruzco, cromóforo protector del ADN) y los feomelanosomas son los compartimentos en los que produce la síntesis del pigmento feomelanina (pigmento amarillo-rojizo, sin capacidad de protección, y favorecedor de daño oxidativo) [58]. El tipo, la cantidad y distribución de los melanosomas en los queratinocitos determinan el color de pelo y piel [59], mientras que los melanosomas retenidos en los melanocitos de los ojos son lo que determinan su color [60].

La piel tiene una determinada pigmentación constitutiva (basal) y una pigmentación facultativa la cual es inducida tras una exposición solar, ambas son diferentes según cada raza e indivíduo y están genéticamente determinadas [61].

La pigmentación es un rasgo complejo a nivel genético, con más de 120 genes implicados en su regulación. De ellos, el receptor de la melanocortina tipo 1 (*MC1R*) tiene un papel clave en el proceso de síntesis. El gen codifica un receptor de membrana acoplada a proteína G-AMPc, de 317–aminoácidos que controla la cantidad relativa de síntesis de los dos tipos de pigmento que producimos, al unirse a su ligando, la hormona melanoestimulante α-MSH [62]. Junto con el gen *MC1R* se han identificado otros genes reguladores de la pigmentación como *ASIP*, *DCT, OCA2*, *SCL24A5*, *MATP*, *TYR* y *TYRP1*.

El proceso de síntesis es iniciado por la activación de *MC1R* mediante la unión a la hormona α-MSH, la cual es secretada por los queratinocitos en respuesta a la RUV. La unión promueve la activación del adenilato ciclasa aumentando la formación de cAMP. El aumento de cAMP conduce a la activación de la proteína Quinasa A (*PKA*) la cual a su vez, promueve la transcripción del gen *MITF* mediante el factor de transcripción *CREB*. *MITF* juega un papel clave en el control de la melanogenesis y promueve la transcripción de múltiples genes como *TYR*, *TYRP1 o DCT* implicados en el control de la cantidad relativa y absoluta de eumelanina y feomelanina [63] (Figura 8). Por lo que la activación de MC1R tiene como consecuencia final la transformación de la tirosina en eumelanina, aumentando así los niveles de este tipo de pigmento y disminuyendo los niveles de feomelanina. El incremento del contenido de eumelanina en la piel produce el bronzeado el cual actúa como un fotoprotector, protegiendo a las células cutáneas del daño inducido por la RUV y en consecuencia evitando la transformación maligna.

De forma interesante, la activación de *MITF* también promueve la transcripción de genes de proliferación celular y antiapoptóticos en el melanocito [64]. Por lo que la activación de MC1R en respuesta a la RUV es también determinante para garantizar la supervivencia del melanocito, al inhibir la apoptosis inducida por la RUV.

Actualmente, el gen *MC1R*, es el mejor caracterizado y validado como gen de riesgo a cáncer cutáneo de baja-media penetrancia. Se ha descrito que la presencia de polimorfismos de *MC1R*, en especial los conocidos como “variantes de pelo-rojo” (RHC), los cuales provocan una proteína no funcional (p.D84E, p.R151C, p.R160W y p.D294H), incrementan el riesgo a desarrollar cáncer cutáneo.

Varios estudios han detectado el efecto del tipo y número de variantes en el riesgo a desarrollar tumores queratinocíticos. Las variantes RHC incrementan entre 2 y 3 veces el riesgo a desarrollar BCCs [65-67] o SSCs [67, 68]. Incluso se ha observado un aumento del riesgo a desarrollar BCCs asociado a variantes comunes como son p.V60L (OR=3,212) y p.V92M (OR=2,87)[69].

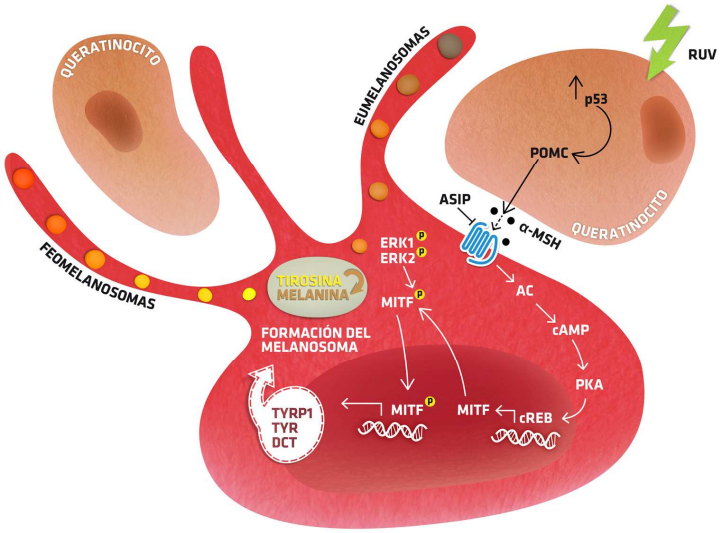


Figura 8: Vía molecular de la síntesis de melanina

El papel del gen *MC1R* en la susceptibilidad a MM ha sido ampliamente analizado y está bien establecido el incremento del riesgo a desarrollarlo causado por las variantes p.D84E, p.R142H, p.R151C, p.I155T, p.R160W y p.D294H. La presencia de múltiples variantes, se asocia a un incremento de 2 a 10 veces el riesgo de desarrollar MM, así como a una disminución de la edad de debut [70].

De forma notable en la mayoría de estudios se observa que el riesgo de las variantes es independiente de las características fenotípicas del individuo (color de pelo y piel), sugiriendo que el gen *MC1R* puede conferir riesgo independientemente de su relación con la pigmentación. En este sentido, en un estudio caso-control con 960 pacientes y 396 casos se detectó que el riesgo a MM causado por variantes del gen era notablemente superior en aquellos individuos con fenotipos protectores (color de pelo o ojos oscuros, buena capacidad de broncearse) o baja exposición al sol [71].

2. El locus *CDKN2A* y predisposición a melanoma

Aproximadamente un 5-10% de los casos de MM tiene lugar en un ámbito familiar y por tanto en individuos con una predisposición genética heredada. En dichas familias, se observa un patrón de herencia autonómica dominante con penetrancia incompleta, con múltiples casos de MM, y habitualmente, asociado a individuos con múltiples nevus melanocíticos o nevus displásicos. Mediante estudios citogenéticos, moleculares y de ligamiento se han identificado dos genes, *CDKN2A* y *CDK4* como genes de susceptibilidad a MM. El locus *CDKN2A* comprende 4 exones y codifica dos proteínas diferentes, las cuales son transcritas en diferentes pautas de lectura y modificadas mediante "splicing" alternativo (Figura 9). El tránscrito alpha que comprende el exón 1 alfa, el exón 2 y el exón 3, codifica para p16INK4A y actúa como un gen supresor de tumores el cual se une a la quinasa dependiente de ciclina 4 (*CDK4*) y a la quinasa dependiente de ciclina 6 (*CDK6*) [72]. Esta unión inhibe la asociación de las quinasas con la ciclina D1, evitando la formación del complejo CDK/Ciclina D. Este complejo fosforila la proteína del

Retinoblastoma permitiendo al melanocito la progresión del ciclo celular atravesando el punto de control de la fase G1 [72, 73]. El transcrito beta el cual comprende el exón 1 beta y el exón 2 codifica para la proteína p14ARF. Esta proteína regula la función de p53 y la proteína del Retinoblastoma., uniéndose e inhibiendo la función de HDM2 [74] y, por lo tanto, favoreciendo la apoptosis y bloqueando la transformación de los melanocitos en células malignas.

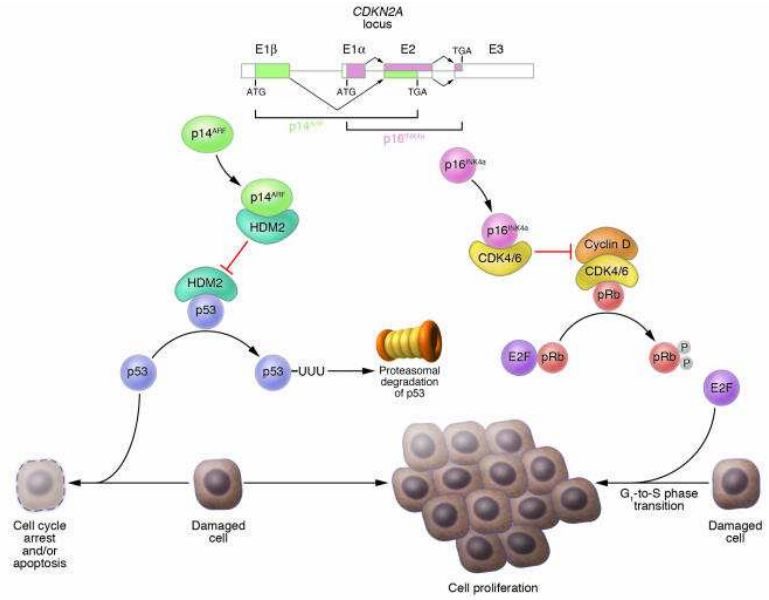


Figura 9: Función de las proteínas p16INK4A y p14ARF ejn el control celular y procesos cancerígenos

Aproximadamente el 20% de las familias afectadas de melanoma familiar tanto en los países de Europa como en América y Australia son portadoras de mutaciones en *CDKN2A*; aunque la frecuencia varía considerablemente entre países (0-50%), siendo la frecuencia más baja la que se observa en familias de países europeos.

Aunque la mayoría de mutaciones se encuentran en los exones que codifican para p16INK4A (1 alfa y exon 2), también se han encontrado mutaciones que tan solo afectan a p14ARF [75]. Bishop DT et al. estimaron que la penetrancia de las mutaciones en *CDKN2A* es, de forma global, de 0, 30 a los 50 años y de 0,67 a los 80 años. De forma interesante, la penetrancia difere considerablemente entre America del Norte, Europa y Australia. En este último país la penetrancia de la mutación es de 0.91 a los 80 años. En el estudio no se observaron diferencias según si la mutación afectaba a un tránscrito o a ambos [76]. Estos datos ponen de manifiesto tanto la existencia de genes modificadores, como el papel de factor ambiental (RUV) en el riesgo y desarrollo del MM.

Del total de mutaciones identificadas, 5 de ellas se encuentran de forma recurrente, debido a un origen fundador. Las familias con elevada susceptibilidad de países del sur de Europa suelen ser portadoras de las variantes p.G101W o p.V59G [76, 77].

Junto con las mutaciones germinales, hay evidencias que sugieren que determinados polimorfismos en *CDKN2A* podrían aumentar el riesgo a MM, aunque en menor medida [78, 79].

Por otro lado, la inactivación de *CDKN2A* es un evento inicial en todos los tumores cutáneos, por lo que se ha postulado que formas familiares de SSC podría deberse a mutaciones germinales en el gen [80]. Sin embargo no hay resultados concluyentes sobre el papel del gen en la susceptibilidad a desarrollar cáncer cutáneo no melanoma. Recientemente, se ha descrito un paciente portador de una mutación en *CDKN2A* con una elevada incidencia de MMs y de SCCs de cabeza y cuello [81], también se ha identificado que la variante rs2151280 localizada cerca de *CDKN2A* incrementa el riesgo a BCC [82], sin embargo no se detectaron alteraciones en *CDKN2A* en una serie de 40 pacientes con SCC de cabeza y cuello y que desarrollaron un segundo tumor primario[83].

6. Eventos moleculares iniciales en el desarrollo del cáncer cutáneo

Mediante estudios de genética molecular o inmunohistoquímicos, se han identificado genes clave en el desarrollo de los diferentes tipos de cáncer cutáneo, considerandose de forma general que su desregulacación son eventos iniciales en la progresión del tumor, como por ejemplo las alteracionaes en genes de la vía de las MAPquinasas (*H-RAS*, *N-RAS*, *K-RAS* y *B-RAF*) y de control del ciclo celular, senescencia y apoptosis (*CDKN2A* y *TP53*).

1. Lesiones y tumores queratinocíticos:

Un 50% de queratosis actínicas presentan mutaciones en el gen *TP53* (la mayoría de mutaciones son substituciones de un solo nucleótido de cisteína a timina) [84] y la activación constitutiva de la vía de las MAPquinasas debido a mutaciones en el gen *RA*S (*H-RAS* y *K-RAS*) se observa en 16% de las lesiones [85].

Se ha postulado que en las queratosis actínicas, la inestabilidad genética podría promover la carcinogénesis aunque no hay resultados concluyentes. Mientras que en población caucásica, aproximadamente un 50% de lesiones presentan de forma recurrente pérdidas de heterozigosidad en las regiones 17p, 17q, 9p, 9q, y 13q [86, 87]; estas alteraciones solamente se detectan en un 8% de lesiones de población asiática [88]. Estas diferencias también podrían ser debidas a que existen diferentes mecanismos patogénicos moleculares entre estas dos poblaciones.

Un evento asociado a la progresión de estas lesiones hacia el desarrollo de un SCC invasivo es la pérdida de *CDKN2A,* mediante pérdidas de heterozigosidad de la región 9p21 [89]; lo que demuestra que la inactivación de p16INK4A es clave en la progresión del cáncer cutáneo. La desregulación de la proteína también puede ser a causa de mutaciones adquiridas en el gen.

También se han identificado mutaciones somáticas en el gen *CDKN2B,* adyacente a *CDKN2A*, y que codifica la proteína de ciclo celular p15 [90].

En el BCC, la mayoría de los tumores presentan alteraciones inactivadoras en el gen *PTCH1*, que codifica una proteína inhibidora de la vía de señalización "Sonic Hegdehog" [91]. Junto con *PTCH1*, la desregulación de los genes *TP53* y *CDKN2A* es también clave en la tumorogénesis del BCCs. De forma interesante, se ha observado que la mayoría de las mutaciones detectadas en estos son causadas por el efecto carcinogénico de la RUV (C->T ó C-->TT) [92, 93] lo que confirma a nivel molecular el efecto de la RUV en el desarrollo de estos tumores.

2. Tumores melanocíticos

La caracterización molecular de los MMs a nivel somático ha supuesto una auténtica revolución, tanto del conocimiento etiopatogénico como en las nuevas terapias del MM metastásico. Curtin Ja et al. observaron que existen diferentes patrones de alteraciones genómicas adquiridas las cuales, se correlacionan con las características del tumor (grado de daño inducido por la RUV, sitio de origen del melanoma) y del paciente (edad, tendencia a asociar nevus o tendencia a presentar fotoenvejecimiento) [94]. Por lo que determinadas alteraciones se pueden relacionar a subtipos histopatológicos concretos de MM. Estos datos muestran como los diferentes tipos de tumores se pueden considerar en gran medida como entidades biológicas diferenciadas.

Las alteraciones más frecuentes y que se consideran un evento inicial en la génesis del tumor, son las mutaciones activadoras en los genes *N-RAS* y del gen *B-RAF.* Se detectan aproximadamente en un 20% y un 50% de tumores respectivamente; y promueven la activación constitutiva de la vía de las MAPquinasas y en consecuencia la proliferación celular [95]. Estas alteraciones son mutualmente excluyentes y su frecuencia difiere entre los subtipos de tumores. Las alteraciones en *N-RAS* son más prevalentes en tumores de áreas crónicamente expuestas a la RUV (como el melanoma lentiginoso maligno) y se localizan en los exones 2 y 3 del gen. Contrariamente, las alteraciones en *B-RAF* se observan mayoritariamente en tumores de zonas del cuerpo con una exposición a la radiación solar intermitente (son frecuentemente detectadas en el melanoma de extensión superficial) y en pacientes de edad de debut temprana. El 90% de mutaciones se localizan en el codón 600 de la proteína (p.V600E) [96, 97].

Por otro lado, la alteración más frecuente en los MM cutáneos no asociados a la RUV, (melanoma lentiginoso acral) son las ganancias de la región 11q13, donde se localiza el gen *CCND1*, el cual es un gen diana de la vía de la MAPquinasas y antagonista de p16INK4A [98].

En base a estas evidencias, se postula que los mecanismos genéticos implicados en el desarrollo de este tipo de tumor difieren de los otros subtipos, los cuales están más relacionados con la RUV. La ciclina D1 mediante su unión a *CDK4* o *CDK6* promueve la fosforilación de la proteína del Retinoblastoma. Se ha observado que la distribución de alteraciones en *CCND1* o incluso en *CDK4* está inversamente relacionada con la presencia de mutaciones en *B-RAF* ó *N-RAS*, por lo que se les atribuye el mismo efecto oncogénico [94]. Más recientemente, se ha detectado que el gen *c-KIT* se encuentra también frecuentemente alterado en este subtipo de tumor.[99]

La pérdida del gen *CDKN2A* es un evento recurrente en cualquier subtipo de MM, indicando que, igual que en los tumores queratinocítos, este evento es crucial para el desarrollo del tumor [100, 101].

Un porcentaje muy elevado de nevus melanocíticos (60-80%) presentan mutaciones en el gen *B-RAF*. Dado que *CDKN2A* tiene un papel crítico en el control del ciclo celular y en la senescencia activada por oncogenes; se ha postulado que la desregulación de *CDKN2A* es necesaria para evitar la senescencia activada por oncogenes y por tanto, evitar que formas como los nevus melanocíticos progresen desarrollando un tumor.

Finalmente, otros genes de la región 9p21 podrían estar implicados en el inicio y progresión del MM ya que se ha identificado tumores en los que la pérdida cromosómica se sitúa fuera del gen *CDKN2A* [102].

HIPÓTESIS

1. El sustrato genético de un individuo está implicado tanto en el riesgo a desarrollar cáncer cutáneo como en las características moleculares que adquiere el tumor.

2. Las diferencias etiopatológicas entres los subtipos de tumores de melanoma pueden ser atribuidas, al menos en parte, a la interacion que se establece entre los factores de riesgo y la exposición a la radiación ultravioleta.

3. Determinadas alteraciones moleculares pueden estar intrínsicamente asociadas a un subtipo de tumores con características etiopatológicas concretas.

OBJETIVO PRINCIPAL

- Analizar e identificar genes y vías moleculares críticas para el desarrollo de cáncer cutáneo, tanto en la predisposición como en el desarrollo mediante el análisis molecular de tipos de tumor con unas características etiopatológicas concretas.

SUB-OBJETIVOS

I. Evaluar el efecto de mutaciones germinales en el locus *CDKN2A* así como variantes no funcionales en el gen *MC1R* en la desregulación del transcriptoma y su papel en la transformación maligna en cáncer cutáneo.

II. Analizar las frecuencias de variantes en el gen *MC1R* y su relación con el subtipo de melanoma.

III. Estudiar tanto las características clínicas del melanoma cutáneo como las características fenotípicas y germinales del paciente que se relacionan con un determinado subtipo histopatológico de tumor (ALM).

IV. Identificar genes y funciones biológicas desreguladas en el campo de cancerización como área promotora del cáncer cutáneo fotoinducido.

PUBLICACIONES

TRABAJO I

RESUMEN TRABAJO I

Capturing the biological impact of *CDKN2A* and *MC1R* genes as an early predisposing event in melanoma and non melanoma skin cancer.

Joan Anton Puig-Butille, , María José Escámez; Francisco Garcia-Garcia; Gemma Tell-Marti, Àngels Fabra, Lucía Martínez-Santamaría, Celia Badenas, Paula Aguilera, Marta Pevida, Joaquín Dopazo, Marcela del Río Marcela, Susana Puig.

Oncotarget: Accepted for publication. *Factor de impacto: 6.636*

Objetivo

Investigar el efecto causado por mutaciones germinales en el locus *CDKN2A* o variantes no funcionales del gen *MC1R* en el transcriptoma de células cutáneas fenotípicamente normales (melanocitos y queratinocitos), procedentes de individuos con elevada susceptibilidad a desarrollar cáncer cutáneo.

Metodología

Se obtuvieron co-cultivos de melanocitos y queratinocitos primarios a partir de biopsias de piel sana de 2 parejas de hermanos, seleccionados en base al estado genómico de *CDKN2A* y *MC1R.* Se aisló el RNA total de los cultivos primarios y se capturó la expresión global de los mismos mediante “arrays” de expresión (Whole Human Genome Oligo Microarray kit (G4112F, Agilent, US). En primer lugar, se analizó la expresión génica diferencial en base al estado germinal de los genes *CDKN2A* y *MC1R,* mediante el paquete bioinformático “limma” incluido en Bioconductor, y se procedió al ajuste por comparaciones multiples de los p-valores obtenidos, con la metodología de Benjamini y Hochberg. La expresión de 13 genes seleccionados en base al análisis de expresión diferencial, así como la del gen *MC1R* se analizaron mediante PCR en tiempo real (RT-PCR).

En segundo lugar, se realizó un análisis de enriquecimiento basado en vías moleculares mediante la herramienta Fatiscan, implementada en el programa bioinformático Babelómics y utilizando la información de la base de datos KEEG (Kyoto Encyclopedia of Genes and Genomes pathways). Mediante este análisis, se procedió a la identificación de vías molecuares sobrerepresentadas en el grupo de genes desregulados.

Finalmente, los patrones de co-regulación de expresión génica detectados en los co-cultivos, se compararon *in-silico* con los observados en melanoma y cáncer de piel no melanoma (carcinoma celular escamoso). Para cada tipo de tumor se determinó el "fold-change "de los genes que formanban parte de los perfiles de expresión detectados en los co-cultivos. Mediante análisis de correlación se cuantificó la relación lineal entre los diferentes estudios.

Resultados

Análisis de expresión asociado a p.G101W en *CDKN2A*:

En el estudio de expresión diferencial se detectan 1536 transcritos diferencialmente expresados en los co-cultivos protadores de mutación en *CDKN2A,* y se identifica una firma de expresión compuesta por 108 genes que discrimina las células portadoras de la mutación y las células con la forma salvaje del gen. Dicha firma incluye un elevado número de genes implicados en metabolismo y componentes celulares (37% de los genes sobreexpresados y 42% de los infraexpresados en células mutadas). El segundo grupo mas prevalente de genes sobreexpresados y el tercero de los infraexpresados lo componen genes inmunomoduladores y de respuesta al interferón. El perfil molecular también incluye genes de adhesión celular, crecimiento celular y proliferación, diferenciación epidérmica y melanogénesis así como varios factores de transcripción.

Análisis de expresión asociado a polimorfismos de cabello rojo en *MC1R*:

Por otro lado, 3570 genes se encuentran diferencialmente expresados en los co-cultivos de células portadoras de variantes no funcionales del gen *MC1R* comparado con los que tienen la forma salvaje del gen. La firma genética de las células con variantes no funcionales la forman 159 genes e incluye una infra-expresión de genes de diferenciación del melanocito y pigmentación. Estos genes de diferenciación del melanocito y de la pigmentación son regulados, mayoritariamente, por la vía de señalización mediada por el propio *MC1R*. Además, se observa una sobre-expresión de genes de respuesta al daño del ADN y de reparación del ADN, genes de ciclo celular y proliferación, y de genes asociados a microtúbulos con una función motora.

Mediante el análisis de enriquecimiento funcional se detecta, entre los genes infra-expresados en las células mutadas en *CDKN2A,* una sobre-representación de genes de la vía de señalización mediada por Notch (hsa_04330; p-valor=0,038). Por otro lado, las células con el gen *MC1R* no funcional presentan una desregulación de 17 vías biológicas alteradas. Se observa una sobre-expresión de genes de las principales vías de reparación del ADN y de la replicación del ADN, de la vía de ciclo celular y la vía de la fosforilación oxidativa. De forma notable, también se detecta una sobre-expressión de las vías asociadas a varias enfermedades neurodegenerativas (enfermedad de Hungtington, Alzheimer y Parkinson). Por otro lado, se observa una infra-expresión de las vías de endocitosis y lisosoma, las cuales están intrínsicamente relacionadas con la transferencia de los melanosomas por parte de los melanocitos a los queratinocitos.

En los estudios de expresión diferencial, 621 genes se encuentran desregulados de forma común independientemente de la alteración germinal del co-cultivo. De ellos, 485 genes se encuentran des-regulados en el mismo sentido (sobreexpresados o infraexpresados) y 136 genes se encuentran inversamente

desregulados (30 sobreexpresados en las células portadoras de mutación en el gen *CDKN2A* y infraexpresada en la células con el *MC1R* no funcional y; 106 genes infraexpresados en las células portadoras de mutación en el gen *CDKN2A* y sobreexpresados en la células con el *MC1R* no funcional. Mediante el análisis de enriquecimento funcional, no se observa una sobrerrepresentación estadísticamente significativa de vías biológicas en estos grupos de genes.

Finalmente, se evalúa si la desregulación del transcriptoma en co-cultivos de melanocitos y queratinocitos fenotípicamente normales se observa también en las formas malignas correspondientes. Se compara el patrón de expresión observado en el estudio con el observado en melanomas y carcinomas escamosos cutáneos previamente reportados y de libre acceso (disponibles en la Base de datos Gene Expression Omnibus; set de datos GSE250 y GSE12391). A causa de las diferencias metodológicas tan solo se evalúan los genes comunes entre los tres estudios (53,7% de los genes más desregulados en las células portadoras de mutaciones en el gen *CDKN2A*, y 76,1% de los genes más desregulados en las células portadoras de variantes en el gen *MC1R).*

Los patrones de expresión detectados tanto en las células con mutación en el gen *CDKN2A* o con variantes no funcionales en el gen *MC1R* correlacionan estadísticamente con el patrón que presentan dichos genes en los tumores cutáneos. Cabe destacar, que el patrón de expresión observado en las células con el *MC1R* no funcional es más similar entre los estudios que el patrón observado en las células portadoras de mutaciones en *CDKN2A* (p-valor<0,005 y p-valor<0,06, respectivamente). Estas diferencias son causadas en parte, a que un subgrupo de 11 genes presentan un patrón inverso entre la expresión observada en carcinomas de células escamosas y la observada en melanomas o células cutáneas portadoras de mutaciones germinales en *CDKN2A*

Capturing the biological impact of *CDKN2A* and *MC1R* genes as an early predisposing event in melanoma and non melanoma skin cancer.

Joan Anton Puig-Butille[1,2], María José Escámez[3,4,5], Francisco Garcia-Garcia[6,7], Gemma Tell-Marti [1], Àngels Fabra[8], Lucía Martínez-Santamaría[3,4,5], Celia Badenas[1,2], Paula Aguilera[1,2], Marta Pevida[5,9], Joaquín Dopazo[6,7,10], Marcela del Río[3,4,5], Susana Puig[1,2]

[1] Melanoma Unit, Hospital Clinic & IDIBAPS (Institut d'Investigacions Biomèdiques Agustí Pi i Sunyer), Barcelona, Spain

[2] Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Barcelona, Spain.

[3] Regenerative Medicine Unit. Epithelial Biomedicine Division. Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas (CIEMAT), Madrid, Spain.

[4] Department of Bioengineering. Universidad Carlos III (UC3M), Madrid, Spain.

[5] Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Madrid, Spain

[6] Functional Genomics Node, National Institute of Bioinformatics, CIPF Valencia, Spain

[7] Department of Bioinformatics, Centro de Investigación Príncipe Felipe, Valencia, Spain

[8] Biological Clues of the Invasive and Metastatic Phenotype Group. Molecular Oncology Lab, IDIBELL, Barcelona, Spain

[9] Tissue Engineering Unit. Centro Comunitario de Sangre y Tejidos del Principado de Asturias (CCST), Oviedo, Spain

[10] Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Valencia, Spain.

***Correspondence to**: Susana Puig, **email**: susipuig@gmail.com*

Keywords: Familial Melanoma, CDKN2A, MC1R, gene expression, p16, mutations

***Received**: September 25, 2013* ***Accepted**: December 16, 2013* ***Published**: December 16, 2013*

ABSTRACT:

Germline mutations in *CDKN2A* and/or red hair color variants in *MC1R* genes are associated with an increased susceptibility to develop cutaneous melanoma or non melanoma skin cancer.

We studied the impact of the *CDKN2A* germinal mutation p.G101W and *MC1R* variants on gene expression and transcription profiles associated with skin cancer. To this end we set-up primary skin cell co-cultures from siblings of melanoma prone-families that were later analyzed using the expression array approach.

As a result, we found that 1535 transcripts were deregulated in *CDKN2A* mutated cells, with over-expression of immunity-related genes (*HLA-DPB1, CLEC2B, IFI44, IFI44L, IFI27, IFIT1, IFIT2, SP110* and *IFNK)* and down-regulation of genes playing a role in the Notch signaling pathway. 3570 transcripts were deregulated in *MC1R* variant carriers. In particular, genes related to oxidative stress and DNA damage pathways were up-regulated as well as genes associated with neurodegenerative diseases such as Parkinson's, Alzheimer and Huntington.

Finally, we observed that the expression signatures indentified in phenotypically normal cells carrying *CDKN2A* mutations or *MC1R* variants are maintained in skin cancer tumors (melanoma and squamous cell carcinoma). These results indicate that transcriptome deregulation represents an early event critical for skin cancer

INTRODUCTION

The worldwide incidence of skin cancer, including non melanoma skin cancer (NMSC) and cutaneous melanoma (CM), is rapidly increasing, being the most common human cancer[1]. While, both entities are influenced by the carcinogenic effect of UV exposure, their incidence and tumor aggressiveness differ considerably, CM being the least frequent and most aggressive form[2] .

Approximately, 10% of total melanoma cases originate in individuals belonging to high-risk melanoma pedigrees. To date, *CDKN2A* is the major gene associated with the high risk inherited in melanoma prone families [3] and in multiple primary melanoma patients [4]. *CDKN2A* acts as a tumor suppressor gene, negatively regulating cell cycle progression and promoting cellular senescence. Recently, a role for *CDKN2A* in cellular oxidative stress regulation has been suggested [5, 6]. Recurrent *CDKN2A* mutations have been identified in melanoma families [7-11], mutation p.G101W being the most frequent one detected in Mediterranean pedigrees [12]. Although heterozygous loss of *CDKN2A* is sufficient to confer a 67% lifetime risk of melanoma [13], the mechanisms responsible for tumor enhancement still have to be clarified [14]. In contrast, the role of high-penetrance *CDKN2A* mutations in NMSC susceptibility has not been clearly elucidated [15, 16].

Skin cancer epidemiology is complex due to the multigenic nature of the disease. In particular, the *MC1R* gene is a key regulator of skin pigmentation and melanocyte differentiation, playing a central role in determining the pigmentation phenotype, sun sensitivity and tanning ability[17]. Certain *MC1R* polymorphisms are responsible for the red hair color (RHC) phenotype (red hair, fair skin and poor tanning response)[18] which is associated with high UV-radiation sensitivity and skin cancer susceptibility (CM and NMSC)[19, 20]. Furthermore, *MC1R* variants are a modifying factor for melanoma risk in *CDKN2A* mutation carriers, [21-23] suggesting that multiple medium- and low-penetrance genes may influence the risk conferred by high penetrance melanoma genes.

To date, there is a lack of information about the mechanisms underlying the increased skin cancer risk in carriers of *CDKN2A* mutations in association with *MC1R* variant*s*. The effect of both genes has been separately investigated using murine-derived melanocytes and/ or focused on mono-cultured melanocytes [6, 24-26]. However, these mono-cultures exhibit phenotypic characteristics closely mimicking those observed in transformed melanocytes, such as elevated growth rate homeostasis [33, 34]. Thus, the establishment and characterization of a melanocyte–keratinocyte co-culture system is essential for elucidating the early molecular events leading to skin cancer.

The aim of the present study was to investigate the global molecular effect of germinal *CDKN2A* mutations (p.G101W) and *MC1R* RHC variants in the transcriptome of primary skin cells from individuals belonging to skin cancer prone families (familial melanoma pedigrees).

RESULTS

Impact of *CDKN2A* germinal mutation p.G101W on Global Transcript Profiles of skin primary cultures:

Whole genome expression profiling was performed on melanoctye-keratinocyte co-cultures from *CDKN2A* mutation carriers (samples A1 and B1 described in Table 1) and wild-type *CDKN2A* individuals (samples A2 and B2). After stringent microarray data filtering, 1536 of the initial 19596 transcripts on the array were found to be differentially expressed in *CDKN2A* mutated cultured cells. Specifically, 60.7% of transcripts were found to be up-regulated (933/1536) and 39.3% of them down-regulated (603/1536).

Class comparison generated a signature of 108 unique genes that significantly discriminated between *CDKN2A* mutation and non-mutation (p-value <0.0001, listed in Table S1). Functional categorization of those transcripts belonging to known genes *(*Figure 1a, Figure 1b*)* showed that the mutant *CDKN2A* signature included deregulation of a vast number of genes involved in cell component and metabolism (37% up-regulated and 42% down-regulated,). Notably, immunomodulation and interferon response genes represented the second most prevalent subset of up-regulated transcripts (24%) and third down-regulated subgroup (13%) which may reflect a constitutively altered cytokine and chemokine profile in mutant cells. The set of deregulated genes involved in immunomodulation and interferon response includes: *CLEC2B, IFI44L, IFIT1, IFI44, IFI27, IFNK, DDX58, RNF182, IFIT2, IL15, SP110, BTN3A2, RFX2, IL17D* and *HLA-DPB1* (Figure 2). The molecular profile also included deregulation of genes which may confer a phenotype with the capability for malignant transformation since they are involved in cell adhesion, cell growth and proliferation. Over-expression of apoptosis related genes *CASP3*, *XAF1* and *SAMD9* was also detected. Transcription factors such

Table 1: Genotype and phenotype features of four individuals from two melanoma pedigrees.

Pedigree A						
Individual	MM*	CDKN2A	MC1R	Skin phototype	Eye color	Hair color
A1	5	p.G101W	p.R160W, p.R151C	II	Green	Red
A2	0	Wild-type	p.R160W, p.R151C	II	Green	Red
Pedigree B						
Individual	MM*	CDKN2A	MC1R	Skin phototype	Eye color	Hair color
B1	0	p.G101W	Wild-type	II	Blue	Black
B2	0	Wild-type	Wild-type	II	Blue	Brown

Two siblings from each pedigree (A and B) were included. Each individual was considered as a "genomic condition" in the study. A total of two and three melanoma cases have been found in pedigree A and B, respectively. Abbreviations: *CDKN2A*: cyclin-dependent kinase inhibitor 2A; *MC1R*: melanocortin 1 receptor; MM: melanomas. * Number of melanomas in each individual.

analysis revealed an over-representation of down-regulated genes from the Notch signaling pathway (hsa_04330; p=0.038) in mutant *CDKN2A* skin cells including *NCOR2, PSEN, DLLC1, DVL3, NOTCH3,*

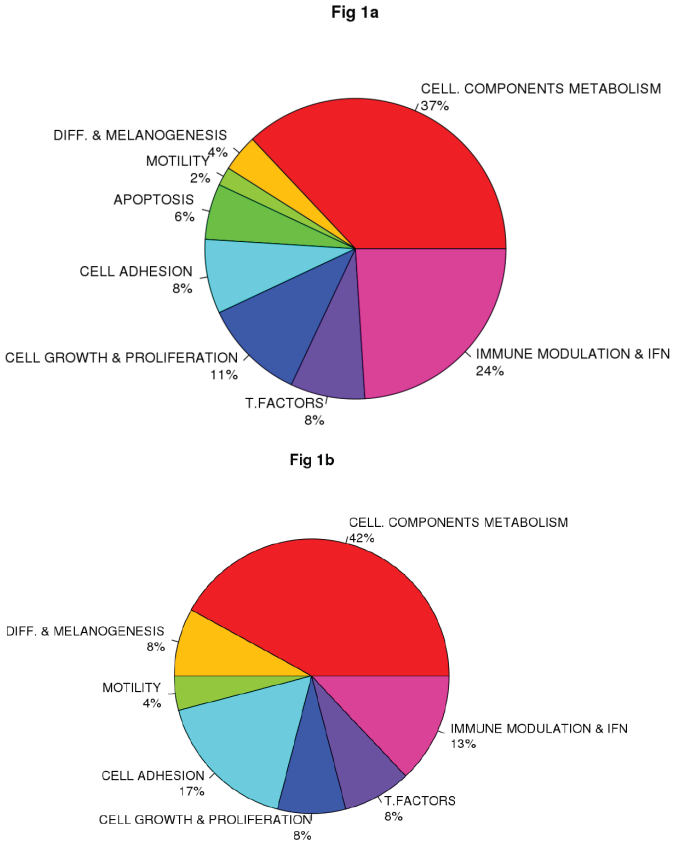


Figure 1: Impact of germinal CDKN2A mutation on global transcript profile in melanocyte-keratinocyte co-culture

RFNG and the co-activator *MAML1* which controls the growth-promoting effect of Notch1.

Impact of non-functional *MC1R* gene on Global Transcript Profiles of skin primary cultures:

MC1R analysis was carried out following the same workflow used in the *CDKN2A* analysis. Whole genome expression profiling displayed 3570 transcripts differentially expressed in RHC variant (A1 and A2) vs. wild-type MC1R skin cells (B1 and B2). Specifically, 54% of transcripts (1954/3570) were up-regulated while 46% (1616/3570) were down-regulated. By class comparison, 159 genes showed highly statistically significant differences ($p < 0.0000001$) (listed in Table S2). RHC skin cells showed a down-regulation of a subset of genes categorized as melanocyte differentiation and pigmentation genes. The transcription of most of these genes is regulated directly via the *MC1R* signaling pathway, or indirectly through keratinocyte differentiation genes *(TYRP1, MLANA, TYR, CRNN, PADI1, GDF15, MLPH, KIT, INHBB, DAB2, KLK6, FOXC1,* and *NDRG4)*. Furthermore, RHC variant skin cells exhibited up-regulation of DNA damage and/or DNA repair response genes (*BUB1, CHEK1, POLQ, RAD51AP1, RAD54B, RAD51C, BLM, UBE2T, GINS2, EXO1, FAM33A, PTTG2, PTTG1, RAD54L, DTL),* cell cycle and proliferation genes *(CDKN3, CDC25, CDK1, CCNA2, CDC20, CCNB2, CCNB1, CKS, CDCA3, PLK1, PRIM, CDCA8, GMNN, CDC6, VRK1, CKS1B, RFC4, CDC45, CENPN, HAUS8, PSRC, MCM6, MELK, AURKA, NCAPG)* and microtubule-based motors kinesin superfamily genes *(KIF11, KIF15, KIF2C, KIF4A,KIF23).*

The functional enrichment analysis showed a considerably higher number of altered pathways in non-functional *MC1R* cells than in *CDKN2A* mutant cells. The RHC skin cells displayed over-expression of genes playing

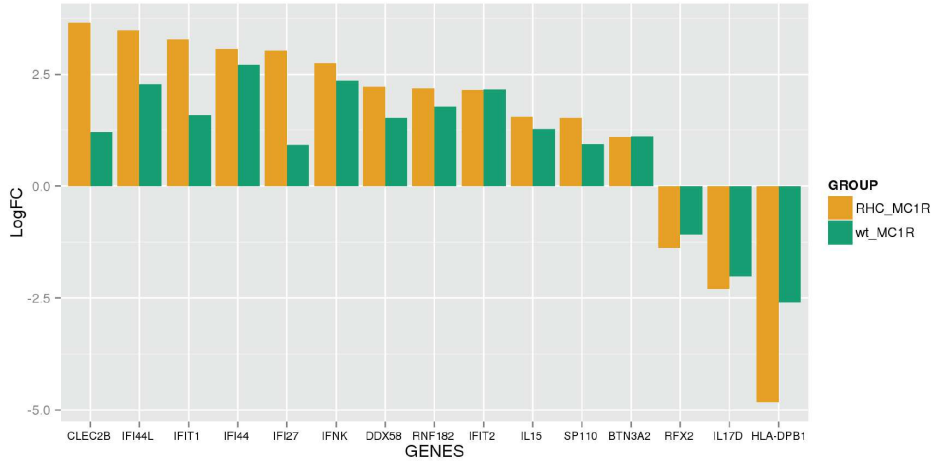


Figure 2: Set of immunomodulation and interferon response deregulated in mutant CDKN2A co-cultures. The logarithm of the Fold change (LogFC) for the set of genes is indicated for wild-type *MC1R* co-cultures (wt_MC1R; green) and for Red Hair Colour *MC1R* variants co-cultures (RHC MC1R; yellow).

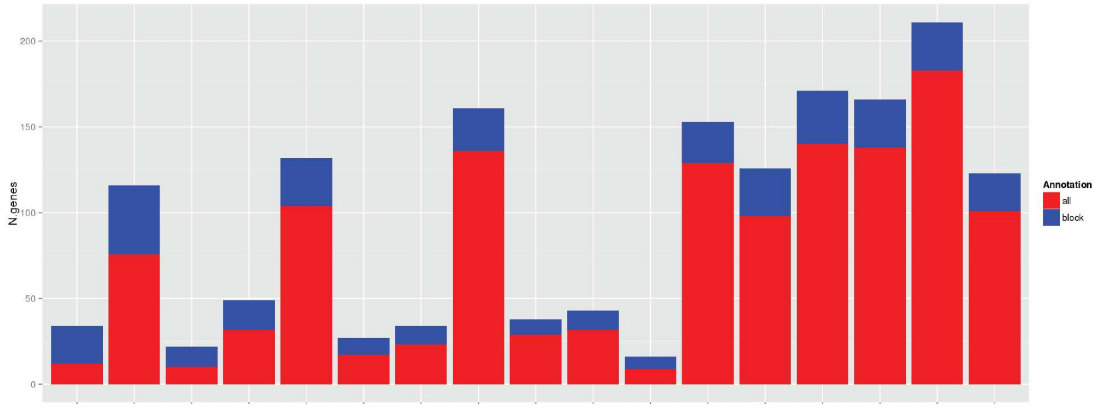


Table 2: Deregulated pathways in RHC MC1R melanocyte-keratinocyte co-culture system.

KEGG Term	STATUS	Adj. P-Value
DNA replication (hsa03030)	UP	3.83E-14
Cell cycle (hsa04110)	UP	7.87E-14
Mismatch repair (hsa 03430)	UP	1.56E-06
Nucleotide excision repair (hsa03420)	UP	7.01E-06
Oxidative phosphorylation (hsa00190)	UP	6.91E-05
Homologous recombination (hsa03440)	UP	6.98E-04
Base excision repair (hsa03410)	UP	1.07E-03
Purine metabolism (hsa00230)	UP	1.80E-02
Pyruvate metabolism (hsa00620)	UP	3.47E-02
Valine. leucine and isoleucine degradation (hsa00280)	UP	8.13E-03
One carbon pool by folate (hsa00670)	UP	2.69E-03
Spliceosome (hsa03040)	UP	1.80E-02
Parkinson's disease (hsa05012)	UP	3.10E-05
Hugtington's disease (hsa05016)	UP	5.25E-04
Alzheimer's disease (hsa 05010)	UP	3.03E-03
Endocytosis (hsa04144)	DOWN	3.83E-02
Lysosome (hsa04142)	DOWN	4.70E-03

The KEGG term is indicated for each pathway (X-axis). Status: UP: pathway overrepresented in the group of up-regulated transcripts in RHC MC1R; DOWN: pathway over-represented in the group of down-regulated transcripts in RHC MC1R.

a role in major DNA repair and cell homeostasis pathways such as DNA replication, cell cycle and also oxidative phosphorylation which leads to production of reactive oxygen species (Figure 3 and Table 2). The lysosome and endocytosis pathways, which are intrinsically related to melanosome transfer from melanocytes to keratinocytes, were over-represented in the set of down-regulated cells also exhibited an up-regulation of genes involved in neurodegenerative disorders such as Parkinson´s disease (hsa05012), Huntington´s disease (hsa5016) and Alzheimer´s disease (hsa05010). Although most of the genes were involved in several of these pathways, some of them were restricted to Huntington's disease (*AP2B1, CREB3L4, POLR2H* and *SOD1*), to Alzheimer´s disease (*NAE1*, *FAS*) or to Parkinson's disease (*PARK7).*

Besides the lack of functionality caused by p.R151C and p.R160W variants, we also analyzed a possible effect on *MC1R* levels, by the relative quantification of *MC1R* expression. We observed a statistically significant reduction in *MC1R* expression in RHC skin cells (ddCt=0.06±0.02) compared to wild-type *MC1R* skin cells (ddCt=0.50±0.07; P-value<0.0001). Furthermore, the expression of *FARP1, SLFN11, GFPT2, COL5A3, TYRP1, LEF1, KRT2, ST6GALNAC3, MLANA, MSMB, SILV, A2M,* and *ALOX5* was evaluated by RT-PCR confirming the results obtained in the array (data not shown).

We further examined the overlap between genes differentially expressed in both mutant *CDKN2A* (n=1536) and in non functional MC1R cells (n=3570). A total of 485 genes were altered in the same fashion in both mutant *CDKN2A* and non functional *MC1R* cells and the expression of 136 genes was found inversely correlated between both groups. Of these 136 genes (listed in Table S3), 30 were up-regulated in mutant *CDKN2A* and down-regulated in non functional MC1R cells and 106 genes were down-regulated in mutant *CDKN2A* and up-regulated in non functional *MC1R* cells (Figure 4). The functional analysis of these 136 genes did not reveal over-representation of any specific pathway.

In silico model evaluation

We hypothesized that if the altered gene signatures observed in non-lesional skin from *CDKN2A* mutation or RHC *MC1R* variant carriers are critical for malignant transformation, these patterns will be maintained across the carcinogenic process. To support our hypothesis, the expression pattern of the most deregulated genes in mutant *CDKN2A* or RHC *MC1R* variant skin cells was compared to their expression observed in two previously published skin cancer datasets. The GSE2503 dataset contained the whole genome expression of five squamous cell carcinomas (SCCs) and six healthy skin samples[35] and the GSE12391 dataset included 23 melanomas (CMs) at different stages (radial growth phase or vertical growth phase) and 18 common nevi[36]. Due to differences in the array platforms used among the studies, only the expression of genes present in all datasets was evaluated

genes. Primary co-cultures with the non-functional *MC1R* gene showed a statistically significant expression pattern (p-value <0.005) similar to those detected in SCCs (Pearson correlation=0.44) and CMs (Pearson correlation=0.25) (Figure 5a).

Because of platform disparities, the mutant *CDKN2A* expression pattern was evaluated analyzing 53.7% of the most deregulated transcripts (58/108). The expression pattern correlated to those observed in SCCs (Pearson correlation=0.35) and CMs (Pearson correlation=0.28) showing a statistical tendency among datasets (p-value <0.06), in which immunomodulation and interferon response genes were also up-regulated (*SP110*, *BTN3A2, IL15, IFIT2, IFI44, IFIT1, IFI44L* and

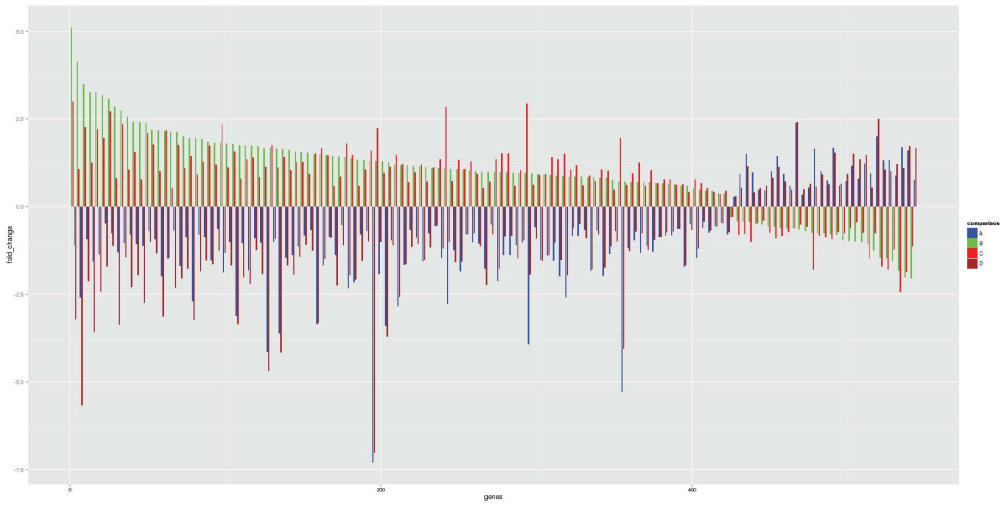


Figure 4: Genes inversely expressed in mutant CDKN2A and RHC MC1R. 136 genes were found inversely expressed between mutant *CDKN2A* and RHC *MC1R* genes (listed in Table S3). A: RHC *MC1R* vs wt *MC1R* (both mutant *CDKN2A*); B: mutant *CDKN2A* vs wt *CDKN2A* (both RHC *MC1R*); C: mutant *CDKN2A* vs wt *CDKN2A* (both wt *MC1R*); D: RHC MC1R vs wt MC1R (both wt *CDKN2A*).

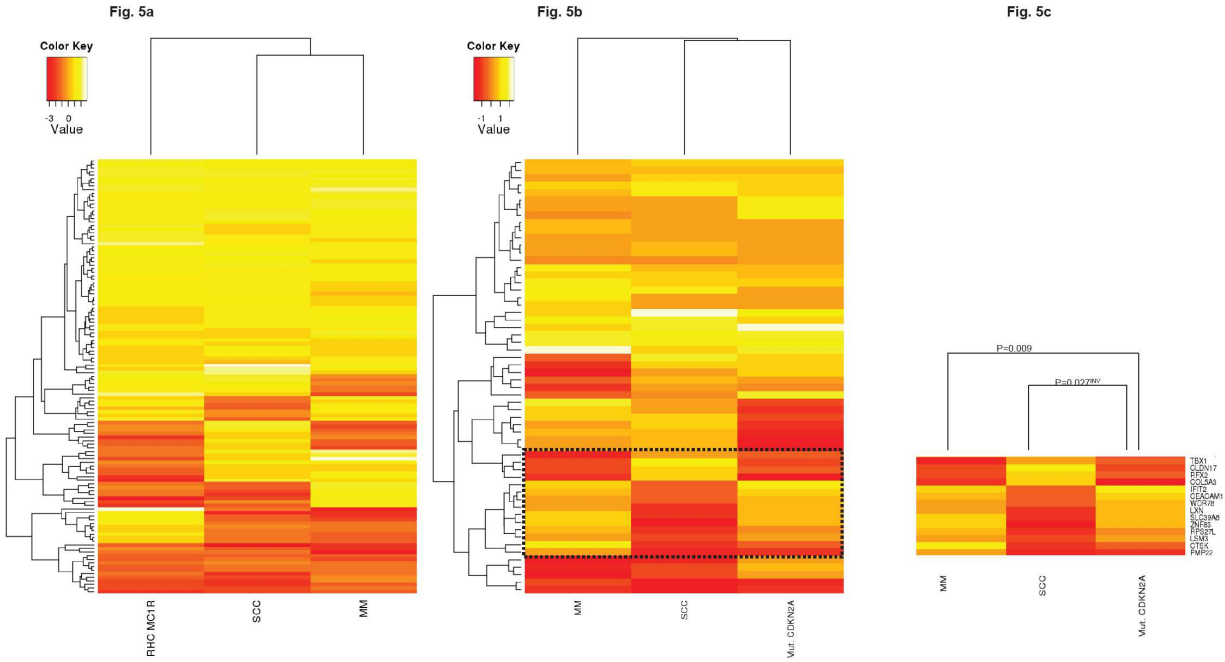


Figure 5: In-silico model evaluation. Comparison of the expression patterns detected in our study to those observed in Squamous cell

CLEC2B; Figure 5b). A correlated expression pattern was found within a subset of genes between CMs and mutant *CDKN2A* skin cells (Pearson Correlation=0.66, P-value = 0.009; Figure 5c). Remarkably, this subset of genes presented an expression pattern that inversely correlated between SCCs and CMs (Pearson correlation= -0.88, p-value<0.001) and also between SCCs and mutant *CDKN2A* skin cells (Pearson correlation= -0.58, p-value=0.027). Specifically, the expression pattern of *TBX1, CLDN17, RFX2, COL5A3, IFIT2, CEACAM1, WDR78, LXN, SLC39A8, ZNF83, RPS27L, LSM3, CTSK*, and *PMP22* was exclusively correlated to MM and mutant *CDKN2A* MM prone skin cells, and inversely with NMSC.

DISCUSSION

The aim of the study was to determine the constitutional effect of germline *CDKN2A* mutations or *MC1R* variants in skin cancer, in order to identify early critical molecular targets implicated in the disease. To do so we analyzed co-cultured melanocyte-keratinocyte systems derived from two siblings belonging to two melanoma prone families with a founder *CDKN2A* mutation and/or carrying non-functional *MC1R* alleles to compare global gene expression profiles.

To date, *CDKN2A* is the major gene responsible for increased melanoma susceptibility in high-risk pedigrees. Germline high-penetrance mutations are found in 10% of melanoma prone families and somatic *CDKN2A* alterations are also recurrent events in primary melanomas[37] and melanoma cell lines[38]. Alterations in gene expression in as yet phenotypically normal cells but bearing single-hit mutations in tumor suppressor genes appear to be the earliest molecular change during cancer development[39].

Altered baseline expression signatures associated with *CDKN2*A mutations in cultured normal skin fibroblasts from familial melanoma patients have already been reported [40]. However, the transcriptome deregulation within keratinocytes and melanocytes where cutaneous carcinomas and melanomas arise have not been previously assessed. We found that transcriptome signatures were altered by single hit *CDKN2A* mutations in co-cultured melanocyte-keratinocyte systems. *CDKN2A* mutant cells exhibit mainly baseline differences in genes related to cell components, metabolism and immune response. Melanoma cells are addicted to oncogene-driven energy production which can be mediated by somatic *BRAF* mutations [41], our findings indicate that non transformed skin cells already have a deregulated metabolic profiling which may be necessary for the skin cancer initiation [44]. Consistent with this hypothesis, our data reflects the important role of innate immunity and immune response pathways which may be deregulated in early steps of melanoma development. Skin cells also exhibited deregulation of epidermal differentiation and melanogenesis genes. Thus, we found down-regulation of the *TYRP1* gene, a melanocyte differentiation marker and over expression of *MFI2* gene, a cell-surface glycoprotein playing a role in melanoma cell proliferation and tumorigenesis [45] and *EEA1* gene, a marker of early endosomes [46]. Interestingly, *EEA1* interacts with *MFI2* regulating endosome fusion and trafficking [47]. Early endosomes are direct precursors of melanosomes, which are the specialized organelles for the biosynthesis and storage of melanins[46]. These findings may indicate that constitutive disorganized melanosomal structures and changes in pigment production may favor malignant transformation which in turn, could be related to autoimmune response deregulation [48, 49].

Via functional analyses, we observed a down-regulation of the Notch signaling pathway in *CDKN2A* mutant cells. This pathway is essential in epidermal-melanocyte interactions [50, 51] and recent evidence suggests Notch pathway as a link between the control of epidermal differentiation and proliferation and skin homeostasis [52, 53]. Notch signaling has a dual action (as oncogene or as tumor suppressor) in skin cancer. While, up-regulation of the Notch pathway is observed in CMs or in SCCs at sun-protected sites, it is down-regulated in UV-related solar keratoses SCCs or in common melanocytic nevi (reviewed in Panelos and Massi, 2009 [54]). A high melanocytic nevi count is the strongest known risk factor for melanoma[55] and a potent predictor of mutant *CDKN2A* gene carrier status[56]. Thus, the baseline down-regulation of the Notch signaling pathway in *CDKN2A* mutation carriers may proffer a melanocyte proliferation advantage, which can trigger common nevi development in human skin.

The pigmentation related gene *MC1R* acts as a moderate melanoma risk gene and variants in this gene are modifying factors for melanoma risk in *CDKN2A* mutation carriers [21-23]. Furthermore, *MC1R* variants have also been clearly associated with elevated NMSC susceptibility [19, 20]. We observed that the number of genes differentially expressed in non-functional *MC1R* was considerably higher than in mutant *CDKN2A*. Both p.R151C and p.R160W alleles halted *MC1R* activity and also induced an altered cell surface molecule expression due to impaired export traffic [57] that could lead to a reduction in *MC1R* gene expression levels as observed in our study. We found that RHC *MC1R* skins cells differ from wild-type in the deregulation of genes involved in

increased DNA damage without UV radiation exposure were up-regulated, indicating that RHC variants contribute to cancer risk in humans increasing the DNA damage mediated by intrinsic UV-independent mechanisms as recently observed in mice models [58]. Notably, RHC *MC1R* skin cells also showed up-regulated pathways related to neurodegenerative diseases. The *MC1R* gene is expressed in cells of the nervous system and its activation decreases oxidative stress and has anti-inflammatory and immunomodulatory effects [59]. Co-occurrence of Parkinson's disease (PD) and CMs [60, 61] has been reported in epidemiological studies. The diagnosis of CM is associated with an approximate 50% increased risk of subsequent PD[61] development whereas individuals with PD have a two-fold increase in risk of subsequent CM[62] development. Previous data suggests that this relation is associated with pigment-related genes[63] rather than Parkinson's-related genes[64, 65]. Our study further supports that *MC1R* is involved in the cross-link between both diseases. Thus, *MC1R* variants may increase the oxidative damage in brain cells and deregulate inflammatory processes which consequently, increase the susceptibility to neurodegenerative disorders.

Previous studies showed that many of the alterations associated with transcriptome and proteome signatures of as yet phenotypically normal cells bearing inherited alterations are also present in the corresponding form of cancer [39]. Thus, in our study, expression patterns detected in MM prone cells from non-lesional areas of the skin were compared with those observed in skin cancer tumors (SCCs and CMs) [35, 36]. Although differences in the experimental designs among studies (normal skin cells *vs* tumoral tissue or cultured cells *vs* fresh-frozen tissue) may interfere in the analysis, the expression patterns detected in our study correlate to those observed in skin tumors. Interestingly, the *MC1R* expression pattern was more similar among studies than the *CDKN2A* pattern (p-value<0.005 and p-value<0.06, respectively). These differences are caused in part by a subset of genes showing a converse pattern which allows us to distinguish between SSCs versus CMs and mutant *CDKN2A* skin cells (MM prone cells). Accordingly, previous studies have detected the inverse expression of two genes from this subset (*CEACAM1* and *CTSK)* between SSCs and MMs [66, 67]. Such differences may underlie the epidemiological differences observed between *MC1R* and *CDKN2A* and skin cancer susceptibility since *MC1R* polymorphisms are involved in both CMM and NMSC susceptibility while *CDKN2A* mutations are closely related to CM susceptibility rather than SCC susceptibility.

In conclusion, we have identified baseline expression signatures in skin cells carrying germline to skin cancer, highlighting the role of genes involved in immune response, in melanosome biogenesis and the Notch signaling pathway Also, our data indicates that non functional *MC1R* variants promote DNA damage by intrinsic UV-independent mechanisms in human skin cells. Furthermore, our study revealed a role for *MC1R* in the susceptibility to neurodegenerative diseases which may be related to its role in oxidative stress and inflammatory processes.

METHODS

Study Design

To detect the effect of *CDKN2A* and *MC1R* genes, two melanoma families (A and B) were selected (Table I). The most frequent mutation, p.G101W, resulting from a common ancestor in Mediterranean pedigrees, was chosen in relation to the *CDKN2A* status. Regarding *MC1R* status, the presence of two red hair variants in each individual (RHC: p.R151C and p.R160W) was analyzed. To reduce the effect of intra-individual variability, two siblings from each family were selected, resulting in four different extreme genotypic conditions: a carrier of variants in both genes (sample A1), an individual without variants in any gene (sample B2) and two carriers of variants in one of these genes (samples A2 and B1), respectively.

The study was approved by the IRB and signed informed consents were obtained from all individuals.

Primary keratinocyte and melanocyte co-culture

Skin biopsies from non-lesional areas were taken and human keratinocytes and melanocytes were obtained by mechanical fragmentation and enzymatic digestion [68]. Briefly, skin biopsy fragments were digested with collagenase type I solution (2mg/ml) (Sigma–Aldrich, Gillingham, UK) for 90 minutes. The collagenase solution was then completely eliminated and the remaining skin biopsy was introduced into a mixture of 0.05% trypsin /0.02% EDTA (T/E) (Sigma). Every 30 min, T/E was changed for a fresh T/E mixture. Collected T/E was inactivated with serum containing culture medium and was centrifuged at 1400 rpm for 10 minutes. Primary keratinocytes and melanocytes obtained by this method were cultured on a feeder layer of lethally irradiated (X-ray; 50 Gy) 3T3-J2 cells (a gift from Dr J. Garlick, SUNY), as previously described [69, 70]. The seeding media was a 3:1 mixture of Dulbecco's Modified Eagle Medium (DMEM) (GIBCO-BRL, Barcelona, Spain)

culture medium was changed every two days. This co-culture system has been extensively proven to preserve stemness of keratinocytes[69-71] as well as to maintain the physiological melanocyte:keratinocyte ratio (1:40)[72, 73]. Moreover, by using the skin cells from this type of co-culture, the donor's phenotypic pigmentation and UV-response features are preserved *in vivo* on a humanized skin mouse model[74].

RNA extraction

Total RNA isolation from primary cultures on passage 3-4 was performed using the Trizol extraction method (Invitrogen Life Technologies, Carlsbad, CA) followed by purification in commercial columns (Qiagen, Valencia, CA). Total isolated RNA was further purified using an RNeasy kit (Qiagen, Valencia, CA). RNA concentration was determined using a NanoDrop Spectrophotometer (Thermo Scientific) and integrity of the RNA was verified by Bioanalyzer 2100 (Agilent, USA). The RNA integrity number was in all cases higher than 8.

Expression array

Analysis of global expression was performed using the Whole Human Genome (4x44k) Oligo Microarray kit (G4112F, Agilent, US). The microarray contains probes from over 41,000 unique human genes and transcripts, all with public OMIM annotations (RefSeq, Goldenpath, Ensembl, Unigene Human Genome and GenBank databases). Overall, 50 ng of RNA were labeled using Low input Quickamp Labeling kit (Agilent, US). In all samples 10 commercial controls probes were added in order to standardize the results (RNA Spike-in kit, one color, Agilent, US). The arrays were scanned using the DNA Microarray Scanner G2565CA (Agilent, US). Finally, Feature Extraction Software (FES, Agilent, USA) was used both to perform the quality control process and to extract the information. Three replicates from each primary culture were analyzed.

Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) of selected genes

To confirm the microarray results, the expression of 13 genes (*FARP1, SLFN11, GFPT2, COL5A3, TYRP1, LEF1, KRT2, ST6GALNAC3, MLANA, MSMB, SILV, A2M,* and *ALOX5*) was validated by RT-PCR. Also, *MC1R* Core Reagent kit (Roche Applied Science, Penzbergf, Germany). Real-time PCR was performed using Taqman Universal PCR master Mix (Roche Applied Science, Penzbergf, Germany). Reaction was performed in an ABI Step One plus RT-PCR sequence detection instrument (Applied Biosystems, CA, US). Primer design and gene-specific probe selection were carried out by the Universal Probe Library software (UPL, Roche, Mannheim, Germany).

Data was evaluated using the relative quantification method of ddCt[75]. Expression values were evaluated by T-test for equality means using the SPSS 17.0. P-values less than or equal to 0.05 were considered statistically significant.

Microarray data and statistical analyses

The Agilent Processed Signal was standardized across arrays using quantile normalization[76]. Differential gene expression analysis was carried out using the limma package from Bioconductor. Multiple testing adjustments of p-values were performed according to Benjamini and Hochberg methodology[77].

Gene set analysis was carried out for the Kyoto Encyclopedia of Genes and Genomes pathways (KEGG) using FatiScan[78] in Babelomics[79]. This tool detects significantly up- or down-regulated blocks of functionally related genes in lists of genes ordered by different criteria such as differential expression, KEGG pathways and others. The core of the method is based on an algorithm to test whether a set of genes, labeled with terms (biological information), contain significant enrichments of one or several of these terms with respect to another set of reference genes. FatiScan uses a Fisher's exact test for 2×2 contingency tables for comparing two groups of genes and extracting a list of KEGG terms whose distribution among the groups is significantly different. Given that many KEGG terms are simultaneously tested, the results of the test are corrected for multiple testing to obtain an adjusted p-value. FatiScan returns adjusted p-values based on the False Discovery Rate (FDR) method[77]. KEGG Pathways for the genes in the microarray where taken from the KEGG web.

In order to gain insight into the co-regulation patterns on transcript expression profiles, data from our microarray experiments were clustered together with expression data obtained from two previous experiments focused on NMSC (GSE2503 dataset[35]) and MM (GSE12391 dataset[36]). Raw data were obtained from the Gene Expression Omnibus. For each dataset we analyzed the differential expression and determined the

scenario, fold-changes were normalized using the quantile method[76] to compare differential expression results in all experiments. We performed a hierarchical clustering analysis of differentially expressed transcripts and the graphical representation showed the relationship between experiments. The correlation analysis quantified the linear relationship between the different studies m

Supplemental Data Description

Supplemental Data includes two tables with the list of the most deregulated genes associated with *CDKN2A* mutations or *MC1R* variants; and one table containing the list of genes inversely deregulated between CDKN2A and MC1R.

ACKNOWLEDGMENTS

The present project was founded by a grant from CIBER de Enfermedades Raras of the Instituto de Salud Carlos III, Spain (INTRA/07/726.2). The research at the Melanoma Unit in Barcelona is partially funded by Grants from Fondo de Investigaciones Sanitarias (P.I. 09/01393 and P.I. 12/00840, co-funded by "Fondo Europeo de Desarrollo Regional (FEDER). Unión Europea. Una manera de hacer Europa"); by the AGAUR 2009 SGR 1337 of the Catalan Government, Spain; by the European Commission under the 6th Framework Programme, Contract nr: LSHC-CT-2006-018702 (GenoMEL) and by the National Cancer Institute (NCI) of the US National Institute of Health (NIH) (CA83115). This work is also partly supported by grants BIO2008-04212 and SAF2010-16976 from the Spanish Ministry of Science and Innovation (MICINN), PROMETEO/2010/001 from the GVA-FEDER and S2010/BMD-2420 (CELLCAM) from Comunidad de Madrid.

Web Resources:

Bioconductor (http://www.bioconductor.org)

Babelomics (http://babelomics.bioinfo.cipf.es/)

Kyoto Encyclopedia of Genes and Genomes pathways (KEGG) (http://www.genome.jp/kegg/)

Ensembl 56 release (http://www.ensembl.org)

Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/)

Accession Numbers The GEO accession number for the expression microarray data reported in this paper is GSE44805.

http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=ptorruosgomwgds&acc=GSE44805

Conflict of Interest:

The authors state no conflict of interest.

REFERENCES

1. Siegel R, Naishadham D and Jemal A. Cancer statistics, 2012. CA Cancer J Clin. 2012; 62(1):10-29.
2. Thompson JF, Scolyer RA and Kefford RF. Cutaneous melanoma in the era of molecular profiling. Lancet. 2009; 374(9687):362-365.
3. Goldstein AM, Chan M, Harland M, Hayward NK, Demenais F, Bishop DT, Azizi E, Bergman W, Bianchi-Scarra G, Bruno W, Calista D, Albright LA, Chaudru V, Chompret A, Cuellar F, Elder DE, et al. Features associated with germline CDKN2A mutations: a GenoMEL study of melanoma-prone families from three continents. J Med Genet. 2007; 44(2):99-106.
4. Puig S, Malvehy J, Badenas C, Ruiz A, Jimenez D, Cuellar F, Azon A, Gonzalez U, Castel T, Campoy A, Herrero J, Marti R, Brunet-Vidal J and Mila M. Role of the CDKN2A locus in patients with multiple primary melanomas. J Clin Oncol. 2005; 23(13):3043-3051.
5. Liontos M, Pateras IS, Evangelou K and Gorgoulis VG. The Tumor Suppressor Gene ARF as a Sensor of Oxidative Stress. Curr Mol Med. 2012.
6. Jenkins NC, Liu T, Cassidy P, Leachman SA, Boucher KM, Goodson AG, Samadashwily G and Grossman D. The p16(INK4A) tumor suppressor regulates cellular oxidative stress. Oncogene. 2011; 30(3):265-274.
7. Lang J, Boxer M and MacKie RM. CDKN2A mutations in Scottish families with cutaneous melanoma: results from 32 newly identified families. Br J Dermatol. 2005; 153(6):1121-1125.
8. Larre Borges A, Cuellar F, Puig-Butille JA, Scarone M, Delgado L, Badenas C, Mila M, Malvehy J, Barquet V, Nunez J, Laporte M, Fernandez G, Levrero P, Martinez-Asuaga M and Puig S. CDKN2A mutations in melanoma families from Uruguay. Br J Dermatol. 2009; 161(3):536-541.
9. Yakobson E, Eisenberg S, Isacson R, Halle D, Levy-Lahad E, Catane R, Safro M, Sobolev V, Huot T, Peters G, Ruiz A, Malvehy J, Puig S, Chompret A, Avril MF, Shafir R, et al. A single Mediterranean, possibly Jewish, origin for the Val59Gly CDKN2A mutation in four melanoma-prone families. Eur J Hum Genet. 2003; 11(4):288-296.
10. Majore S, De Simone P, Crisi A, Eibenschutz L, Binni F, Antigoni I, De Bernardo C, Catricala C and Grammatico P. CDKN2A/CDK4 molecular study on 155 Italian subjects with familial and/or primary multiple melanoma. Pigment

in Utah melanoma families. J Invest Dermatol. 2006; 126(3):660-666.

12. Ciotti P, Struewing JP, Mantelli M, Chompret A, Avril MF, Santi PL, Tucker MA, Bianchi-Scarra G, Bressac-de Paillerets B and Goldstein AM. A single genetic origin for the G101W CDKN2A mutation in 20 melanoma-prone families. Am J Hum Genet. 2000; 67(2):311-319.
13. Bishop DT, Demenais F, Goldstein AM, Bergman W, Bishop JN, Bressac-de Paillerets B, Chompret A, Ghiorzo P, Gruis N, Hansson J, Harland M, Hayward N, Holland EA, Mann GJ, Mantelli M, Nancarrow D, et al. Geographical variation in the penetrance of CDKN2A mutations for melanoma. J Natl Cancer Inst. 2002; 94(12):894-903.
14. Talantov D, Mazumder A, Yu JX, Briggs T, Jiang Y, Backus J, Atkins D and Wang Y. Novel genes associated with malignant melanoma but not benign melanocytic lesions. Clin Cancer Res. 2005; 11(20):7234-7242.
15. Jefferies S, Edwards SM, Hamoudi RA, A'Hern R, Foulkes W, Goldgar D and Eeles R. No germline mutations in CDKN2A (p16) in patients with squamous cell cancer of the head and neck and second primary tumours. Br J Cancer. 2001; 85(9):1383-1386.
16. Cabanillas R, Astudillo A, Valle M, de la Rosa J, Alvarez R, Duran NS and Cadinanos J. Novel germline CDKN2A mutation associated with head and neck squamous cell carcinomas and melanomas. Head Neck. 2011.
17. Rees JL. The genetics of sun sensitivity in humans. Am J Hum Genet. 2004; 75(5):739-751.
18. Valverde P, Healy E, Jackson I, Rees JL and Thody AJ. Variants of the melanocyte-stimulating hormone receptor gene are associated with red hair and fair skin in humans. Nat Genet. 1995; 11(3):328-330.
19. Bastiaens MT, ter Huurne JA, Kielich C, Gruis NA, Westendorp RG, Vermeer BJ and Bavinck JN. Melanocortin-1 receptor gene variants determine the risk of nonmelanoma skin cancer independently of fair skin and red hair. Am J Hum Genet. 2001; 68(4):884-894.
20. Box NF, Duffy DL, Irving RE, Russell A, Chen W, Griffyths LR, Parsons PG, Green AC and Sturm RA. Melanocortin-1 receptor genotype is a risk factor for basal and squamous cell carcinoma. J Invest Dermatol. 2001; 116(2):224-229.
21. Box NF, Duffy DL, Chen W, Stark M, Martin NG, Sturm RA and Hayward NK. MC1R genotype modifies risk of melanoma in families segregating CDKN2A mutations. Am J Hum Genet. 2001; 69(4):765-773.
22. Goldstein AM, Chaudru V, Ghiorzo P, Badenas C, Malvehy J, Pastorino L, Laud K, Hulley B, Avril MF, Puig-Butille JA, Miniere A, Marti R, Chompret A, Cuellar F, Kolm I, Mila M, et al. Cutaneous phenotype and MC1R variants Newton Bishop JA, Bishop DT, Kanetsky PA, Hayward NK, Gillanders E, Elder DE, Avril MF, Azizi E, van Belle P, Bergman W, Bianchi-Scarra G, Bressac-de Paillerets B, et al. Association of MC1R variants and host phenotypes with melanoma risk in CDKN2A mutation carriers: a GenoMEL study. J Natl Cancer Inst. 2010; 102(20):1568-1583.
24. Herraiz C, Jimenez-Cervantes C, Zanna P and Garcia-Borron JC. Melanocortin 1 receptor mutations impact differentially on signalling to the cAMP and the ERK mitogen-activated protein kinase pathways. FEBS Lett. 2009; 583(19):3269-3274.
25. Le Pape E, Passeron T, Giubellino A, Valencia JC, Wolber R and Hearing VJ. Microarray analysis sheds light on the dedifferentiating role of agouti signal protein in murine melanocytes via the Mc1r. Proc Natl Acad Sci U S A. 2009; 106(6):1802-1807.
26. Sviderskaya EV, Hill SP, Evans-Whipp TJ, Chin L, Orlow SJ, Easty DJ, Cheong SC, Beach D, DePinho RA and Bennett DC. p16(Ink4a) in melanocyte senescence and differentiation. J Natl Cancer Inst. 2002; 94(6):446-454.
27. Eisinger M and Marko O. Selective proliferation of normal human melanocytes in vitro in the presence of phorbol ester and cholera toxin. Proc Natl Acad Sci U S A. 1982; 79(6):2018-2022.
28. Herlyn M, Rodeck U, Mancianti M, Cardillo FM, Lang A, Ross AH, Jambrosic J and Koprowski H. Expression of melanoma-associated antigens in rapidly dividing human melanocytes in culture. Cancer Res. 1987; 47(12):3057-3061.
29. Elder DE, Rodeck U, Thurin J, Cardillo F, Clark WH, Stewart R and Herlyn M. Antigenic profile of tumor progression stages in human melanocytic nevi and melanomas. Cancer Res. 1989; 49(18):5091-5096.
30. Valyi-Nagy IT, Hirka G, Jensen PJ, Shih IM, Juhasz I and Herlyn M. Undifferentiated keratinocytes control growth, morphology, and antigen expression of normal melanocytes through cell-cell contact. Lab Invest. 1993; 69(2):152-159.
31. Hsu MY, Meier FE, Nesbit M, Hsu JY, Van Belle P, Elder DE and Herlyn M. E-cadherin expression in melanoma cells restores keratinocyte-mediated growth control and down-regulates expression of invasion-related adhesion receptors. Am J Pathol. 2000; 156(5):1515-1525.
32. Phillips J, Gawkrodger DJ, Caddy CM, Hedley S, Dawson RA, Smith-Thomas L, Freedlander E and Mac Neil S. Keratinocytes suppress TRP-1 expression and reduce cell number of co-cultured melanocytes - implications for grafting of patients with vitiligo. Pigment Cell Res. 2001; 14(2):116-125.
33. Haass NK, Smalley KS, Li L and Herlyn M. Adhesion,

mammalian epidermal melanocytes. Pigment Cell Res. 2005; 18(1):2-12.

35. Nindl I, Dang C, Forschner T, Kuban RJ, Meyer T, Sterry W and Stockfleth E. Identification of differentially expressed genes in cutaneous squamous cell carcinoma by microarray expression profiling. Mol Cancer. 2006; 5:30.
36. Scatolini M, Grand MM, Grosso E, Venesio T, Pisacane A, Balsamo A, Sirovich R, Risio M and Chiorino G. Altered molecular pathways in melanocytic lesions. Int J Cancer. 2010; 126(8):1869-1881.
37. de Snoo FA and Hayward NK. Cutaneous melanoma susceptibility and progression genes. Cancer Lett. 2005; 230(2):153-186.
38. Ogbah Z, Puig-Butille JA, Simonetta F, Badenas C, Cervera R, Mila J, Benitez D, Malvehy J, Vilella R and Puig S. Molecular characterization of human cutaneous melanoma-derived cell lines. Anticancer Res. 2012; 32(4):1245-1251.
39. Herbert BS, Chanoux RA, Liu Y, Baenziger PH, Goswami CP, McClintick JN, Edenberg HJ, Pennington RE, Lipkin SM and Kopelovich L. A molecular signature of normal breast epithelial and stromal cells from Li-Fraumeni syndrome mutation carriers. Oncotarget. 2012; 1(6):405-422.
40. Fan M, Pfeffer SR, Lynch HT, Cassidy P, Leachman S, Pfeffer LM and Kopelovich L. Altered transcriptome signature of phenotypically normal skin fibroblasts heterozygous for CDKN2A in familial melanoma: relevance to early intervention. Oncotarget. 2013; 4(1):128-141.
41. Hall A, Meyle KD, Lange MK, Klima M, Sanderhoff M, Dahl C, Abildgaard C, Thorup K, Moghimi SM, Jensen PB, Bartek J, Guldberg P and Christensen C. Dysfunctional oxidative phosphorylation makes malignant melanoma cells addicted to glycolysis driven by the (V600E)BRAF oncogene. Oncotarget. 2013; 4(4):584-599.
42. Gu F, Qureshi AA, Niu T, Kraft P, Guo Q, Hunter DJ and Han J. Interleukin and interleukin receptor gene polymorphisms and susceptibility to melanoma. Melanoma Res. 2008; 18(5):330-335.
43. Howell WM, Turner SJ, Bateman AC and Theaker JM. IL-10 promoter polymorphisms influence tumour development in cutaneous malignant melanoma. Genes Immun. 2001; 2(1):25-31.
44. Yang XR, Pfeiffer RM, Wheeler W, Yeager M, Chanock S, Tucker MA and Goldstein AM. Identification of modifier genes for cutaneous malignant melanoma in melanoma-prone families with and without CDKN2A mutations. Int J Cancer. 2009; 125(12):2912-2917.
45. Guo J, Wen DR, Huang RR, Paul E, Wunsch P, Itakura E and Cochran AJ. Detection of multiple melanoma-associated markers in melanoma cell lines by RT in situ PCR. Exp Mol Pathol. 2003; 74(2):140-147.
47. Ramanathan HN and Ye Y. The p97 ATPase associates with EEA1 to regulate the size of early endosomes. Cell Res. 2011; 22(2):346-359.
48. Sakai C, Kawakami Y, Law LW, Furumura M and Hearing VJ, Jr. Melanosomal proteins as melanoma-specific immune targets. Melanoma Res. 1997; 7(2):83-95.
49. Hearing VJ. Biochemical control of melanogenesis and melanosomal organization. J Investig Dermatol Symp Proc. 1999; 4(1):24-28.
50. Schouwey K, Delmas V, Larue L, Zimber-Strobl U, Strobl LJ, Radtke F and Beermann F. Notch1 and Notch2 receptors influence progressive hair graying in a dose-dependent manner. Dev Dyn. 2007; 236(1):282-289.
51. Okuyama R, Tagami H and Aiba S. Notch signaling: its role in epidermal homeostasis and in the pathogenesis of skin diseases. J Dermatol Sci. 2008; 49(3):187-194.
52. Xin Y, Lu Q and Li Q. IKK1 control of epidermal differentiation is modulated by notch signaling. Am J Pathol. 2011; 178(4):1568-1577.
53. Lin HY, Kao CH, Lin KM, Kaartinen V and Yang LT. Notch signaling regulates late-stage epidermal differentiation and maintains postnatal hair cycle homeostasis. PLoS One. 2011; 6(1):e15842.
54. Panelos J and Massi D. Emerging role of Notch signaling in epidermal differentiation and skin cancer. Cancer Biol Ther. 2009; 8(21):1986-1993.
55. Chang YM, Newton-Bishop JA, Bishop DT, Armstrong BK, Bataille V, Bergman W, Berwick M, Bracci PM, Elwood JM, Ernstoff MS, Green AC, Gruis NA, Holly EA, Ingvar C, Kanetsky PA, Karagas MR, et al. A pooled analysis of melanocytic nevus phenotype and the risk of cutaneous melanoma at different latitudes. Int J Cancer. 2009; 124(2):420-428.
56. Bishop JA, Wachsmuth RC, Harland M, Bataille V, Pinney E, Mac KP, Baglietto L, Cuzick J and Bishop DT. Genotype/phenotype and penetrance studies in melanoma families with germline CDKN2A mutations. J Invest Dermatol. 2000; 114(1):28-33.
57. Sanchez-Laorden BL, Herraiz C, Valencia JC, Hearing VJ, Jimenez-Cervantes C and Garcia-Borron JC. Aberrant trafficking of human melanocortin 1 receptor variants associated with red hair and skin cancer: Steady-state retention of mutant forms in the proximal golgi. J Cell Physiol. 2009; 220(3):640-654.
58. Mitra D, Luo X, Morgan A, Wang J, Hoang MP, Lo J, Guerrero CR, Lennerz JK, Mihm MC, Wargo JA, Robinson KC, Devi SP, Vanover JC, D'Orazio JA, McMahon M, Bosenberg MW, et al. An ultraviolet-radiation-independent pathway to melanoma carcinogenesis in the red hair/fair skin background. Nature. 2012; 491(7424):449-453.
59. Catania A. Neuroprotective actions of melanocortins: a

relationship between Parkinson disease and melanoma. Neurology. 2011; 76(23):2002-2009.

61. Olsen JH, Friis S and Frederiksen K. Malignant melanoma and other types of cancer preceding Parkinson disease. Epidemiology. 2006; 17(5):582-587.
62. Olsen JH, Friis S, Frederiksen K, McLaughlin JK, Mellemkjaer L and Moller H. Atypical cancer pattern in patients with Parkinson's disease. Br J Cancer. 2005; 92(1):201-205.
63. Gao X, Simon KC, Han J, Schwarzschild MA and Ascherio A. Genetic determinants of hair color and Parkinson's disease risk. Ann Neurol. 2009; 65(1):76-82.
64. Meng S, Song F, Chen H, Gao X, Amos CI, Lee JE, Wei Q, Qureshi AA and Han J. No association between Parkinson disease alleles and the risk of melanoma. Cancer Epidemiol Biomarkers Prev. 2011; 21(1):243-245.
65. Hassin-Baer S, Laitman Y, Azizi E, Molchadski I, Galore-Haskel G, Barak F, Cohen OS and Friedman E. The leucine rich repeat kinase 2 (LRRK2) G2019S substitution mutation. Association with Parkinson disease, malignant melanoma and prevalence in ethnic groups in Israel. J Neurol. 2009; 256(3):483-487.
66. Shinozuka K, Uzawa K, Fushimi K, Yamano Y, Shiiba M, Bukawa H, Yokoe H and Tanzawa H. Downregulation of carcinoembryonic antigen-related cell adhesion molecule 1 in oral squamous cell carcinoma: correlation with tumor progression and poor prognosis. Oncology. 2009; 76(6):387-397.
67. Quintanilla-Dieck MJ, Codriansky K, Keady M, Bhawan J and Runger TM. Cathepsin K in melanoma invasion. J Invest Dermatol. 2008; 128(9):2281-2288.
68. Rheinwald JG and Green H. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. Cell. 1975; 6(3):331-343.
69. Llames SG, Del Rio M, Larcher F, Garcia E, Garcia M, Escamez MJ, Jorcano JL, Holguin P and Meana A. Human plasma as a dermal scaffold for the generation of a completely autologous bioengineered skin. Transplantation. 2004; 77(3):350-355.
70. Del Rio M, Larcher F, Serrano F, Meana A, Munoz M, Garcia M, Munoz E, Martin C, Bernad A and Jorcano JL. A preclinical model for the analysis of genetically modified human skin in vivo. Hum Gene Ther. 2002; 13(8):959-968.
71. Larcher F, Dellambra E, Rico L, Bondanza S, Murillas R, Cattoglio C, Mavilio F, Jorcano JL, Zambruno G and Del Rio M. Long-term engraftment of single genetically modified human epidermal holoclones enables safety pre-assessment of cutaneous gene therapy. Mol Ther. 2007; 15(9):1670-1676.

cultured epithelium. Eur J Cell Biol. 1988; 46(1):176-180.

73. Guerra L, Capurro S, Melchi F, Primavera G, Bondanza S, Cancedda R, Luci A, De Luca M and Pellegrini G. Treatment of "stable" vitiligo by Timedsurgery and transplantation of cultured epidermal autografts. Arch Dermatol. 2000; 136(11):1380-1389.
74. Garcia M, Llames S, Garcia E, Meana A, Cuadrado N, Recasens M, Puig S, Nagore E, Illera N, Jorcano JL, Del Rio M and Larcher F. In vivo assessment of acute UVB responses in normal and Xeroderma Pigmentosum (XP-C) skin-humanized mouse models. Am J Pathol. 2010; 177(2):865-872.
75. Livak KJ and Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001; 25(4):402-408.
76. Bolstad BM, Irizarry RA, Astrand M and Speed TP. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics. 2003; 19(2):185-193.
77. Benjamini Y, Drai D, Elmer G, Kafkafi N and Golani I. Controlling the false discovery rate in behavior genetics research. Behav Brain Res. 2001; 125(1-2):279-284.
78. Al-Shahrour F, Arbiza L, Dopazo H, Huerta-Cepas J, Minguez P, Montaner D and Dopazo J. From genes to functional classes in the study of biological systems. BMC Bioinformatics. 2007; 8:114.
79. Medina I, Carbonell J, Pulido L, Madeira SC, Goetz S, Conesa A, Tarraga J, Pascual-Montano A, Nogales-Cadenas R, Santoyo J, Garcia F, Marba M, Montaner D and Dopazo J. Babelomics: an integrative platform for the analysis of transcriptomics, proteomics and genomic data with advanced functional profiling. Nucleic Acids Res. 2010; 38(Web Server issue):W210-213.

Supplementary Material:

Table S1: List of the most significant deregulated transcripts regarding *CDKN2A* status (p.G101W vs wild-type), adjusted P-value< 0.0001. Only genes identified in both *MC1R* wild-type and *MC1R* RHC variant carriers are included (N=108)
Abbreviations: FC: Fold Change; P-value: adjusted P-Value of such comparison; wt: wild-type.
*Statistical values of mutant *CDKN2A* vs wild-type in *MC1R* RHC skin cells (individual A1 vs individual A2).
**Statistical values of mutant *CDKN2A* vs wild-type in wild-type *MC1R* skin cells (individual B1 vs individual B2)

Gene ID	Gene Name	*FC* in *MC1R RHC* *	P-value in *MC1R RHC* *	*FC* in wt *MC1R***	P-value in wt *MC1R***
NM_002121	*HLA-DPB1*	-4.83	4.05E-13	-2.60	5.79E-10
NM_005766	*FARP1*	-3.6	1.32E-11	-1.53	5.80E-07
NM_152270	*SLFN11*	-3.4	1.87E-06	-2.08	7.47E-07
NM_005110	*GFPT2*	-3.2	5.49E-06	-2.50	5.64E-05
NM_015719	*COL5A3*	-3.1	1.87E-06	-2.28	3.91E-05
NM_000550	*TYRP1*	-2.76	1.43E-06	-2.34	6.40E-06
NM_016269	*LEF1*	-2.69	1.73E-09	-1.64	6.46E-07
NM_000423	*KRT2*	-2.45	7.93E-05	-2.70	1.58E-05
NM_000304	*PMP22*	-2.41	9.22E-10	-1.80	3.50E-08
NM_152996	*ST6GALNAC3*	-2.38	1.38E-08	-1.44	4.65E-06
NM_138284	*IL17D*	-2.31	7.53E-06	-2.02	2.08E-05
AF086044	*unknown*	-2.23	1.74E-10	-3.42	5.23E-13
NM_198951	*TGM2*	-2.08	4.10E-06	-2.42	4.33E-07
NM_138375	*CABLES1*	-2.08	5.78E-09	-3.31	8.92E-12
NM_006426	*DPYSL4*	-2.03	3.01E-05	-3.92	6.81E-09
NM_032048	*EMILIN2*	-1.83	5.82E-06	-2.04	1.00E-06
AK074473	*unknown*	-1.83	6.09E-06	-2.43	1.29E-07
NM_001129	*AEBP1*	-1.81	2.43E-07	-1.40	3.91E-06
NM_014070	*unknown*	-1.78	2.71E-07	-1.31	8.02E-06
BC004343	*C21orf122*	-1.66	5.99E-06	-1.47	1.48E-05
NM_148897	*SDR9C7*	-1.63	3.91E-05	-1.83	6.51E-06
AF131834	*unknown*	-1.53	2.07E-06	-2.60	1.81E-09
NM_080647	*TBX1*	-1.53	4.46E-06	-1.21	3.80E-05
NM_012131	*CLDN17*	-1.47	4.17E-05	-1.79	2.57E-06
NM_000635	*RFX2*	-1.38	2.98E-06	-1.08	2.98E-05
ENST00000224809	*unknown*	-1.28	6.59E-06	-1.81	6.37E-08
NM_032534	*KRBA1*	-1.21	2.92E-06	-0.97	2.25E-05
NM_152359	*CPT1C*	-1.08	1.08E-05	-1.48	1.50E-07
NM_001077263	*TMPRSS13*	-0.87	4.43E-05	-2.81	9.51E-12
NM_000396	*CTSK*	-0.74	7.83E-05	-1.81	1.05E-09
NM_003798	*CTNNAL1*	0.71	6.15E-05	0.81	9.91E-06
NM_015920	*RPS27L*	0.77	4.43E-05	0.86	8.34E-06
NM_001015509	*unknown*	0.79	1.13E-05	0.68	3.46E-05
THC2657938	*unknown*	0.81	4.28E-05	2.08	2.37E-10
NM_000022	*ADA*	0.87	8.65E-06	1.08	3.92E-07
THC2698177	*unknown*	0.89	6.21E-05	1.75	1.38E-08
NM_005760	*CEBPZ*	0.90	3.14E-05	2.20	3.14E-10
NM_004346	*CASP3*	0.91	1.50E-05	1.31	1.23E-07

NM_004487	*GOLGB1*	0.95	5.49E-06	1.17	2.84E-07
BC027178	*PRPF40A*	0.99	8.74E-05	2.62	3.74E-10
NM_004280	*EEF1E1*	1.00	7.97E-05	1.06	2.48E-05
NM_004986	*KTN1*	1.04	2.90E-06	2.53	1.59E-11
ENST00000377156	*unknown*	1.07	1.97E-05	1.09	9.97E-06
NM_007047	*BTN3A2*	1.09	1.77E-05	1.11	9.31E-06
NM_020169	*LXN*	1.11	3.10E-05	1.97	1.97E-08
ENST00000306024	*LSM3*	1.12	2.45E-05	0.99	5.33E-05
NM_001033719	*ZNF404*	1.13	4.49E-05	2.13	1.58E-08
NM_016010	*FAM164A*	1.13	1.23E-05	0.99	3.29E-05
NM_001007234	*unknown*	1.15	1.63E-06	1.42	7.87E-08
NM_001012334	*MDK*	1.16	6.19E-05	1.20	2.48E-05
NM_181453	*GCC2*	1.19	6.48E-05	1.56	1.83E-06
NM_033514	*LIMS3*	1.20	8.85E-07	1.16	8.49E-07
NM_033316	*MFI2*	1.20	9.60E-05	1.87	3.91E-07
NM_002890	*RASA1*	1.22	1.18E-06	0.86	3.86E-05
NM_018300	*ZNF83*	1.23	5.73E-05	1.70	8.63E-07
NM_201612	*unknown*	1.23	1.19E-05	2.28	4.38E-09
NM_207014	*WDR78*	1.30	6.81E-05	1.50	8.56E-06
NM_002078	*GOLGA4*	1.31	4.03E-05	1.21	5.59E-05
AK075484	*SAMD4B*	1.31	1.52E-05	3.52	3.01E-11
NM_003566	*EEA1*	1.33	9.48E-05	2.36	7.47E-08
NM_144777	*SCEL*	1.33	5.55E-07	1.01	9.54E-06
CB050071	*unknown*	1.41	5.52E-05	1.53	1.31E-05
NM_022154	*SLC39A8*	1.43	8.09E-05	1.54	2.19E-05
CR603437	*unknown*	1.44	1.33E-06	1.35	1.75E-06
NM_018169	*C12orf35*	1.48	2.17E-05	2.02	3.71E-07
NM_207331	*unknown*	1.49	9.36E-06	1.28	3.07E-05
AB075826	*FAM171B*	1.49	5.31E-05	1.47	3.83E-05
NM_004510	*SP110*	1.53	1.66E-07	0.94	3.63E-05
NM_172174	*IL15*	1.55	5.30E-08	1.29	4.08E-07
NM_000888	*ITGB6*	1.55	3.99E-05	2.52	8.05E-08
NM_005059	*RLN2*	1.58	1.97E-05	1.38	5.37E-05
NM_025114	*CEP290*	1.67	1.90E-05	2.70	3.88E-08
AK096536	*unknown*	1.70	3.16E-06	1.36	2.52E-05
THC2683448	*unknown*	1.72	2.01E-06	1.91	3.87E-07
NM_001013728	*unknown*	1.74	1.34E-05	2.23	4.47E-07
BC029919	*unknown*	1.78	4.62E-06	1.40	4.30E-05
NM_001712	*CEACAM1*	1.80	1.35E-06	1.56	4.49E-06
NM_003020	*SCG5*	1.83	2.10E-08	2.37	5.14E-10
NM_004921	*unknown*	1.86	8.41E-05	1.74	9.87E-05
NM_018429	*BDP1*	1.90	1.48E-05	2.05	3.60E-06
THC2676635	*unknown*	1.93	2.93E-08	1.27	3.49E-06
NM_178445	*CCRL1*	1.99	1.68E-05	1.98	1.08E-05
THC2740317	*unknown*	2.04	7.85E-06	1.75	2.63E-05
NM_182762	*MACC1*	2.05	5.66E-09	0.89	8.55E-05
XR_015158	*unknown*	2.15	6.40E-10	1.91	2.32E-09
NM_001547	*IFIT2*	2.16	4.68E-08	2.17	3.56E-08
NM_152737	*RNF182*	2.19	1.05E-05	1.78	5.97E-05
NM_014314	*DDX58*	2.22	3.99E-08	1.53	2.66E-06
AK022351	*unknown*	2.26	4.01E-05	2.74	2.66E-06
THC2526509	*unknown*	2.26	6.31E-08	1.19	7.99E-05

NM_181501	*ITGA1*	2.38	1.07E-08	1.09	8.38E-05
NM_018284	*GBP3*	2.40	1.80E-07	2.10	6.58E-07
AI078143	*unknown*	2.60	2.11E-07	1.52	7.46E-05
THC2510656	*unknown*	2.62	8.54E-06	2.91	1.51E-06
THC2549494	*unknown*	2.72	4.74E-06	2.48	8.65E-06
NM_020124	*IFNK*	2.74	7.98E-07	2.36	3.02E-06
NM_152703	*SAMD9L*	2.98	8.72E-06	2.94	6.15E-06
NM_005532	*IFI27*	3.03	3.42E-11	0.92	5.22E-05
NM_017654	*SAMD9*	3.04	1.08E-07	1.98	1.26E-05
NM_006417	*IFI44*	3.07	3.28E-12	2.71	7.74E-12
THC2679528	*unknown*	3.18	4.40E-07	2.89	9.26E-07
AK023743	*unknown*	3.18	1.52E-10	1.95	7.56E-08
NM_001548	*IFIT1*	3.28	6.99E-11	1.59	5.54E-07
BF213738	*unknown*	3.46	3.06E-11	1.34	2.66E-06
NM_006820	*IFI44L*	3.49	6.99E-11	2.28	1.49E-08
D89479	*SULT1B1*	3.56	1.70E-08	3.24	4.36E-08
NM_005127	*CLEC2B*	3.66	1.61E-13	1.20	1.33E-07
NM_017523	*XAF1*	5.11	1.98E-08	3.00	9.40E-06

Table S2: List of the most significant deregulated transcripts regarding MC1R status (p. R106W and p.R151C vs wild-type) identified in both comparisons.
Adjusted P-value < 0.0000001. Only genes identified in both analyses are included (N=159)
Abbreviations: FC: Fold Change; P-value: adjusted P-Value of such comparison; wt: wild-type.
*Statistical values of MC1R RHC vs wild-type in mutant CDKN2A skin cell (individual A1 vs individual B1).
**Statistical values of MC1R RHC vs wild-type in wild-type CDKN2A skin cell (individual A2 vs individual B2).

Gene ID	Gene Name	FC in mutant *CDKN2A**	P-value in mutant *CDKN2A**	FC in wt *CDKN2A***	P-value in wt *CDKN2A***
NM_005672	*PSCA*	-7.30	2.39E-12	-7.02	1.20E-12
NM_000550	*TYRP1*	-7.24	2.85E-12	-6.82	2.14E-12
NM_000095	*COMP*	-6.79	3.34E-13	-5.08	2.95E-12
NM_005511	*MLANA*	-5.74	2.51E-09	-6.96	7.05E-11
NM_000372	*TYR.TYRL*	-5.59	1.29E-12	-7.19	1.62E-14
NM_002443	*MSMB*	-5.30	1.03E-14	-4.05	1.47E-13
NM_006928	*SILV*	-4.79	1.66E-08	-6.05	3.02E-10
NM_000166	*GJB1*	-4.72	2.43E-10	-2.76	9.95E-08
NM_000014	*A2M*	-4.52	3.06E-08	-7.21	2.40E-11
NM_000698	*ALOX5*	-4.17	3.63E-11	-2.19	6.05E-08
NM_016190	*CRNN*	-4.14	8.50E-10	-4.68	5.89E-11
THC2711870	*unknown*	-3.98	8.16E-09	-6.00	1.27E-11
THC2675163	*unknown*	-3.73	6.81E-12	-6.37	5.96E-15
NM_032588	*TRIM63*	-3.70	6.23E-10	-5.36	1.70E-12
NM_004750	*CRLF1*	-3.70	1.31E-10	-2.14	6.30E-08
NM_013358	*PADI1*	-3.61	1.08E-08	-4.16	6.97E-10
ENST00000366930	*TGFB2*	-3.56	3.31E-10	-2.27	4.78E-08
NM_004864	*GDF15*	-3.31	8.19E-11	-4.23	1.09E-12
NM_020182	*PMEPA1*	-3.18	2.39E-12	-1.98	4.44E-10
NM_001712	*CEACAM1*	-3.12	6.03E-10	-3.36	7.97E-11

NM_005512	*LRRC32*	-3.11	8.78E-08	-3.21	2.37E-08
NM_144497	*AKAP12*	-3.10	8.04E-08	-2.91	7.23E-08
NM_130808	*CPNE4*	-2.97	4.58E-09	-2.69	6.33E-09
NM_001901	*CTGF*	-2.94	9.98E-10	-4.13	4.12E-12
NM_001039212	*unknown*	-2.87	9.12E-11	-3.14	9.05E-12
NM_024101	*MLPH*	-2.86	1.13E-08	-4.17	3.06E-11
ENST00000264554	*SHC2*	-2.80	1.77E-08	-2.97	3.32E-09
NM_000201	*ICAM1*	-2.75	2.83E-09	-2.45	4.99E-09
NM_000222	*KIT*	-2.75	6.97E-09	-5.47	3.15E-13
NM_201631	*TGM5*	-2.69	6.67E-09	-2.57	4.59E-09
NM_002193	*INHBB*	-2.61	3.47E-10	-2.46	2.87E-10
NM_001001547	*CD36*	-2.60	1.37E-08	-2.58	6.28E-09
NM_001945	*HBEGF*	-2.59	4.84E-08	-2.56	2.20E-08
NM_002463	*MX2*	-2.59	2.72E-09	-5.66	4.69E-14
NM_173198	*NR4A3*	-2.45	2.44E-10	-4.42	5.15E-14
NM_001343	*DAB2*	-2.42	2.64E-08	-3.27	1.92E-10
NM_138375	*CABLES1*	-2.23	1.35E-09	-3.46	1.49E-12
NM_001012964	*KLK6*	-2.19	2.71E-09	-4.55	8.27E-14
NM_016463	*CXXC5*	-2.10	3.00E-08	-2.26	4.70E-09
NM_001453	*FOXC1*	-1.94	1.02E-09	-1.72	2.04E-09
NM_003793	*CTSF*	-1.92	4.54E-08	-3.15	2.38E-11
NM_022910	*NDRG4*	-1.84	9.53E-09	-1.55	3.44E-08
NM_006989	*RASA4*	-1.67	7.49E-08	-2.15	1.08E-09
NM_145804	*ABTB2*	-1.45	7.91E-08	-2.40	3.58E-11
NM_003565	*ULK1*	-1.39	7.35E-08	-1.75	1.36E-09
NM_030912	*TRIM8*	-1.25	5.80E-08	-1.13	8.84E-08
NM_206836	*PECI*	1.33	6.12E-08	1.23	6.13E-08
NM_005915	*MCM6*	1.33	9.39E-08	1.69	1.62E-09
AY163812	*C17orf80*	1.34	6.62E-08	1.47	7.72E-09
CR600585	*unknown*	1.45	8.28E-09	1.35	7.88E-09
NM_033514	*LIMS3*	1.46	3.69E-08	1.42	2.13E-08
NM_016126	*HSPB11*	1.47	6.42E-08	1.48	2.29E-08
NM_001079525	*PAICS*	1.50	1.12E-08	2.03	7.45E-11
NM_016448	*DTL*	1.53	5.00E-09	1.85	1.41E-10
NM_003579	*RAD54L*	1.54	6.67E-09	1.87	1.86E-10
NM_032636	*PSRC1*	1.59	5.94E-08	1.67	1.21E-08
NM_033417	*HAUS8*	1.59	6.77E-08	1.71	1.05E-08
NM_018455	*CENPN*	1.61	2.25E-08	2.40	4.13E-11
NM_030771	*CCDC34*	1.64	3.02E-09	3.26	1.47E-13
NM_017868	*TTC12*	1.67	7.74E-10	2.18	7.74E-12
NM_003258	*TK1*	1.72	8.63E-08	1.83	1.53E-08
NM_003504	*CDC45L*	1.75	6.55E-09	1.99	4.57E-10
NM_002916	*RFC4*	1.76	1.32E-09	2.19	2.50E-11
ENST00000367003	*C1orf97*	1.77	2.25E-08	2.65	4.11E-11
NM_145231	*C14orf143*	1.84	1.04E-08	1.86	3.46E-09
NM_003798	*CTNNAL1*	1.85	2.44E-10	1.94	4.44E-11
NM_014317	*PDSS1*	1.86	3.58E-08	2.19	1.71E-09
NM_004219	*PTTG1*	1.87	9.03E-09	1.92	2.48E-09
NM_001159	*AOX1*	1.87	8.16E-09	2.23	3.05E-10
NM_018186	*C1orf112*	1.94	5.99E-09	1.94	2.12E-09
NM_001826	*CKS1B*	1.96	5.59E-09	2.00	1.55E-09
BX640843	*unknown*	1.99	5.94E-09	2.51	9.73E-11

NM_182620	*FAM33A*	2.01	1.76E-09	1.72	5.18E-09
NM_003686	*EXO1*	2.01	2.93E-10	2.19	3.22E-11
NM_004282	*BAG2*	2.02	9.45E-10	2.61	1.14E-11
NM_014051	*TMEM14A*	2.09	1.45E-08	2.14	4.46E-09
NM_016095	*GINS2*	2.12	7.82E-10	2.14	2.35E-10
NM_145014	*HYLS1*	2.13	5.04E-08	2.66	1.08E-09
NM_024037	*C1orf135*	2.13	8.41E-10	2.22	1.78E-10
NM_003384	*VRK1*	2.15	6.03E-10	2.37	5.77E-11
BC040051	*unknown*	2.15	5.79E-08	2.00	5.83E-08
NM_001254	*CDC6*	2.20	8.16E-08	2.57	4.42E-09
NM_014176	*UBE2T*	2.23	1.12E-11	2.18	5.39E-12
NM_203467	*PPIL5*	2.23	9.58E-10	1.82	5.22E-09
NM_000057	*BLM*	2.26	7.59E-08	2.85	1.45E-09
NM_024808	*C13orf34*	2.27	8.38E-08	2.54	7.74E-09
NM_006607	*PTTG2*	2.27	2.24E-10	1.92	7.20E-10
NM_001928	*CFD*	2.30	3.69E-08	2.45	6.65E-09
NM_015895	*GMNN*	2.31	1.29E-10	1.77	1.51E-09
NM_018101	*CDCA8*	2.34	7.82E-10	2.36	2.52E-10
AK027541	*unknown*	2.35	2.05E-08	2.34	8.84E-09
NM_001766	*CD1D*	2.36	6.03E-09	2.53	9.33E-10
NM_002876	*RAD51C*	2.37	4.47E-10	2.20	4.50E-10
NM_000946	*PRIM1*	2.38	2.14E-09	2.22	1.94E-09
NM_005030	*PLK1*	2.40	8.38E-08	2.41	3.33E-08
NM_031299	*CDCA3*	2.41	2.46E-09	2.09	6.11E-09
NM_012415	*RAD54B*	2.43	6.39E-08	2.34	4.24E-08
NM_006479	*RAD51AP1*	2.46	3.02E-08	3.80	3.48E-11
NM_138555	*KIF23*	2.48	8.16E-09	2.87	4.69E-10
NM_018193	*FANCI*	2.50	4.23E-11	2.38	2.70E-11
NM_014791	*MELK*	2.50	1.91E-09	2.50	7.39E-10
NM_175617	*MT1E*	2.52	6.20E-10	2.12	2.36E-09
NM_012177	*FBXO5*	2.54	1.32E-09	2.45	8.26E-10
NM_001012271	*BIRC5*	2.55	9.58E-10	2.63	2.35E-10
NM_001012267	*CENPP*	2.56	2.85E-12	1.76	1.78E-10
NM_001827	*CKS2*	2.57	1.36E-10	2.22	3.37E-10
BC044246	*TMEM200A*	2.57	7.02E-09	2.04	5.61E-08
NM_012310	*KIF4A*	2.57	3.81E-09	2.48	2.30E-09
NM_199420	*POLQ*	2.58	6.02E-08	3.45	5.20E-10
NM_001274	*CHEK1*	2.58	1.29E-10	3.00	5.77E-12
NM_001005414	*unknown*	2.62	6.03E-10	1.95	1.10E-08
NM_182751	*MCM10*	2.62	2.54E-08	3.10	1.09E-09
NM_001010897	*SERP2*	2.65	3.23E-09	2.27	9.59E-09
NM_001034	*RRM2*	2.67	1.05E-08	2.47	1.16E-08
NM_145018	*C11orf82*	2.71	1.68E-10	2.95	1.85E-11
NM_031966	*CCNB1*	2.74	2.63E-11	2.84	5.39E-12
NM_005059	*RLN2*	2.78	9.81E-09	2.58	1.05E-08
NM_006845	*KIF2C*	2.80	1.74E-09	2.78	7.12E-10
NM_001211	*BUB1B*	2.81	4.88E-08	3.45	1.32E-09
NM_198433	*AURKA*	2.85	1.71E-08	2.81	8.31E-09
NM_004701	*CCNB2*	2.92	2.85E-12	3.11	5.71E-13
NM_020242	*KIF15*	2.93	5.10E-09	4.62	4.38E-12
NM_004523	*KIF11*	2.96	1.80E-08	3.18	2.81E-09
NM_001255	*CDC20*	3.05	4.70E-09	2.92	3.13E-09

BC013418	*C13orf3*	3.10	3.10E-09	3.42	3.24E-10
AL834537	*CCDC150*	3.11	3.65E-08	4.61	7.45E-11
NM_004336	*BUB1*	3.13	2.15E-10	3.42	2.35E-11
NM_004117	*FKBP5*	3.14	4.53E-08	3.43	5.67E-09
NM_052913	*TMEM200A*	3.14	8.40E-08	2.96	7.23E-08
NM_001067	*TOP2A*	3.14	9.65E-11	3.93	1.70E-12
NM_005733	*KIF20A*	3.15	1.78E-11	2.96	1.38E-11
NM_001813	*CENPE*	3.18	6.03E-10	4.69	1.24E-12
NM_152515	*CKAP2L*	3.18	8.97E-08	3.74	4.49E-09
NM_001237	*CCNA2*	3.19	2.93E-10	3.25	8.24E-11
NM_018136	*ASPM*	3.20	1.92E-09	3.55	1.78E-10
NM_007280	*OIP5*	3.21	1.51E-11	2.87	2.37E-11
NM_138419	*FAM54A*	3.23	3.04E-11	3.07	2.01E-11
NM_020675	*SPC25*	3.26	1.59E-12	3.45	1.87E-13
NM_018492	*PBK*	3.28	3.26E-09	3.95	9.92E-11
NM_022346	*NCAPG*	3.29	5.95E-13	3.50	7.81E-14
NM_024094	*DSCC1*	3.31	3.47E-10	3.99	1.02E-11
NM_032117	*MND1*	3.35	6.71E-12	3.77	5.71E-13
NM_001809	*CENPA*	3.38	7.61E-11	3.90	3.72E-12
NM_001786	*CDC2*	3.38	2.39E-12	3.27	1.24E-12
NM_145697	*NUF2*	3.40	8.28E-09	4.81	3.22E-11
NM_021186	*ZP4*	3.49	1.03E-14	4.91	3.21E-17
NM_016343	*CENPF*	3.52	1.25E-09	2.85	7.88E-09
NM_001790	*CDC25C*	3.60	3.47E-08	3.67	1.06E-08
NM_018131	*CEP55*	3.63	4.13E-10	3.89	5.89E-11
NM_016195	*KIF20B*	3.68	4.08E-10	2.62	1.36E-08
NM_014750	*DLGAP5*	3.71	2.39E-12	4.17	1.87E-13
NM_005192	*CDKN3*	4.04	7.94E-08	4.03	3.28E-08
NM_018365	*MNS1*	4.09	5.55E-10	3.31	3.38E-09
NM_012484	*HMMR*	4.51	2.85E-12	5.25	1.87E-13
NM_080672	*PHACTR3*	4.53	8.83E-10	3.81	3.34E-09
NM_001956	*EDN2*	4.78	2.38E-09	4.67	1.19E-09
NM_153046	*TDRD9*	4.83	1.87E-12	4.59	1.08E-12
NM_032935	*MT4*	5.04	2.85E-12	5.54	4.26E-13
BC004565	*unknown*	5.05	1.16E-11	2.65	2.23E-08

Table S3: List of genes inversely deregulated between *CDKN2A* and *MC1R* analyses. Abbreviations: F.C: Fold Change; P-value: adjusted P-Value of such comparison; wt: wild-type.

		mutant CDKN2A vs wt CDKN2A (both RHC MC1R)		mutant CDKN2A vs wt CDKN2A (both wt MC1R)		RHC MC1R vs wt MC1R (both mutant CDKN2A)		RHC MC1R vs wt MC1R (both wt CDKN2A)	
HGNC_symbol	**Ensembl_link**	**log F.C**	**adj.p.value**	**log F.C**	**adj.p.value**	**log F.C**	**adj.p.value**	**log F.C**	**adj.p.value**
XAF1	NM_017523	5.11	1.98E-08	3.00	9.40E-06	-1.11	0.041921124	-3.22	1.59E-06
MX2	NM_002463	4.14	1.32E-11	1.06	0.000133758	-2.59	2.72E-09	-5.66	4.69E-14
IFI44L	NM_006820	3.49	6.99E-11	2.28	1.49E-08	-0.93	0.000400236	-2.14	9.59E-09
IFIT3	NM_001549	3.26	8.18E-09	1.26	0.00036059	-1.57	3.37E-05	-3.57	5.21E-10
CAPN14	AK092257	3.25	3.91E-05	2.20	0.00113099	-1.37	0.032844763	-2.42	0.0002558
	AK023743	3.18	1.52E-10	1.95	7.56E-08	-0.49	0.039372674	-1.71	1.27E-07
IFI44	NM_006417	3.07	3.28E-12	2.71	7.74E-12	-0.76	5.31E-05	-1.12	2.60E-07
KRT19	NM_002276	2.86	3.59E-10	0.81	0.000744197	-1.31	4.49E-06	-3.36	8.78E-12
IFNK	NM_020124	2.74	7.98E-07	2.36	3.02E-06	-1.04	0.007725554	-1.43	0.000326988
IFI6	NM_022873	2.56	3.45E-08	1.05	0.000601236	-0.79	0.00606156	-2.30	2.96E-08
BTN3A3	NM_006994	2.42	1.07E-05	1.55	0.000669858	-1.08	0.011856791	-1.95	3.31E-05
LMO2	NM_005574	2.41	1.43E-10	0.79	1.00E-04	-1.12	1.67E-06	-2.75	5.51E-12
GBP3	NM_018284	2.40	1.80E-07	2.10	6.58E-07	-0.70	0.020231435	-1.00	0.000961856
RNF182	NM_152737	2.19	1.05E-05	1.78	5.97E-05	-0.93	0.015597153	-1.33	0.000585284
RHOBTB3	NM_014899	2.17	1.04E-05	1.01	0.008209935	-1.98	1.59E-05	-3.14	2.33E-08
IFIT2	NM_001547	2.16	4.68E-08	2.17	3.56E-08	-1.48	3.08E-06	-1.47	1.46E-06
USP18	NM_017414	2.14	1.55E-08	0.52	0.018990885	-0.69	0.002714314	-2.31	1.21E-09
EPSTI1	NM_033255	2.12	3.99E-05	1.77	0.000160602	-1.70	0.0002321	-2.05	1.60E-05
	A_32_P53976	2.01	3.17E-05	1.10	0.005270514	-0.86	0.026819981	-1.77	3.55E-05
RSAD2	NM_080657	1.96	0.001691807	1.44	0.012103521	-2.71	4.60E-05	-3.23	2.96E-06
	AK023660	1.96	4.65E-08	0.92	0.000195518	-0.81	0.000669435	-1.85	2.23E-08
	THC2676635	1.93	2.93E-08	1.27	3.49E-06	-0.86	0.000221405	-1.52	1.43E-07
CLCA3	NM_004921	1.86	8.41E-05	1.74	9.87E-05	-1.54	0.000333218	-1.65	8.58E-05
PARP9	NM_031458	1.83	2.21E-06	1.21	0.000138412	-0.63	0.024137843	-1.26	4.46E-05
SCG5	NM_003020	1.83	2.10E-08	2.37	5.14E-10	-1.87	8.28E-09	-1.32	2.93E-07
HS3ST1	NM_005114	1.80	2.00E-05	1.12	0.001411596	-1.00	0.003458263	-1.69	1.14E-05
CEACAM1	NM_001712	1.80	1.35E-06	1.56	4.49E-06	-3.12	6.03E-10	-3.36	7.97E-11
OAS1	NM_002534	1.76	0.000121473	0.79	0.039773363	-1.04	0.007622258	-2.01	7.90E-06
	L06175	1.75	0.000889078	1.36	0.004950943	-1.82	0.000403092	-2.22	2.73E-05
	AK124281	1.75	5.68E-05	1.40	0.00033138	-0.90	0.011971445	-1.25	0.000538014
C5orf56	ENST00000378953	1.73	0.000157045	0.84	0.030463445	-1.03	0.008749111	-1.93	1.47E-05
CRNN	NM_016190	1.67	0.000105303	1.13	0.002501087	-4.14	8.50E-10	-4.68	5.89E-11
	AK025669	1.67	0.000315954	1.77	0.000112469	-1.00	0.013197622	-0.90	0.017900911
PADI1	NM_013358	1.66	0.000201315	1.12	0.004289502	-3.61	1.08E-08	-4.16	6.97E-10
	THC2640099	1.63	0.003962305	1.41	0.008167973	-1.45	0.006843443	-1.67	0.001407815
TNFSF10	NM_003810	1.61	3.76E-05	1.04	0.001525958	-1.37	0.000112711	-1.93	1.06E-06
F3	NM_001993	1.56	0.007308907	1.28	0.019400875	-1.14	0.039539591	-1.42	0.007227404
IL15	NM_172174	1.55	5.30E-08	1.29	4.08E-07	-0.82	5.92E-05	-1.09	1.10E-06
SP110	NM_004510	1.53	1.66E-07	0.94	3.63E-05	-0.67	0.000916414	-1.26	4.53E-07
BST2	NM_004335	1.49	0.00349873	1.52	0.00211498	-3.36	4.60E-07	-3.33	2.09E-07
CLCA4	NM_012128	1.49	0.001612926	1.67	0.000362464	-1.67	0.000354497	-1.48	0.000637245

	BX090412	1.48	0.001281109	1.47	0.000930605	-0.87	0.037340541	-0.88	0.025448576
	BC045163	1.45	3.62E-05	0.58	0.040103551	-1.39	3.12E-05	-2.25	3.73E-08
HOXA10	NM_018951	1.43	4.44E-06	0.86	0.000579266	-0.53	0.022134445	-1.10	2.33E-05
TLR2	NM_003264	1.42	0.000310032	1.80	1.61E-05	-2.33	7.17E-07	-1.95	2.78E-06
	THC2657493	1.38	0.000134482	1.47	4.25E-05	-2.16	4.46E-07	-2.07	3.14E-07
OASL	NM_003733	1.34	1.66E-05	0.58	0.01676382	-0.78	0.001967307	-1.54	7.42E-07
	CR625594	1.34	0.000464881	1.05	0.002484941	-0.70	0.03890031	-0.98	0.002738176
PSCA	NM_005672	1.32	0.002415497	1.59	0.000301512	-7.30	2.39E-12	-7.02	1.20E-12
	A_24_P476718	1.31	0.000870782	2.24	1.89E-06	-1.93	9.86E-06	-1.00	0.00360047
SPINK1	NM_003122	1.29	0.005034924	0.96	0.024954425	-3.40	1.10E-07	-3.73	1.34E-08
	CR612518	1.28	0.006785661	1.14	0.011308172	-0.96	0.032090928	-1.11	0.009489293
	A_32_P169353	1.20	0.045002273	1.48	0.010727271	-2.84	2.98E-05	-2.56	4.67E-05
	BC062324	1.19	0.01579769	1.22	0.010153956	-1.67	0.00082046	-1.64	0.000563989
DHX58	NM_024119	1.18	5.99E-06	0.71	0.000741093	-0.68	0.001151375	-1.15	2.09E-06
	BM989848	1.16	0.011329228	0.98	0.023865075	-0.89	0.042562201	-1.07	0.010191815
MDK	NM_001012334	1.16	6.19E-05	1.20	2.48E-05	-1.55	1.14E-06	-1.51	7.03E-07
S100A4	NM_002961	1.12	0.000446466	0.72	0.010561535	-0.77	0.007225432	-1.17	0.000100805
BTN3A2	NM_007047	1.09	1.77E-05	1.11	9.31E-06	-0.57	0.00559599	-0.55	0.004476681
ALDH1L1	NM_012190	1.09	0.006504537	1.35	0.000801896	-1.46	0.000386161	-1.20	0.001386589
SEPP1	NM_005410	1.08	0.017795719	2.85	1.26E-06	-2.78	1.49E-06	-1.00	0.01532286
	THC2671679	1.07	3.76E-05	0.73	0.001017835	-1.24	3.55E-06	-1.58	7.50E-08
HCP5	NM_006674	1.06	0.007759651	1.33	0.000898615	-1.84	3.33E-05	-1.57	9.87E-05
	AK026517	1.05	0.002902491	1.06	0.001900336	-0.80	0.015527585	-0.79	0.011579443
	THC2754005	1.02	0.000456872	1.29	2.63E-05	-1.03	0.000244525	-0.77	0.002241279
ITGA2	NM_002203	1.01	0.022552265	0.94	0.026376749	-1.06	0.012631859	-1.13	0.005479826
WNT7A	NM_004625	0.99	0.000464881	0.53	0.033957447	-1.77	4.27E-07	-2.24	8.20E-09
NEBL	NM_006393	0.99	3.91E-05	0.73	0.000558386	-0.52	0.008729268	-0.78	0.000141682
PLAT	NM_000930	0.98	0.000875467	1.35	2.25E-05	-2.14	8.14E-08	-1.77	3.78E-07
EIF2AK2	NM_002759	0.98	0.020075442	1.52	0.000512242	-1.38	0.001188256	-0.84	0.027870165
	AF086052	0.98	0.009230344	1.53	0.000138634	-1.38	0.000353702	-0.83	0.013843783
ALDH1A3	NM_000693	0.96	0.000531565	0.60	0.014677759	-1.10	8.25E-05	-1.46	1.54E-06
	BC056662	0.96	0.014386956	1.04	0.006439966	-1.04	0.006569774	-0.96	0.007518063
FMO2	NM_001460	0.96	0.037434212	2.94	1.09E-06	-3.92	2.36E-08	-1.94	5.37E-05
	A_32_P35031	0.95	0.000214111	0.62	0.005152449	-0.59	0.00820823	-0.91	0.000104052
	K03200	0.93	0.005429992	0.89	0.005220606	-1.51	3.96E-05	-1.55	1.55E-05
	AK130514	0.91	0.017250913	0.93	0.011913737	-1.39	0.000462002	-1.38	0.00028677
ZSCAN4	NM_152677	0.90	0.014937773	1.41	0.000279708	-1.54	0.000105108	-1.03	0.002697114
APOL6	NM_030641	0.89	0.018487069	1.35	0.000505697	-1.98	8.89E-06	-1.51	9.06E-05
STS	NM_000351	0.87	0.014650689	1.50	0.000108672	-2.58	1.92E-07	-1.95	2.61E-06
	THC2543120	0.86	0.009788415	1.06	0.001361535	-0.83	0.009051891	-0.63	0.035366686
PPARG	NM_138711	0.86	0.001039611	1.19	2.54E-05	-0.84	0.000819453	-0.50	0.023893587
IFITM1	NM_003641	0.85	0.002663548	0.62	0.018223872	-0.67	0.012012196	-0.90	0.000693909
	CA314451	0.84	0.016043784	0.89	0.00853883	-1.83	9.45E-06	-1.79	6.00E-06
	THC2551769	0.84	0.004358863	0.73	0.008331505	-0.69	0.012821531	-0.79	0.003004577
ENC1	NM_003633	0.83	0.002044084	1.05	0.000153929	-1.96	1.05E-07	-1.74	2.01E-07
KCNN4	NM_002250	0.82	0.006830609	1.03	0.000785576	-1.34	5.13E-05	-1.14	0.000158723
SPINK5	NM_006846	0.76	0.001825778	0.47	0.033705902	-0.70	0.002501485	-0.99	5.08E-05

MSMB	NM_002443	0.73	0.000832073	1.96	6.81E-09	-5.28	1.03E-14	-4.05	1.47E-13
PARP12	NM_022750	0.73	0.001230432	0.63	0.002667999	-1.18	4.95E-06	-1.27	8.66E-07
	THC2545454	0.71	0.02260588	0.95	0.002276893	-0.95	0.002311169	-0.71	0.012721232
LYN	NM_002350	0.71	0.003114957	1.27	7.07E-06	-1.32	3.92E-06	-0.76	0.000708175
	AK022020	0.71	0.004745127	0.59	0.012998879	-1.12	4.23E-05	-1.25	6.53E-06
LAYN	NM_178834	0.71	0.01514181	1.04	0.000500006	-1.28	5.82E-05	-0.95	0.000706483
PPP1R12B	NM_032105	0.68	0.009014906	0.68	0.00660204	-0.86	0.00096841	-0.86	0.000563989
ATP1B1	NM_001677	0.67	0.040190654	0.78	0.013887884	-0.84	0.008541899	-0.73	0.014330567
CAMK2G	NM_172171	0.66	0.009630083	0.77	0.001946373	-0.83	0.001095948	-0.71	0.002542575
	AK023696	0.64	0.030845413	0.63	0.025358095	-0.64	0.025179173	-0.64	0.017239992
TMEM132A	NM_017870	0.60	0.015740285	0.64	0.007383258	-1.72	3.16E-07	-1.68	1.76E-07
TRIM2	NM_015271	0.59	0.004377494	0.42	0.029710532	-0.50	0.009901857	-0.67	0.000579911
LRAT	NM_004744	0.51	0.043998571	0.78	0.00198053	-1.46	2.88E-06	-1.19	1.50E-05
CCNDBP1	NM_037370	0.48	0.009949949	0.67	0.00049834	-0.61	0.001068743	-0.43	0.01171219
SMAD3	U68019	0.45	0.047091148	0.52	0.017762701	-0.74	0.001205253	-0.68	0.001635126
TMEM51	NM_018022	0.45	0.028015892	0.43	0.029372403	-0.56	0.005094908	-0.58	0.002388652
	AK025166	0.39	0.028250766	0.37	0.030166685	-0.45	0.008515843	-0.47	0.004029612
NPEPPS	NM_006310	0.38	0.044356931	0.45	0.014074734	-0.79	0.000114668	-0.72	0.000155666
ALG9	NM_001077691	-0.31	0.031798838	-0.29	0.034627663	0.28	0.044702714	0.30	0.023463075
MPP1	NM_002436	-0.42	0.047666353	-0.78	0.000483518	1.51	2.71E-07	1.16	3.00E-06
MYO19	NM_001033580	-0.43	0.026532957	-1.00	1.21E-05	0.98	1.38E-05	0.41	0.02114696
FANCC	NM_000136	-0.51	0.011609739	-0.47	0.014584719	0.48	0.012959276	0.52	0.004986322
OVOL2	NM_021220	-0.52	0.001084197	-0.39	0.007292061	0.47	0.001844654	0.60	0.000100716
SIN3B	BC025026	-0.57	0.011569948	-0.75	0.00103124	1.01	5.47E-05	0.83	0.000227367
CDT1	NM_030928	-0.58	0.019198248	-0.90	0.000479465	1.44	2.66E-06	1.13	2.30E-05
PON3	NM_000940	-0.62	0.005105333	-0.84	0.000276722	0.93	8.48E-05	0.72	0.000647845
ADRB2	NM_000024	-0.63	0.001524882	-0.73	0.000236914	0.59	0.001792781	0.48	0.005629305
PLK1	NM_005030	-0.63	0.042808398	-0.63	0.034901844	2.40	8.38E-08	2.41	3.33E-08
CTSK	NM_000396	-0.74	7.83E-05	-1.81	1.05E-09	1.64	2.75E-09	0.58	0.000331696
ASF1B	NM_018154	-0.76	0.048590758	-0.83	0.022912812	1.00	0.007299284	0.92	0.008832468
NMNAT3	NM_178177	-0.76	0.000552736	-0.87	9.23E-05	0.76	0.000345671	0.65	0.000881113
REEP2	NM_016606	-0.78	0.005759911	-0.93	0.001039901	1.69	1.66E-06	1.54	2.21E-06
ABCF2	NM_007189	-0.80	0.000944624	-0.74	0.001305162	0.60	0.00667932	0.66	0.001912744
LMCD1	NM_014583	-0.95	0.002645754	-0.76	0.010312363	0.74	0.012418953	0.93	0.001307708
NRP2	NM_201264	-0.97	0.002390052	-0.63	0.031835409	1.17	0.000297324	1.51	9.74E-06
ST7OT1	NR_002330	-0.99	0.000144723	-0.44	0.048099669	0.80	0.00068922	1.36	1.07E-06
PKMYT1	NM_182687	-0.99	0.001983455	-0.74	0.012491031	1.23	0.000180492	1.48	1.20E-05
CPT1C	NM_152359	-1.08	1.08E-05	-1.48	1.50E-07	0.94	2.56E-05	0.54	0.003391166
BEX5	NM_001012978	-1.26	0.001348714	-0.76	0.032521061	2.00	7.09E-06	2.50	2.08E-07
DHRS13	NM_144683	-1.45	0.00088615	-1.72	0.000114616	1.32	0.001366222	1.05	0.005333513
CLDN17	NM_012131	-1.47	4.17E-05	-1.79	2.57E-06	1.34	6.23E-05	1.01	0.000593411
STAC	NM_003149	-1.57	0.001093743	-1.23	0.005257075	0.88	0.043609023	1.22	0.003788264
CD248	NM_020404	-2.04	2.85E-05	-1.14	0.004130168	0.77	0.049370648	1.67	7.16E-05

TRABAJO II

RESUMEN TRABAJO II

Distribution of *MC1R* variants among melanoma subtypes: p.R163Q is associated with Lentigo Maligna Melanoma in a Mediterranean population.

Joan Anton Puig-Butillé, Cristina Carrera, Rajive Kumar, Zaida Garcia-Casado, Celia Badenas, Paula Aguilera, Josep Malvehy, Eduardo Nagore, Susana Puig.

Br J Dermatol. 2013 Oct;169(4):804-11. *Factor de impacto: 3.759*

Objetivo

Analizar el papel de variantes del gen *MC1R* en el riesgo a desarrollar un tipo de melanoma con características histopatológicas determinadas.

Metodología

Es un estudio retrospectivo observacional de pacientes de melanoma de dos series de hospitales de los cuales se tenía confirmación histopatológica del tumor. Se incluyeron pacientes con múltiples melanomas primarios de los cuales se conocía el subtipo histopatológico del total de tumores que desarrollaron y el tiempo transcurrido entre los diagnósticos de los distintos melanomas primarios. De todos los pacientes se disponía de ADN germinal y se recogieron la información fenotípica, sexo y edad de debut.

En todos los pacientes se procedió al análisis molecular del gen *MC1R* mediante PCR y secuenciación Sanger. El impacto de las variantes detectadas se analizó *in-silico* mediante el programa de predicción PolyPhen-2 v2.2.2; y se clasificaron según los criterios publicados previamente en: “variantes de color de pelo rojo” y “variantes no de color de pelor rojo”.

En el análisis del número total de variantes y el subtipo histopatológico, de los pacientes con múltiples melanomas primarios se incluyó exclusivamente la información del primer melanoma. Por el contrario, en el análisis especifico para cada variante, estos pacientes se reclasificaron según si habían desarrollado o no, como mínimo un melanoma del subtipo que se analizaba. Para cada variante se

analizó si cumplían el equilibrio de Hardy-Weinberg y se calcularon las frecuencias alélicas y genotípicas. La correlación entre el número de variantes y las variables a estudiar se calculó mediante tablas de contingencia y el test $\chi 2$ de Pearson. Mediante modelos de regresión múltiple se determinó las variantes asociadas a las características fenotípicas y el riesgo a desarrollar un subtipo especifico de melanoma.

Resultados

Se incluyeron 1679 pacientes de los cuales 85% desarrollaron un único melanoma y 15% desarrollaron múltiples melanomas primarios. El subgrupo de pacientes con un único melanoma incluye, según el subtipo histopatológico, 979 pacientes que desarrollaron melanoma de extensión superficial (SSM; 68,6%), 249 pacientes melanoma nodular (NM; 17,4%), 118 pacientes melanoma lentiginoso maligno (LMM; 8,3%) y 82 pacientes desarrollaron un melanoma lentiginoso acral (ALM; 5,7%). El subgrupo de pacientes que desarrollaron múltiples melanomas primarios comprende un total de 588 tumores, de los cuales, 83,5% son SSM, 6,8% NM, 8,2% LMM y 1,5% ALM.

De forma global, se identifican 53 variantes alélicas en el gen *MC1R* (11 variantes sinónimas y 42 variantes no sinónimas); la mayoría de ellas detectadas en pocos o en un único paciente. Se han identificado 13 variantes no descritas previamente. Del total de variantes, 10 variantes no sinónimas tienen una frecuencia superior al 1% de casos: p.V60L (30%), p.V92M (11,7%), p.D294H (9,4%), p.R151C (8,8%), p.R160W (6,2%), p.R163Q (4,2%), p.R142H (3,3%), p.I155T (3,8%), p.V122M (1,5%) y p.D84E (1,0%). Los análisis de las características fenotípicas o subtipo histopatológico del tumor se llevan a cabo exclusivamente en éstas 10 variantes.

En el estudio se han detectado asociaciones previamente descritas como, el riesgo a color de pelo rojo y/o pieles claras de portadores de las variantes p.R142H, p.R151C, p.R160W y p.D294H o en individuos portadores de 2 o más variantes totales. Por otro lado, se detecta un incremento del riesgo color de ojos claro causado por la variante p.R142H, bajo un modelo de herencia dominante (OR=2,07; IC95%=1,18-3,65; p-valor=0,011).

El estudio revela que los subtipos histopatológicos de melanoma difieren en cuanto al número de variantes totales del gen *MC1R* (p-valor=0,028) o el número de variantes de color rojo (p-valor=0,035). Los subtipos de melanoma LMM y SSM presentan un mayor número de variantes totales o variantes de pelo rojo comparado con los subtipos NM o ALM. Por último, se observa que los portadores de la variante p.R163Q tienen, aproximadamente el doble de riesgo a desarrollar LMM bajo un modelo de herencia dominante (OR= 2,16; IC95%= 1,07-4,37; p-valor=0,044).

BJD
British Journal of Dermatology

Distribution of *MC1R* variants among melanoma subtypes: p.R163Q is associated with lentigo maligna melanoma in a Mediterranean population

J.A. Puig-Butillé,[1,2] C. Carrera,[1,3] R. Kumar,[4] Z. Garcia-Casado,[5] C. Badenas,[1,2] P. Aguilera,[1,3] J. Malvehy,[1,3] E. Nagore[6] and S. Puig[1,3]

[1]Centro Investigación Biomédica en Red en Enfermedades Raras (CIBERER), Instituto de Salud Carlos III, Barcelona, Spain
[2]Biochemical and Molecular Genetics Service and [3]Department of Dermatology, Melanoma Unit, Hospital Clinic and IDIBAPS (Institut d'Investigacions Biomèdiques Agustí Pi i Sunyer), Barcelona, Spain
[4]Division of Molecular Genetic Epidemiology, German Cancer Research Center, Heidelberg, Germany
[5]Department of Molecular Biology and [6]Department of Dermatology, Instituto Valenciano de Oncología, Valencia, Spain

Correspondence
Susana Puig.
E-mails: susipuig@gmail.com; spuig@clinic.ub.es

Accepted for publication
30 April 2013

Funding sources
The research at the Melanoma Unit in Barcelona was partially funded by Grants 03/0019, 05/0302, 06/0265 and 09/01393 from Fondo de Investigaciones Sanitarias, Spain; by the CIBER de Enfermedades Raras of the Instituto de Salud Carlos III, Spain; by the AGAUR 2009 SGR 1337 of the Catalan Government, Spain; by the European Commission under the 6th Framework Programme, contract no. LSHC-CT-2006-018702 (GenoMEL) and by the National Cancer Institute of the U.S. National Institutes of Health (CA83115). The work was carried out at the Esther Koplowitz Centre, Barcelona, Spain. The samples from the Instituto Valenciano de Oncología were collected from the Biobanco del Instituto Valenciano de Oncología.

Conflicts of interest
None declared.

DOI 10.1111/bjd.12418

Summary

Background Cutaneous melanoma tumour is classified into clinicohistopathological subtypes that may be associated with different genetic and host factors. Variation in the MC1R gene is one of the main factors of risk variation in sporadic melanoma. The relationship between MC1R variants and the risk of developing a specific subtype of melanoma has not been previously explored.
Objectives To analyse whether certain MC1R variants are associated with particular melanoma subtypes with specific clinicohistopathological features.
Methods An association study was performed between MC1R gene variants and clinicopathological subtypes of primary melanoma derived from 1679 patients.
Results We detected 53 MC1R variants (11 synonymous and 42 nonsynonymous). Recurrent nonsynonymous variants were p.V60L (30·0%), p.V92M (11·7%), p.D294H (9·4%), p.R151C (8·8%), p.R160W (6·2%), p.R163Q (4·2%) p.R142H (3·3%), p.I155T (3·8%), p.V122M (1·5%) and p.D84E (1·0%). Melanoma subtypes showed differences in the total number of MC1R variants ($P = 0·028$) and the number of red hair colour variants ($P = 0·035$). Furthermore, an association between p.R163Q and lentigo maligna melanoma was detected under a dominant model of heritance (odds ratio 2·16, 95% confidence interval 1·07–4·37; $P = 0·044$). No association was found between p.R163Q and Fitzpatrick skin phototype, eye colour or skin colour, indicating that the association was independent of the role of MC1R in pigmentation. No association was observed between MC1R polymorphisms and other melanoma subtypes.
Conclusions Our findings suggest that certain MC1R variants could increase melanoma risk due to their impact on pathways other than pigmentation, and may therefore be linked to specific melanoma subtypes.

What's already known about this topic?

- The MC1R gene plays a role in pigmentation synthesis and inflammatory processes, and activates the mitogen-activated protein kinase pathway.
- MC1R variants associated with pigmentation increase the risk of developing melanoma and nonmelanoma skin cancer.
- The p.R163Q variant, not associated with pigmentation, is associated with nonmel-

What does this study add?

- The p.R163Q variant, which is not directly associated with phenotype variation, is associated for the first time with the risk of developing lentigo maligna melanoma.

Cutaneous malignant melanoma (MM) has previously been classified into distinct subtypes based on histological appearance, biological behaviour and epidemiological features.[1,2] Later this classification lost relevance because there was often a significant overlap between types, and it lacked prognostic value. Nevertheless, some of these variants show characteristic clinical features and may be associated with different risk factors. Differences according to the anatomical location of the primary tumour, the ultraviolet (UV) pattern of exposure and somatic genetic alterations have also been identified.[3,4] Overall, superficial spreading melanomas (SSMs) develop mostly on the trunk and extremities, associated with acute–intermittent sun-exposure patterns. In contrast, lentigo malignant melanomas (LMMs) usually originate on the face or chronically exposed areas. The incidence of both subtypes of melanoma increases continuously over time in populations of European origin.[5] Acral lentiginous melanomas (ALMs), located on the palms, soles and subungual sites, are not associated with sun exposure, their incidence being similar in dark- and fair-skinned populations. The epidemiology of nodular melanomas (NMs) is not clearly associated with sun exposure, maintaining a stable incidence and mortality.[6] These associations may explain the epidemiological differences detected in different populations and studies. While in most studies intermittent or recreational sun exposure and sunburns are consistently associated with melanoma risk (probably with SSM), in a few studies melanoma has also been associated with occupational sun exposure, cumulative lifetime sun exposure or markers of such exposure.[7,8] This risk seems to be associated mostly with LMM as, in areas with high levels of sun exposure, LMM becomes the more frequent subtype of melanoma.[5]

Risk factors for melanoma development also include genetic and host characteristics such as fair skin, family history of melanoma, and eye and hair pigmentation.[9–11]

Pigmentation-related melanocortin receptor 1 (MC1R), which is the major contributor to pigmentation diversity in humans, is also a risk factor for melanoma.[12] This gene is highly polymorphic, with more than 100 variants, many being nonsynonymous.[13] A meta-analysis identified five *MC1R* variants (p.D84E, p.R142H, p.R151C, p.R160W and p.D294H) associated with the red hair colour phenotype, which is characterized by fair pigmentation (fair skin, red hair and freckles) and sun sensitivity (poor tanning response and solar lentigines).[14] Functional studies have revealed the complexity of *MC1R* genomic variation. Allelic variants show differences in loss of function among red and non-red hair colour variants.[13] Furthermore, distribution of the allelic frequency of recurrent p.V60L and p.D294H variants are different even when comparing different dark-pigmented populations.[15]

Few studies have focused on the role of *MC1R* variants in melanoma beyond the study of melanoma risk in individuals. An association between germline *MC1R* status and the presence of a somatic *BRAF* mutation in melanoma was found.[16,17] However, these findings have not been confirmed by other studies,[18–20] illustrating the complexity of cross-talk between *MC1R* variants, UV exposure pattern, melanoma subtype and somatic alterations, which can be over-represented in certain combinations.

The aim of this study was to analyse whether some *MC1R* variants are associated with particular clinicohistopathological melanoma subtypes.

Materials and methods

Samples

An observational retrospective study was designed including a series of 1679 patients with melanoma from two hospital-based series. The inclusion criterion was patients having confirmed histopathological information on the tumour. In patients with multiple primary melanomas (MPMs), only those with histopathological subtype information for all tumours and information available on the time of occurrence for each were included. All patients were treated and controlled at the Melanoma Unit of the Hospital Clinic of Barcelona (Barcelona, Spain) and the Instituto Valenciano de Oncologia (Valencia, Spain).

The study of *MC1R* variants was approved by the institutional review board of both hospitals, and informed consent from all study participants was obtained.

The outcome variable of the study was the histopathological melanoma subtype. For the purpose of the study only the following subtypes were considered: LMM, SSM, NM and ALM. Patients with other unknown or unclassified tumours were excluded from the study.

In the analysis of *MC1R* variants and histopathological subtypes, the patients with MPM were included only once in each analysis. In the analysis of the total number and type of variants, patients were included based on the histopathological subtype of the first developed melanoma. In the analyses of each of the 10 most frequent variants and melanoma subtypes (LMM, SSM or NM), patients with MPM were reclassified according to whether they had had, or not, the specific melanoma subtype at any time. Such a strategy avoids the inclu-

As potential confounders, the following variables were considered: sex, age at onset, hair colour (red, blonde or brown/black), skin phototype according to the classification by Fitzpatrick (I–II vs. III–V)[21] and eye colour (dark/brown vs. green/blue).

MC1R molecular screening

Samples from the Melanoma Unit of the Hospital Clinic of Barcelona were amplified using the primers described by Chaudru *et al.*[22] Polymerase chain reaction conditions were: initial denaturizing step at 95 °C for 5 min; followed by 35 cycles of 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min; and a final extension at 72 °C for 10 min and maintaining at 4 °C. Specific internal MC1R primers were designed to analyse the entire coding sequence (INT-F: TACATCTCCATCTTCTAC GC and INT-R: GTGCTGAAGACGACACTG). Samples from the Instituto Valenciano de Oncologia, Valencia, were genotyped as described by Scherer *et al.*[23]

Statistical analysis

MC1R variants were classified as red hair colour (R) or non-red hair colour (r) according to previously reported criteria.[14] Therefore, MC1R variants classified as R were p.D84E, p.R142H, p.R151C, p.R160W and p.D294H. All other nonsynonymous MC1R variants were classified as r. Synonymous variants were considered as wild-type MC1R alleles. For the purpose of this study, only variants with an observed frequency of at least 1% were analysed. *In silico* analysis of each variant to predict the effect of the amino acid change in both protein structure and MC1R function was carried out using PolyPhen-2 version 2.2.2 (http://genetics.bwh.harvard.edu/pph2/).[24] Correlation between the number of MC1R variants and confounding variables was calculated by cross-tabulations and Pearson's χ^2 test using IBM SPSS Statistics 20 (IBM, Armonk, NY, U.S.A.). Genetic data were analysed using snpStats software (http://www.bioconductor.org/).[25] Multiple logistic regression models (codominant, dominant, recessive, overdominant and log additive) were performed for odds ratio (OR), 95% confidence interval (CI) and P-value. Both the Akaike information criterion and Bayesian information criterion were used to choose the model of inheritance that fit the data best. In some analyses, the model was adjusted by confounding variables that are associated with the MC1R variant of interest. P-values < 0·05 were considered statistically significant. All tests were two sided, and Bonferroni correction for multiple comparisons was applied to all P-values.

Results

MC1R genotyping was carried out in 1679 patients who met the selection criteria, 1428 (85%) were cases of single

The MPM subset included 198 patients (78·9%) who developed two MMs, 36 patients (14·3%) who developed three MMs and eight (3·2%) and nine (3·6%) patients who developed four and at least five MMs, respectively. Patients with MPM displayed a total of 588 MMs, 83·5% of which were SSM (491/588), 8·2% (48) were LMM, 6·8% (40) were NM and 1·5% (nine) were ALM. The frequencies for each subtype in the subset of first MMs in patients with MPM (n = 251; LMM 6·8%, SSM 82·9%, NM 8·3% and ALM 2%) were not statistically different from those observed in the subset of subsequent MMs in the same patients (n = 337; LMM 9·2%, SSM 83·8%, NM 5·6% and ALM 1·4%).

The rate of the histopathology subtype concordance was evaluated in patients with MPM. The rate of concordance was 85·6% in patients with SSM (178/208), 35% in LMM (6/17) and 5% in NM (1/21). None of the five patients with a first ALM developed other ALMs.

The study identified 53 MC1R variants (11 synonymous and 42 nonsynonymous), most being detected in a small number of patients or restricted to one (Table 1). Thirteen MC1R variants had not been identified in previous studies. Among synonymous variants, the highest frequency was observed for p.T314T (17·6% of patients). In the 1679 patients with melanoma, recurrent nonsynonymous variants with a frequency of at least 1% were p.V60L (30·0%), p.V92M (11·7%), p.D294H (9·4%), p.R151C (8·8%), p.R160W (6·2%), p.R163Q (4·2%), p.R142H (3·3%), p.I155T (3·8%), p.V122M (1·5%) and p.D84E (1·0%). Analyses of MC1R variants, phenotypic features and histopathological subtypes of the tumours were carried out only for these 10 variants.

Overall, skin phototype information was available in 94·8% of cases, and eye and hair colour in 90·5% and 93·4%, respectively. The p.R142H, p.R151C, p.R160W and p.D294H variants were statistically significantly associated with red hair colour and fair skin (phototype I or II) under a codominant model of heritance (Table 2). The association of MC1R variants and eye colour was restricted to p.R142H, which was associated with fair eye colour (green or blue) under a dominant model of heritance (OR 2·07, 95% CI 1·18–3·65; P = 0·011).

Differences in terms of number and type of MC1R variant (r or R) were analysed. Skin phototype and hair colour showed differences in the total number of variants (P < 0·001). When the analysis was focused on the number of r variants, there was no statistically significant association with phenotypic characteristics. However, a trend was observed between fair skin and the presence of at least two variants (P = 0·041). In contrast, the number of R variants was associated with both hair colour and skin phototype (P < 0·001). Overall, 89% of patients with red hair carried at least one R variant, and 54% of them carried at least two R variants.

According to the number of MC1R variants and histopatho-

Table 1 Frequency of *MC1R* variants detected in patients with melanoma

Nucleotide change	Amino acid change	Polyphen-2 score[a]	Genomic status	n (%),[b] from total N = 1679
c.5C>T	p.*A2V*	0·000	Het	1 (0·1)
c.112G>A	p.V38M	0·006	Het	1 (0·1)
c.121T>A	p.*S41C*	0·067	Het	1 (0·1)
c.175C>T	p.*V59M*	1·00	Het	1 (0·1)
c.178T>G	p.V60L	0·988	Het	459 (27·3)
			Hom	44 (2·6)
c.190G>A	p.A64T	0·988	Het	1 (0·1)
c.248C>T	p.S83L	0·998	Het	3 (0·2)
c.247T>C	p.S83P	0·998	Het	5 (0·3)
c.252C>A	p.D84E[c]	0·999	Het	17 (1·0)
c.251C>C	p.*D84H*	1·00	Het	4 (0·2)
c.265G>A	p.G89R	0·737	Het	1 (0·1)
c.274G>A	p.V92M	0·015	Het	195 (11·6)
			Hom	2 (0·1)
c.284C>T	p.T95M	0·889	Het	3 (0·2)
c.357C>A	p.V119V	–	Het	2 (0·1)
c.364G>A	p.V122M	0·126	Het	25 (1·5)
c.383T>C	p.M128T	0·235	Het	3 (0·2)
c.425G>A	p.R142H[c]	1·000	Het	54 (3·2)
			Hom	1 (0·1)
c.424C>A	p.R142S	1·000	Het	1 (0·1)
c.424C>T	p.R142C	1·000	Het	1 (0·1)
c.434C>T	p.S145F	0·989	Het	1 (0·1)
c.438C>T	p.*A146A*	–	Het	1 (0·1)
c.445G>A	p.A149T	1·000	Het	2 (0·1)
c.446C>T	p.*A149V*	1·00	Het	1 (0·1)
c.451C>T	p.R151C[c]	1·000	Het	139 (8·3)
			Hom	9 (0·5)
c.464T>C	p.I155T	0·986	Het	60 (3·6)
			Hom	4 (0·2)
c.466C>G	p.V156L	0·567	Het	1 (0·1)
c.467T>C	p.V156A	0·784	Het	1 (0·1)
c.478C>T	p.R160W[c]	0·861	Het	103 (6·1)
			Hom	1 (0·1)
c.488G>A	p.R163Q	0·004	Het	69 (4·1)
			Hom	1 (0·1)
c.504C>T	p.I168I	–	Het	5 (0·3)
c.546C>T	p.Y182Y	–	Het	2 (0·1)
c.550G>A	p.D184N	0·001	Het	1 (0·1)
c.586T>C	p.F196L	0·997	Het	1 (0·1)
c.637C>T	p.R213W	0·019	Het	1 (0·1)
c.699G>A	p.Q233Q	–	Het	30 (1·8)
c.741G>A	p.L247L	–	Het	1 (0·1)
c.766C>T	p.P256S	1·000	Het	1 (0·1)
c.788T>C	p.L263P	1·00	Het	1 (0·1)
c.792C>T	p.I264I	–	Het	1 (0·1)
c.793G>A	p.V265I	0·067	Het	1 (0·1)
c.813C>T	p.P271P	–	Het	1 (0·1)
c.814A>G	p.T272A	0·006	Het	2 (0·1)
c.815C>T	p.T272M	0·974	Het	1 (0·1)
c.815C>A	p.T272K	0·944	Het	2 (0·1)
c.835A>G	p.N279D	0·979	Het	1 (0·1)
c.850C>T	p.L284F	0·965	Het	1 (0·1)
c.892 C>T	p.R298R	–	Het	1 (0·1)
c.923C>T	p.T308M	0·979	Het	1 (0·1)
g.942A>G	p.T314T	–	Het	287 (17·1)
			Hom	8 (0·5)
g.948C>T	p.S316S	–	Het	2 (0·1)

Het, heterozygous variant; Hom, homozygous variant. Novel variants are indicated in italics. [a]*In silico* impact prediction of each nonsynonymous variant on the structure and function of *MC1R* (values close to 0·000, benign; values close to 1·00, damaging). [b]Homozygous values given only where variant observed. [c]Variant associated with red hair colour phenotype.

variants was observed (P = 0·035), showing a lower number of variants in both the ALM and NM subtypes.

Association of histopathological subtype and specific recurrent *MC1R* variants was restricted to those subtypes associated with a sun-exposure pattern (SSM, LMM and NM). The logistic regression model was adjusted by the number of primary tumours, sex, age at onset and phenotypic characteristics. No statistically significant association was found between certain *MC1R* variants and SSM or NM. In contrast, an association was detected between the p.R163Q variant and LMM development under a dominant model of heritance (OR 2·16, 95% CI 1·07–4·37; P = 0·044) (Table 4).

Discussion

Since Clark *et al.* classified melanomas into three distinct subtypes[2] and, thereafter, a fourth subgroup was proposed,[26] several studies have elucidated epidemiological and clinical features that are more associated with a particular histopathological subtype.[27–30] Some of these differences can be attributed to variation of UV exposure (chronic sun exposure or intermittent).[4]

Currently, polymorphisms in the *MC1R* gene are known to be major determinants of hair and skin colour.[31] Furthermore, *MC1R* polymorphisms play a role in sun sensitivity and low tanning ability in response to UV radiation, independently of skin colour.[32] Thus, certain *MC1R* variants could be related to a particular histopathological group of melanomas associated with different patterns of UV radiation.

In the present study, the genomic status of *MC1R* from 1679 patients with melanoma was analysed according to their histopathological melanoma subtypes. Patients with MPMs were also included, as different melanomas from the same patient may be considered as independent occurrences of the disease.[33] In the association studies for the number of variants,

Table 2 Analysis of MC1R and phenotypic traits

Association of recurrent MC1R with phenotypic traits[a]					
Hair colour		Brown/black	Red		
MC1R variant	Genotype	n (%)	n (%)	OR (95% CI)[b]	P-value
p.R142H	G/G	1168 (97·9)	58 (78)	1·00	
	G/A	25 (2·1)	15 (20)	12·40 (6·18–24·89)	< 0·0001[c]
	A/A	0 (0)	1 (1)	NA	
p.R151C	C/C	1118 (93·7)	41 (55)	1·00	
	C/T	75 (6·3)	28 (38)	10·27 (6·01–17·56)	< 0·0001[c]
	T/T	0 (0)	5 (7)	NA	
p.R160W	C/C	1132 (94·9)	58 (78)	1·00	
	C/T	61 (5·1)	16 (22)	5·2 (2·7–9·4)	< 0·0001[c]
	T/T	0 (0)	0 (0)	NA	
p.D294H	G/G	1102 (92·4)	43 (58)	1·00	
	G/C	91 (7·6)	25 (34)	6·96 (4·06–11·93)	< 0·0001[c]
	C/C	0 (0)	6 (8)	NA	
Skin phototype		III–IV	I–II		
MC1R variant	Genotype	n (%)	n (%)	OR (95% CI)[b]	P-value
p.R142H	G/G	930 (97·6)	608 (95·3)	1·00	
	G/A	23 (2·4)	29 (4·5)	1·95 (1·12–3·41)	0·027[c]
	A/A	0 (0)	1 (0·2)	NA	
p.R151C	C/C	897 (94·1)	552 (86·5)	1·00	
	C/T	55 (5·8)	79 (12·4)	2·36 (1·65–3·39)	< 0·0001[c]
	T/T	1 (0·1)	7 (1·1)	10·71 (1·31–87·42)	
p.R160W	C/C	906 (95·1)	584 (91·5)	1·00	
	C/T	47 (4·9)	53 (8·3)	1·73 (1·15–2·60)	0·0018[c]
	T/T	0 (0)	1 (0·2)	NA	
p.D294H	G/G	883 (92·7)	553 (86·7)	1·00	
	G/C	70 (7·3)	78 (12·2)	1·76 (1·25–2·48)	< 0·0001[c]
	C/C	0 (0)	7 (1·1)	NA	
Eye colour		Dark	Green/blue		
MC1R variant	Genotype	n (%)	n (%)	OR (95% CI)[b]	P-value
p.R142H	G/G	896 (97·6)	571 (95·2)	1·00	
	G/A or A/A	22 (2·4)	29 (4·8)	2·07 (1·18–3·65)	0·011[d]

Association of number of MC1R variants with phenotypic traits					
	Number of variants, n (%)				
	0	1	≥ 2	Total	P-value
Hair colour					
Black/brown	565 (47·4)	439 (36·8)	189 (15·8)	1193	
Red	4 (5)	12 (16)	58 (78)	74	< 0·00001
Phototype					
I–II	219 (34·3)	235 (36·8)	184 (28·8)	638	
III–IV	472 (49·5)	338 (35·5)	143 (15·0)	953	< 0·00001
Eye colour					
Fair	268 (44·7)	207 (34·5)	125 (20·8)	600	
Dark	392 (42·7)	336 (36·6)	190 (20·7)	918	NS
	Number of non-red hair colour variants, n (%)				
	0	1	≥ 2	Total	P-value
Hair colour					

Table 2 (continued)

	Number of non-red hair colour variants, n (%)				
	0	1	≥ 2	Total	P-value
Phototype					
I–II	354 (55·5)	231 (36·2)	53 (8·3)	638	
III–IV	596 (62·5)	293 (30·7)	64 (6·7)	953	0·041
Eye colour					
Fair	361 (60·2)	199 (33·2)	40 (6·7)	600	
Dark	544 (59·3)	299 (32·6)	75 (8·2)	918	NS
	Number of red hair colour variants, n (%)				
	0	1	≥ 2	Total	P-value
Hair colour					
Black/brown	939 (78·7)	249 (20·9)	5 (0·4)	1193	
Red	8 (11)	26 (35)	40 (54)	74	< 0·00001
Phototype					
I–II	417 (65·4)	174 (27·3)	47 (7·4)	638	
III–IV	756 (79·3)	188 (19·7)	9 (0·9)	953	< 0·00001
Eye colour					
Fair	442 (73·7)	137 (22·8)	21 (3·5)	600	
Dark	680 (74·1)	205 (22·3)	33 (3·6)	918	NS

OR, odds ratio; CI, confidence interval; NA, not analysed; NS, not significant. [a]Analysis was performed for each recurrent MC1R variant (p.V60L, p.V92M, p.D294H, p.R151C, p.R160W, p.R163Q, p.R142H, p.I155T, p.V122M and p.D84E); only variants with statistically significant P-values are shown. [b]ORs are adjusted by age at onset, sex and hospital of recruitment. Model of heritance was chosen according to the Akaike information criterion and Bayesian information criterion values. [c]Codominant model. [d]Dominant model.

Table 3 Number of variants and histopathological subtype of melanoma

	Number of variants				
	0	1	≥ 2	Total	P-value
Total MC1R variants, n (%)					
LMM	57 (41.9)	53 (39.0)	26 (19.1)	136	0.028
SSM	503 (42.4)	433 (36.5)	251 (21.1)	1187	NS
NM	122 (45.4)	96 (35.7)	51 (19.0)	269	NS
ALM	54 (62)	23 (26)	10 (11)	87	NS
Number of non-red hair colour variants, n (%)					
LMM	82 (60.3)	47 (34.6)	7 (5.1)	136	0.382
SSM	698 (58.8)	401 (33.8)	88 (7.4)	1187	NS
NM	163 (60.6)	87 (32.3)	19 (7.1)	269	NS
ALM	61 (70)	19 (22)	7 (8)	87	NS
Number of red hair colour variants, n (%)					
LMM	98 (72.1)	32 (23.5)	6 (4.4)	136	0.035
SSM	875 (73.7)	264 (22.2)	48 (4.0)	1187	NS
NM	199 (74.0)	67 (24.9)	3 (1.1)	269	NS
ALM	75 (86)	11 (13)	1 (1)	87	NS

(i.e. if a patient had developed two LMMs, in the LMM analysis the patient was recorded once). The systematic exclusion of 15% of patients with MPM in genetic studies could hide important data concerning differences in genetic background,

Table 4 Association of MC1R variant p.R163Q and lentigo maligna melanoma (LMM) tumours

Genotype	No LMM, n (%)[a]	LMM, n (%)[b]	OR (95% CI)[c]	P-value
G/G	1466 (96·1)	143 (92·9)	1·00	
G/A or A/A	59 (3·9)	11 (7·1)	2·16 (1·07–4·37)	0·044[d]

OR, odds ratio; CI, confidence interval. [a]Number of patients who did not develop LMM. [b]Number of patients who developed at least one tumour classified as LMM. [c]ORs adjusted by age at onset, sex, number of malignant melanomas, number of MC1R variants, skin phototype, hair colour and hospital of recruitment. Model of heritance was chosen according to the Akaike information criterion and Bayesian information criterion values. [d]Dominant model.

Analysis of MC1R variants and phenotypic characteristics was carried out to find previous well-established associations[14] and to consider them in the later analysis. The variants p.R142H, p.R151C, p.R160W and p.D294H were associated with red hair and fair skin. The p.R142H variant was also associated with patients with green or blue eyes. Previous studies have found no effect of MC1R genotype on eye colour. However, an epistatic interaction between MC1R and OCA2, which is a

In the present study, the total number of MC1R variants and number of red hair colour variants were higher in both the LMM and SMM subtypes. An increased prevalence of MC1R variants in tumours on intermittently exposed sites has been frequently observed. Unfortunately, the MC1R distribution according to anatomical site was not addressed in our study.

The most relevant finding was the association between p.R163Q and LMM, independent of phenotypic features (it was not associated with Fitzpatrick skin phototype, or eye or hair colour), which suggests that certain variants could be linked to specific melanoma subtypes.

Different functional influence among MC1R variants has been shown in terms of cell-surface expression, functional ability or dominant negative activity in a pigment-related pathway.[13,38–40] In addition to adenylate cyclase signalling, stimulation of MC1R also activates the mitogen-activated protein kinase (MAPK) pathway,[41] and regulates target genes involved in inflammation through the nuclear factor-κB pathway.[42] Thus, interpretation of the effect of MC1R alleles in melanoma beyond its role in pigmentation, such as the relation between p.R163Q and LMM subtype, is complex. Furthermore, differences in frequency and type of variants between populations could result in variations in genotype–phenotype correlation.[23] The frequency of the p.R163Q variant is highly variable with ethnicity, being higher in populations of Asian origin compared with Europeans, but there are differences also among European populations.[15] In the Japanese population, p.R163Q and p.V92M have been related to skin lesions associated with UV damage, such as freckles and solar lentigines.[43] Previous studies have suggested a relationship between these variants and chronic UV radiation in populations of European origin. The p.V92M variant has been associated with severe photoageing of facial skin, independent of the presence of other minor and major variants, in European women.[44] Furthermore, p.R163Q has been related to nonmelanoma skin cancer development in Europeans, underlying its possible role in tumours related to chronic sun exposure.[45] Thus, our finding that p.R163Q is related to LMM susceptibility in our population may be a consequence of the role of this variant in skin photodamage and photoageing, as a propensity to solar lentigines is a strong predictor of LMM and it is not associated with any other subtype of melanoma.[46]

Interestingly, both p.R163Q and p.V92M presented a benign score in the *in silico* analysis (Table 1) and are considered 'pseudoalleles' with no significant effect on eumelanin synthesis.[47] Thus, the biological relevance of these variants could be related to a noncanonical MC1R pathway. Although the p.R163Q variant does not display changes in either surface expression or cyclic adenosine monophosphate signalling, a selective decrease in MAPK activation has recently been described.[48] Thus, the cross-talk between specific MC1R variants and MAPK pathway activation could be responsible in part for the differing results reported for correlation between previous study suggested,[49] our data support that these differences could be due to unique effects of specific MC1R variants, the frequencies of which differ somewhat among populations.

In conclusion, the MC1R variant p.R163Q showed differences among histopathological melanoma subtypes, showing a positive association with LMM. Moreover, these findings suggest that differences exist beyond the role of MC1R variants in the pigment synthesis process. Thus, common variants could be responsible in part for the risk of LMM in populations without fair skin. Further studies should be directed to elucidate the mechanisms by which MC1R variants play a role in susceptibility to melanoma, independent of their relationship with phenotypic traits.

Acknowledgments

This work is dedicated to all our patients and their families who have always collaborated with us and who are the aim of our work. We are indebted to our colleagues, to our nurses Pablo Iglesias, Daniel Gabriel and Eugenia Moliner and our technicians Estefania Martinez and Laura Martín, who work together on a daily basis and whose effort is not always reflected in investigative papers. We also thank Helena Kruyer for her help with the text edition.

References

1 Marks R. Epidemiology of melanoma. *Clin Exp Dermatol* 2000; **25**:459–63.
2 Clark WH Jr, From L, Bernardino EA, Mihm MC. The histogenesis and biologic behavior of primary human malignant melanomas of the skin. *Cancer Res* 1969; **29**:705–27.
3 Elwood JM, Hislop TG. Solar radiation in the etiology of cutaneous malignant melanoma in Caucasians. *Natl Cancer Inst Monogr* 1982; **62**:167–71.
4 Holman CD, Armstrong BK. Cutaneous malignant melanoma and indicators of total accumulated exposure to the sun: an analysis separating histogenetic types. *J Natl Cancer Inst* 1984; **73**:75–82.
5 Forman SB, Ferringer TC, Peckham SJ *et al.* Is superficial spreading melanoma still the most common form of malignant melanoma? *J Am Acad Dermatol* 2008; **58**:1013–20.
6 Shaikh WR, Xiong M, Weinstock MA. The contribution of nodular subtype to melanoma mortality in the United States, 1978 to 2007. *Arch Dermatol* 2011; **148**:30–6.
7 Rodenas JM, Delgado-Rodriguez M, Herranz MT *et al.* Sun exposure, pigmentary traits, and risk of cutaneous malignant melanoma: a case–control study in a Mediterranean population. *Cancer Causes Control* 1996; **7**:275–83.
8 Ballester I, Oliver V, Bañuls J *et al.* Multicenter case–control study of risk factors for cutaneous melanoma in Valencia, Spain. *Actas Dermosifiliogr* 2012; **103**:790–7.
9 Tucker MA, Goldstein AM. Melanoma etiology: where are we? *Oncogene* 2003; **22**:3042–52.
10 Gandini S, Sera F, Cattaruzza MS *et al.* Meta-analysis of risk factors for cutaneous melanoma: II. Sun exposure. *Eur J Cancer* 2005;

12 Rees JL. Genetics of hair and skin color. *Annu Rev Genet* 2003; **37**:67–90.
13 García-Borrón JC, Sánchez-Laorden BL, Jiménez-Cervantes C. Melanocortin-1 receptor structure and functional regulation. *Pigment Cell Res* 2005; **18**:393–410.
14 Raimondi S, Sera F, Gandini S *et al.* MC1R variants, melanoma and red hair color phenotype: a meta-analysis. *Int J Cancer* 2008; **122**:2753–60.
15 Gerstenblith MR, Goldstein AM, Fargnoli MC *et al.* Comprehensive evaluation of allele frequency differences of *MC1R* variants across populations. *Hum Mutat* 2007; **28**:495–505.
16 Landi MT, Bauer J, Pfeiffer RM *et al.* MC1R germline variants confer risk for *BRAF*-mutant melanoma. *Science* 2006; **313**:521–2.
17 Fargnoli MC, Pike K, Pfeiffer RM *et al.* MC1R variants increase risk of melanomas harboring *BRAF* mutations. *J Invest Dermatol* 2008; **128**:2485–90.
18 Hacker E, Hayward NK, Dumenil T *et al.* The association between MC1R genotype and *BRAF* mutation status in cutaneous melanoma: findings from an Australian population. *J Invest Dermatol* 2010; **130**:241–8.
19 Thomas NE, Kanetsky PA, Edmiston SN *et al.* Relationship between germline *MC1R* variants and *BRAF*-mutant melanoma in a North Carolina population-based study. *J Invest Dermatol* 2010; **130**:1463–5.
20 Scherer D, Rachakonda PS, Angelini S *et al.* Association between the germline *MC1R* variants and somatic *BRAF*/*NRAS* mutations in melanoma tumors. *J Invest Dermatol* 2010; **130**:2844–8.
21 Fitzpatrick TB. The validity and practicality of sun-reactive skin types I through VI. *Arch Dermatol* 1988; **124**:869–71.
22 Chaudru V, Laud K, Avril MF *et al.* Melanocortin-1 receptor (MC1R) gene variants and dysplastic nevi modify penetrance of *CDKN2A* mutations in French melanoma-prone pedigrees. *Cancer Epidemiol Biomarkers Prev* 2005; **14**:2384–90.
23 Scherer D, Nagore E, Bermejo JL *et al.* Melanocortin receptor 1 variants and melanoma risk: a study of 2 European populations. *Int J Cancer* 2009; **125**:1868–75.
24 Adzhubei IA, Schmidt S, Peshkin L *et al.* A method and server for predicting damaging missense mutations. *Nat Methods* 2010; **7**:248–9.
25 Solé X, Guinó E, Valls J *et al.* SNPStats: a web tool for the analysis of association studies. *Bioinformatics* 2006; **22**:1928–9.
26 Arrington JH 3rd, Reed RJ, Ichinose H, Krementz ET. Plantar lentiginous melanoma: a distinctive variant of human cutaneous malignant melanoma. *Am J Surg Pathol* 1977; **1**:131–43.
27 Clark WH Jr, Mihm MC Jr. Lentigo maligna and lentigo-maligna melanoma. *Am J Pathol* 1969; **55**:39–67.
28 Clark WH Jr, Elder DE, Van Horn M. The biologic forms of malignant melanoma. *Hum Pathol* 1986; **17**:443–50.
29 Porras BH, Cockerell CJ. Cutaneous malignant melanoma: classification and clinical diagnosis. *Semin Cutan Med Surg* 1997; **16**:88–96.
30 Berwick M, Wiggins C. The current epidemiology of cutaneous malignant melanoma. *Front Biosci* 2006; **11**:1244–54.
31 Valverde P, Healy E, Jackson I *et al.* Variants of the melanocyte-stimulating hormone receptor gene are associated with red hair and fair skin in humans. *Nat Genet* 1995; **11**:328–30.
32 Healy E, Flannagan N, Ray A *et al.* Melanocortin-1-receptor gene and sun sensitivity in individuals without red hair. *Lancet* 2000; **355**:1072–3.
33 Orlow I, Tommasi DV, Bloom B *et al.* Evaluation of the clonal origin of multiple primary melanomas using molecular profiling. *J Invest Dermatol* 2009; **129**:1972–82.
34 Murali R, Goumas C, Kricker A *et al.* Clinicopathologic features of incident and subsequent tumors in patients with multiple primary cutaneous melanomas. *Ann Surg Oncol* 2012; **19**:1024–33.
35 Titus-Ernstoff L, Perry AE, Spencer SK *et al.* Multiple primary melanoma: two-year results from a population-based study. *Arch Dermatol* 2006; **142**:433–8.
36 Duffy DL, Box NF, Chen W *et al.* Interactive effects of MC1R and OCA2 on melanoma risk phenotypes. *Hum Mol Genet* 2004; **13**:447–61.
37 Akey JM, Wang H, Xiong M *et al.* Interaction between the melanocortin-1 receptor and P genes contributes to inter-individual variation in skin pigmentation phenotypes in a Tibetan population. *Hum Genet* 2001; **108**:516–20.
38 Beaumont KA, Newton RA, Smit DJ *et al.* Altered cell surface expression of human MC1R variant receptor alleles associated with red hair and skin cancer risk. *Hum Mol Genet* 2005; **14**:2145–54.
39 Beaumont KA, Shekar SN, Newton RA *et al.* Receptor function, dominant negative activity and phenotype correlations for MC1R variant alleles. *Hum Mol Genet* 2007; **16**:2249–60.
40 Dessinioti C, Antoniou C, Katsambas A, Stratigos AJ. Melanocortin 1 receptor variants: functional role and pigmentary associations. *Photochem Photobiol* 2011; **87**:978–87.
41 Busca R, Abbe P, Mantoux F *et al.* Ras mediates the cAMP-dependent activation of extracellular signal-regulated kinases (ERKs) in melanocytes. *EMBO J* 2000; **19**:2900–10.
42 Wikberg JE, Muceniece R, Mandrika I *et al.* New aspects on the melanocortins and their receptors. *Pharmacol Res* 2000; **42**:393–420.
43 Motokawa T, Kato T, Hashimoto Y, Katagiri T. Effect of Val92Met and Arg163Gln variants of the MC1R gene on freckles and solar lentigines in Japanese. *Pigment Cell Res* 2007; **20**:140–3.
44 Elfakir A, Ezzedine K, Latreille J *et al.* Functional *MC1R*-gene variants are associated with increased risk for severe photoaging of facial skin. *J Invest Dermatol* 2010; **130**:1107–15.
45 Scherer D, Bermejo JL, Rudnai P *et al.* MC1R variants associated susceptibility to basal cell carcinoma of skin: interaction with host factors and XRCC3 polymorphism. *Int J Cancer* 2008; **122**:1787–93.
46 Kvaskoff M, Siskind V, Green AC. Risk factors for lentigo maligna melanoma compared with superficial spreading melanoma: a case–control study in Australia. *Arch Dermatol* 2012; **148**:164–70.
47 Wakamatsu K, Kavanagh R, Kadekaro AL *et al.* Diversity of pigmentation in cultured human melanocytes is due to differences in the type as well as quantity of melanin. *Pigment Cell Res* 2006; **19**:154–62.
48 Doyle JR, Fortin JP, Beinborn M, Kopin AS. Selected melanocortin 1 receptor single-nucleotide polymorphisms differentially alter multiple signaling pathways. *J Pharmacol Exp Ther* 2012; **342**:318–26.
49 Thomas NE, Kanetsky PA, Begg CB *et al.* Melanoma molecular subtypes: unifying and paradoxical results. *J Invest Dermatol* 2010; **130**:12–14.

TRABAJO III

RESUMEN TRABAJO III

Genetic alterations in RAS-regulated pathway in acral lentiginous melanoma

Joan Anton Puig-Butille, Celia Badenas, Zighereda Ogbah, Cristina Carrera, Paula Aguilera, Josep Malvehy, Susana Puig

Exp Dermatol. 2013 Feb;22(2):148-50 *Factor de impacto: 3.578.*

Objetivo

Caracterizar las alteraciones genómicas adquiridas en el melanoma lentiginoso acral, mediante el análisis de regiones candidatas donde se localizan genes implicados en vías de señalización, progresión del ciclo celular o apoptosis.

Metodología

Se seleccionaron un serie de tumores clasificados patológicamente como melanomas lentiginosos acrales (ALM; N=17) de los cuales se disponía de tejido congelado. La caracterización molecular de los tumores comprendía el análisis de los genes *B-RAF* (exones 11 y 15), *N-RAS* (exones 1 y 2), *CDKN2A* (exones 1 alfa, 1 beta, 2 y 3) y *MC1R* mediante PCR y secuenciación Sanger; y la caracterización del número de copias (pérdidas o ganancias) mediante la técnica Multiplex ligadura-dependiente de la sonda de amplificación (MLPA) de la región 9p21 y regiones candidatas en las cuales se localizan oncogenes candidatos en cáncer cutáneo**.** La presencia de mutaciones somáticas en los genes *B-RAF, N-RAS, CDKN2A* y variantes en el gen *MC1R,* así como deleciones en la región 9p21 se evaluaron con estadísticos descriptivos; la distribución de las alteraciones de número de copia en las regiones candidatas se analizó mediante agrupación jerárquica sin supervisión. Por último se evaluó la expresión de la proteína Aurora A mediante inmunohistoquímica en los bloques parafinados correspondientes a la serie de ALM analizados.

Resultados

En el grup de ALM analizados, el 62,5% presentaban variantes en el gen *MC1R*. En 3 de los tumores se identificaron mutaciones somáticas en el gen *N-RAS* y en ningún tumor mutaciones en los genes *B-RAF* o *CDKN2A,* aunque sí se identificaron deleciones de la región 9p21 en un 69% de casos, de los cuales 5 tumores presentaban deleciones focalizadas en los genes *CDKN2A*, *CDKN2B* y *MTAP* y 6 deleción completa de la toda la región. Se detectaron ganancias del número de copias de 12 regiones cromosómicas (1p13.2, 5p15.33, 5q13, 7q21.3, 8q11, 11q13, 11q22, 12q14, 12q14.3, 7q25, 19q12, 20q13.3) en un rango de 37,5% a 6,2% de casos.

Todos los tumores presentan como mínimo una región amplificada, 43.7% dos amplificaciones y 25% de tumores presentaban 3 o más regiones alteradas. En total, 4 regiones se encuentran alteradas de forma recurrente en al menos un 25% de los ALMs: 1p13.2 (gen *N-RAS*), 5p15.33 (gen *TERT*), 20q13.3 (gen *AURKA*) y 11q13 (gen *CCND1* y genes contiguos). La distribución de estas alteraciones en los tumores analizados sugiere la existencia de tres diferentes patrones moleculares en los melanomas tipo ALM, aunque el análisis no alcanza la significación estadística.

La alteraciones de 20q13.3 (37,5% de los casos) y la de 11q13 (31,25% de casos) son mutualmente excluyente; y la alteración 1p13.2 (25% de casos) se observa más frecuentemente en tumores con ganancias de 5p15.33 (18,7% del total de tumores presentan ambas).

Dado que la alteración más recurrente es la ganancia del gen *AURKA,* se evalúa la expresión de la proteína, Aurora quinasa A en 12 tumores de la serie (6 con la alteración y 6 sin la alteración). El resultado fué evaluable en 4 de los 6 tumores con la alteración. En 3 de ellos se observa expresión de Aurora quinasa A (rango de 10% a más de un 75% de células positivas). En contra, no se detecta expresión de la proteína en ninguno de los 6 tumores sin la amplificación genómica del gen.

DOI: 10.1111/exd.12080
www.blackwellpublishing.com/EXD

Letter to the Editor

Genetic alterations in RAS-regulated pathway in acral lentiginous melanoma

Joan A. Puig-Butillé[1,2], Celia Badenas[1,2], Zighereda Ogbah[3], Cristina Carrera [1,3], Paula Aguilera[1,3], Josep Malvehy[1,3] and Susana Puig[1,3]

[1]Centro Investigación Biomédica en Enfermedades Raras (CIBERER), ISCIII, Barcelona, Spain; [2]Biochemical and Molecular Genetics Service, Melanoma Unit, Hospital Clinic & IDIBAPS (Institut d'Investigacions Biomèdiques August Pi i Sunyer), Barcelona, Spain; [3]Department of Dermatology, Melanoma Unit, Hospital Clinic & IDIBAPS (Institut d'Investigacions Biomèdiques August Pi i Sunyer), Barcelona, Spain
Correspondence: Susana Puig, MD, Consultant, Melanoma Unit, Dermatology Department, Hospital Clinic Barcelona, Villarroel 170. 08036 Barcelona, Spain, Tel.: +34-93-2275400 ext. 2893, Fax: +34-93-2275438, e-mails: susipuig@gmail.com; spuig@clinic.ub.es

Abstract: Studies integrating clinicopathological and genetic features have revealed distinct patterns of genomic aberrations in Melanoma. Distributions of *BRAF* or *NRAS* mutations and gains of several oncogenes differ among melanoma subgroups, while 9p21 deletions are found in all melanoma subtypes. In the study, status of genes involved in cell cycle progression and apoptosis was evaluated in a panel of 17 frozen primary acral melanomas. *NRAS* mutations were found in 17% of the tumors. In contrast, *BRAF* mutations were not found. Gains of *AURKA* gene (20q13.3) were detected in 37.5% of samples, gains of *CCND1* gene (11q13) or *TERT* gene (5p15.33) in 31.2% and gains of *NRAS* gene (1p13.2) in 25%. Alterations in 9p21 were identified in 69% of tumors. Gains of 11q13 and 20q13 were mutually exclusive, and 1p13.2 gain was associated with 5p15.33. Our findings showed that alterations in RAS-related pathways are present in 87.5% of acral lentiginous melanomas.

Key words: acral lentiginous melanoma – *AURKA* – melanoma – *MLPA* – *NRAS*

Accepted for publication 20 December 2012

Introduction

Molecular studies have revealed the existence of different biological subsets of melanomas based on the patterns of alterations identified (1–3), some of which correlated with degree of chronic sun-induced damage and site of origin (3). Furthermore, such molecular differences could result in clinical and histopathological differences among lesions (4,5).

Melanomas classified as acral lentiginous melanomas (ALM) develop on volar skin, usually unexposed to UV radiation and are characterized by the presence of an atypical lentiginous proliferation. ALM carry a high number of genomic alterations compared with other melanoma subtypes and most of them account for a smaller proportion of genome (1,3). The molecular hallmarks of ALM are *CCND1* amplifications (1,6,7) or somatic mutations in *c-KIT* (8).

Deletions in the 9p21 region where the *CDKN2A* gene is located are widely detected (9–11). However, other genes from this region could be implicated in melanoma because retention of the *CDKN2A* locus has been found in tumors with deletions at one or both sides of *CDKN2A* (10). Other reported aberrations include large amplifications of 12q (1,3), 7q or 20q and gains localized at 5p15, 11q13, 11q14 (3) and 22q11-13 (1).

Questions addressed

To characterize acquired molecular genomic alterations in a set of ALM from Spanish patients. The study was focused on specific chromosomal regions where genes involved in signalling pathways, cell cycle progression and apoptosis are located.

Experimental design

Seventeen fresh-frozen histopathologically confirmed primary ALMs based on Clark's classification were included. Sampling was guided diagnosis following the step-sectioning protocol for melanoma. Clinical data are described in Table S1. Genomic characterization of the *BRAF*, *NRAS*, *CDKN2A* and *MC1R* genes was performed by PCR-direct sequencing. Deletions of the 9p21 region and gains of regions wherein oncogenes of interest are localized were carried out by the multiplex ligation-dependent probe amplification (MLPA) approach (Data S1). Significance of *AURKA* gain into protein was evaluated by immunohistochemistry method (Data S1).

Unsupervised hierarchical clustering of amplified regions detected was carried out using Cluster 3.0 developed by Eisen Lab (University of California, Berkeley, CA, USA) using Pearson's correlations distance and average linkage clustering. The study was approved by the institutional review board of Hospital Clinic of Barcelona (Spain) and tumors were from the sample collection of Melanoma Unit at the Hospital Clínic of Barcelona.

Results

MC1R variants were found in 62.5% of patients (Table S1). *NRAS* missense mutations were detected in three of 17 ALMs. In contrast, activating *BRAF* mutations were not present (Table S1).

9p21 alterations were detected in 69% of tumors, one sample failed to yield a result (Table S1). While five tumors carried focal deletions of *CDKN2A*, *CDKN2B* and/or *MTAP*, 6 showed loss of the 9p21 region. No mutations were detected in *CDKN2A*.

Copy number gains were detected in 12 different loci (Table 1). All melanomas showed at least one altered locus; 43.7% of tumors presented two and 25% presented ≥ 3 loci. Four alterations were detected in at least 25% of samples: 1p13.2 (*NRAS* gene), 5p15.33 (*TERT* gene), 20q13.3 (*AURKA* gene) and 11q13 (*CCND1* and contiguous genes).

Unsupervised hierarchical clustering was carried out to analyse

Table 1. List of oncogenes analysed by the MLPA approach

Chromosomic region	Oncogenes	Frequency of tumors with amplifications
r01p13.2	*NRAS*	25%
r01p22.1	*BCAR3*	0
r01q32	*MDM4*	0
r03q27	*BCL6*	0
r05p15.33	*TERT*	31.2%
r05q13	*BIRC1*	12.5%
r06p21	*CCND3*	0
r07q21.3	*CDK6*	6.2%
r08q11	*MOS*	18.8%
r11q13	*RELA, GSTP1, CCND1, EMS1, FGF3,*	31.2%[1]
r11q22	*BIRC3*	6.2%
r12p13.32	*CCND2*	0
r12p13.2	*BCLG*	0
r12q14	*CDK4*	12.5%
r12q14.3	*MDM2*	12.5%
r13q12.3	*CCNA1*	0
r14q32.33	*AKT1*	0
r17q25	*BIRC5*	6.2%
r18q21.3	*BCL2*	0
r19q12	*CCNE1*	6.2%
r20q11.1	*BCL2L1*	0
r20q13.1	*PTPN1*	0
r20q13.3	*AURKA*	37.5%
r20q13.33	*FLJ20517*	0
Xq25	*BIRC4*	0

[1]Frequency regarding gains of the *CCND1* gene.

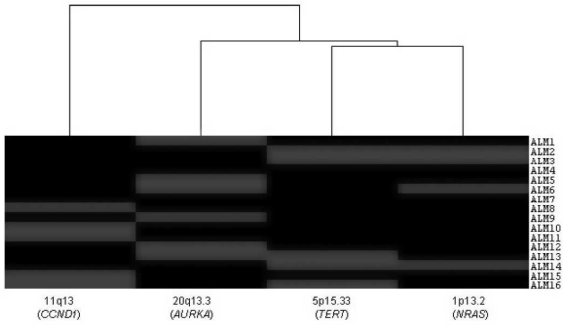


Figure 1. Hierarchical clustering subgroups of acral lentiginous melanoma: distribution of recurrent gains (11q13, 20q13.3, 5p15.33 and 1p13.2). Amplification of a given region is indicated by grey box.

(37.5%) presented 11q13 gain (31.25%). Furthermore, gains of 1p13.2 (25%) were more associated with 5p15.33 gains. Deletions in 9p21 were associated with any specific gained region.

Aurora A protein was evaluated in the corresponding FFPE biopsies of 12 samples. Among samples harbouring *AURKA* gains (six tumors), immunohistochemistry failed in two. Protein expression was detected in three of four tumors in a range of 10% of cells up to 75% of cells (Figure S1). In contrast, Aurora A expression was not detected in samples without alteration.

Conclusion

Although the study showed a high proportion of patients harbouring germinal *MC1R* variants, the frequency of them did not differ from the frequency detected in the control population (data not shown). Activating *NRAS* mutations were detected in ALM as previously described (7,13–15). *BRAF* mutations were not present in any sample, which could be explained by the large proportion of samples carrying other deregulated genes located downstream of the MAPK pathway (*CCND1* or *CDK4)* as described by Curtin et al. (3).

Focal amplifications have been described as molecular markers of ALM (1,3). The most frequently gained loci were at 1p13.2, 5p15.33, 11q13 and 20q13.3. Our data suggest the existence of different ALM subgroups based on the distribution of these alterations. Although a large set of tumors should be analysed to obtain statistical power to detect such profiles, there is plausible biological evidence to support the existence of such patterns. A group of tumors carried 20q13.3 gains (*AURKA* gene), which has been reported previously in melanoma (16). *AURKA* overexpression, which has been closely related to gene amplification or genetic instability (17,18), could be implicated in promoting cancer cell survival, activating Akt and stimulating the PI3K pathway (19). Interestingly, *AURKA* may converge upon oncogenic Ras signalling through the RALGEF pathway (20,21). Another subgroup presented 11q13 gains including *CCND1,* which is a frequent initial molecular event in ALM (2,6,7,22). Gains of *AURKA* and *CCND1* were mutually exclusive, suggesting that both genes would lead to the deregulation of cell proliferation in the same way. Revalidation of these results has been carried out by FISH methodology in a large set of ALM obtaining concordant findings (manuscript in preparation).

The third subgroup presented 1p13.2 gains (*NRAS*), frequently associated with 5p15.33 (*TERT*) alterations. Increased copy number of *NRAS* has been described previously (9,23,24).

In summary, based on these results, we hypothesize that alterations in cell progression genes (*NRAS*, *AURKA* or *CCND1)* could play similar roles as driver alterations in ALM. Further studies have to be performed on a large set of Acral lentiginous melanoma to elucidate the existence of these patterns and also to evaluate the cross-talk between downstream NRAS pathways and melanoma development.

Acknowledgements

S.P and J.M designed the research study. C.C and P.A collected tumor biopsies and obtained the clinical data. J.A.P.B and Z.O performed the research. J.A.P.B and C.B analysed the results. J.A.P.B wrote the paper. This work was supported by a grant from Fondo de Investigaciones Sanitarias (03/0019). Melanoma Unit in Barcelona is partially funded by Grants from Fondo de Investigaciones Sanitarias (09/01393) Spain; by the AGAUR 2009 SGR 1337 of the Catalan Government, Spain; by the CIBER de Enfermedades Raras, ISCIII, Madrid, Spain; by the European Commission under the 6th Framework Programme, Contract nr: LSHC-CT-2006-018702 (GenoMEL); and by the National Cancer Institute (NCI) of the US National Institute of Health (NIH) (CA83115).

Conflict of interests

The authors have declared no conflicting interests.

References

1. Bastian B.C, Kashani-Sabet M, Hamm H, et al.
3. Curtin J.A, Fridlyand J, Kageshita T, et al. N Engl
5. Whiteman D.C, Pavan W.J, Bastian B.C, Pig-

7 Takata M, Goto Y, Ichii N *et al.* J Invest Dermatol 2005: **125**: 318–322.
8 Curtin J A, Busam K, Pinkel D *et al.* J Clin Oncol 2006: **24**: 4340–4346.
9 Jonsson G, Dahl C, Staaf J *et al.* Oncogene 2007: **26**: 4738–4748.
10 Ruiz A, Puig S, Lynch M *et al.* Int J Cancer 1998: **76**: 312–316.
11 Kumar R, Smeds J. Melanoma Res 1999: **9**: 138–147.
12 Scope A, Busam K J, Malvehy J *et al.* Arch Dermatol 2007: **143**: 1548–1552.
13 Reifenberger J, Knobbe C B, Sterzinger A A *et al.* Int J Cancer 2004: **109**: 377–384.
14 Takata M, Lin J, Takayanagi S *et al.* Br J Dermatol 2007: **156**: 1287–1294.
15 Chernoff K A, Bordone L, Horst B *et al.* Clin Cancer Res 2009: **15**: 4288–4291.
16 Koynova D K, Jordanova E S, Milev A D *et al.* Melanoma Res 2007: **17**: 37–41.
17 Zhou H, Kuang J, Zhong L *et al.* Nat Genet 1998: **20**: 189–193.
18 Tanaka E, Hashimoto Y, Ito T *et al.* Clin Cancer Res 2005: **11**: 1827–1834.
19 Yao J E, Yan M, Guan Z *et al.* Mol Cancer 2009: **8**: 95.
20 Lim K H, Brady D C, Kashatus D F *et al.* Mol Cell Biol 2009: **30**: 508–523.
21 Lim K H, Baines A T, Fiordalisi J J *et al.* Cancer Cell 2005: **7**: 533–545.
22 Glatz-Krieger K, Pache M, Tapia C *et al.* Virchows Arch 2006: **449**: 328–333.
23 Stark M, Hayward N. Cancer Res 2007: **67**: 2632–2642.
24 Maldonado J L, Fridlyand J, Patel H *et al.* J Natl Cancer Inst 2003: **95**: 1878–1890.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Inmunohistochemistry of AURKA protein.

Table S1. Clinical and molecular data of acral lentiginous melanomas from Mediterranean patients.

Data S1. Supplementary methods.

Supplementary Methods

Genomic DNA Isolation, PCR amplification and Mutation analysis: The QIAamp DNA mini kit (Qiagen, USA) was used to isolate genomic DNA from melanoma tumours according to the manufacturer's instructions.

Promoter (-34G>T variant), intronic (IVS2-105) and coding regions of the *CDKN2A* gene (exons 1α, 2 and 3 of the p16INK4A protein and exon 1β corresponding to p14ARF protein) were studied. *MC1R* was amplified using primers described by Chaudru *et al*. [103]. The primers for *BRAF* exons 11 and 15, *NRAS* exons 1 and 2, primers were designed to amplify the exons where the most common mutations are detected. All PCRs were carried out using the PCR Master Mix from Promega Co (Madison, WI) following the manufacturer's instructions.

PCR conditions were: initial denaturizing step at 95ºC for 5 min, followed by 35 cycles (95ºC for 1 min, Tm (*BRAF* 56°C, *NRAS* 57°C and *MC1R* 55°C) for 1 min, 72ºC for 1 min), and a final extension at 72ºC for 10 min and maintaining at 4ºC until SSCP or sequencing studies were carried out. Primers for BRAF exon 11F: TTTCTTTTTCTGTTTGGCTTG, 11R: TGTGGTGACATTGTGACAAGT, exon 15F: TGCTTGCTCTGATAGGAAAA, exon 15R: TGAGGCTATTTTTCCACTGA. Primers for NRAS exon 1F: CGCCAATTAACCCTGATTAC, exon 1R: GCTGACCTGATCCTGTCTCT, exon 2F: CCCCTTACCCTCCACACC, exon 2R: TCTGAAAGGATGATCTTTGTGT. Mutation screening for *BRAF, NRAS,* and *CDKN2A* loci was performed by SSCP. *MC1R* was directly sequenced. Specific internal *MC1R* primers were designed to analyze the entire coding sequence (*INT-F:* TACATCTCCATCTTCTACGC and *INT-R:* GTGCTGAAGACGACACTG).

SSCP analysis was carried out. Samples with abnormal migration products were sequenced as follows: PCR products were purified using the GFX^{TM} PCR DNA and Gel Band purification kit (Amersham Biosciences) and automatically sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) and an ABI3100 automatic sequencer (Applied Biosystems).

Multiplex Ligation-dependent Probe Amplification (MLPA) : For mapping the 9p21 region we used the SALSA MLPA P024B CDKN2A/2B region (MRC-Holland Amsterdam, The Netherlands). Analysis of genomic regions wherein oncogenes of interest are localized was carried out using the SALSA MLPA KIT P172 Gain2 (MRC-Holland Amsterdam, Netherlands)

Analysis of MLPA PCR products was then performed using an ABI3100 Genetic Analyzer with a mixture of 6.5 µl deionised formamide, 1 µl PCR product and 0.5 µl ROX-500 Genescan (Applied Biosystems) and analysed using the GeneMapper software (Applied Biosystems) to quantify the peaks generated.

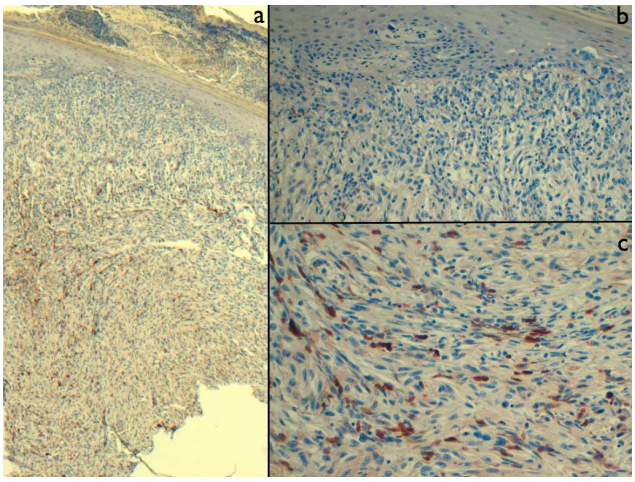
For each fragment, the peak area was calculated and normalized for the mean peak area of control samples (three controls were used). The presence of stroma cells in tumour samples makes it impossible to discern between homozygous deletion or loss of heterozygosity. For that reason, a difference was considered significant if the ratio was less than 0.75 (deletion) or higher than 1.35 (copy number gain). A ratio close to 1.0 indicates two copies present (i.e. wild-type. The MLPA assay was performed twice in some samples in order to confirm the obtained results.

AURKA inmunohistochemistry

The inmunohistochemistry (IHC) was performed using a Leica BOND-MAX™ system (Wetzlar, Germany) which is a fully automated system for IHC. Sections (3-micron) from formalin fixed and paraffin embedded tissues were obtained by using a sliding microtome. Immunohistochemical studies were performed with the automated immunohistochemical system TechMate 500® (Dako Co, Carpinteria, CA), using the EnVision system (Dako). Briefly, 4 μm sections were deparaffinized and hydrated through graded alcohols and water. Peroxidase was blocked for 7.5 minutes in ChemMate peroxidase-blocking solution (Dako). Then, the slides were incubated with the primary antibodies for 30 minutes and washed in ChemMate buffer solution (Dako). The peroxidase-labeled polymer was then applied for 30 minutes. After washing in ChemMate buffer solution, the slides were incubated with the AEC substrate chromogen solution, washed in water, counterstained with hematoxylin and mounted. The primary antibody used in the study was a polyclonal antibody against Aurora A (dilution 1:200; Abcam, Cambridge, UK). Aurora A expression of normal epithelium of the colon served as positive control. Slides were scanned and Pannoramic viewer 1.15 (3D Histech) software was used to evaluate the tumors. All slides were evaluated in a blind manner. Specimens were considered positive for Aurora A when the cytoplasm or the nucleus or both were stained in more than 10% of tumor cells.

Image S1. Inmunohistochemistry of AURKA protein:

This tumour with molecular gains of 20q13.33, showed 20% of positive cells for AURKA nuclear staining. a) overview of the tumour with negative cells for AURKA expression in the superficial tumoral cells and positive cells in the deeper part of the tumour (x10 magnification); b) detail of the superficial part of the tumour, negative for AURKA expression (x20); c) detail of the positive nuclear expression of tumoral cells in the deeper part of the tumour (x40).


a
b
c

TRABAJO II

RESUMEN TRABAJO IV

Role of CPI-17 in restoring skin homeostasis in cutaneous field of cancerization: effects of topical application of a film-forming medical device containing photolyase and UV filters.

Joan A. Puig-Butillè, Josep Malvehy, Miriam Potrony, Carles Trullas, Francisco García-García, Joaquín Dopazo, Susana Puig

Exp Dermatol. 2013 Jul;22(7):494-6 *Factor de Impacto: 3.578*

Objetivo

Explorar los efectos moleculares de la aplicación tópica de un compuesto que contiene filtros UV y fotoliasa (reparador natural de dímeros de pirimidina y ciclobutano y fotoproductos 6-4) en pacientes con campo de cancerización. Para ello se utilizó Eryfotona® AK-NMSC (Eryf-AK),

Metodología

Es un estudio piloto clínico intervecionista y prospectivo. El criterio de inclusión era pacientes con una área con múltiples lesiones de queratosis actínicas (campo de cancerización) mayor de 3.6x3.6cm de una zona de piel expuesta a la radiación UV. Las lesiones del campo de cancerización se clasificaron como queratosis actínicas en base al examen de microscopía confocal *in-vivo* y confirmadas por biopsia antes de iniciar la intervención. Se incluyeron 5 pacientes varones con fotoenvejecimiento severo en áreas de alopecia androgenética así como dos pacientes afectos de Xeroderma Pigmentosum (antebrazos). El producto se aplicó en el campo de cancerización dos veces al día (mañana y 4-6 horas más tarde) durante 4 semanas. El campo de cancerización se subdividió en 4 areas de 0,8x0,8 cm. A lo largo del estudio se obtuvo un "punch" de 3 mm de cada una de las subáreas para la evaluación histopatológica y para la extracción del RNA antes (áreas 1 y 2) y después de las 4 semanas de tratamiento (áreas 3 y 4). La evaluación molecular se realizó mediante el "array" de expresión Whole Human Genome (4x44k) Oligo Microarray kit (G4112F, Agilent, EUA).

El conjunto de datos normalizados se analizó mediante el método de Análisis de Componentes Principales (PCA) para explorar diferencies en cuanto a las características de los pacientes puesto que se incluyeron pacientes afectos del Síndrome de Xeroderma Pigmentosum. Se realizó el análisis de expresión diferencial mediante el paquete bioinformático "limma" incluido en Bioconductor y se procedió al ajuste por comparaciones múltiples de los p-valores obtenidos con la metodología de Benjamini y Hochberg. Se realizó un análisis de enriquecimiento basado en ontologías genómicas mediante la herramienta Fatiscan implementada en el programa bioinformático Babelómics para identificar funciones biológicas sobre-representadas en el campo de cancerizacion, en las lesiones después del tratamiento con fotoliasa y según el tipo de respuesta.

En base a los resultados generados mediante el "array de expresión" se analizó la expresión génica de *CPI-17*, *WDR72, TNF* y *IL-1B* mediante PCR en tiempo rea (RT-PCR).

Resultados

Del total de pacientes, 3 de ellos presentaron una respuesta histologica completa, 1 paciente presentó una mejoria en un 80% del área evaluada y 3 tres casos presentaron una mejoría histopatologia parcial asociada a la presencia de inflamación. En base a los datos histopatologicos, los pacientes se clasificaron como pacientes con respuesta rápida (FR; N=3) y pacientes con una respuesta lenta o parcial (PR; N=4).

En el análisis de expresión génica diferencial comparando las lesiones antes del tratamiento y después del tratamiento, independientemente de la respuesta, no se observó ningún gen significativamente desregulado. El Análisis de Componentes Principales no detectó componentes asociados intrínsicamente a los pacientes afectos de Xeroderma Pigmentosum, y no se observó una correcta agrupación entre lesiones pre-tratadas y lesiones post-tratadas con fotolíasa. En cambio, agrupaba las lesiones post-tratadas según el tipo de respuesta obtenida (FR vs PR; Anexo V). En base a estos resultados, se evaluó la respuesta al tratamiento analizando la expresión global entre los pacientes FR y los pacientes PR después del tratamiento, y se detectó una mayor expresión del gen *CPI-17* (p-valor=0,039) y del gen *WDR72* (p-valor=0,040) en las lesiones con una respuesta rápida. Ambos genes se analizaron de nuevo mediante RT-PCR para confirmar su sobreexpresión. Se confirmó una mayor expresión del gen *CPI-17* en las lesiones con una respuesta rápida (p-valor=0,0001). Mediante esta metodología se determinó molecularmente el grado de inflamación existente en las lesiones antes de la aplicación del compuesto y después del tratamiento mediante el análisis de la expresión de los genes *TNF* y *IL-1B*. Se observó una menor expresión de *TNF* (p=0,012) y *IL1B* (p=0,07) en las lesiones de los pacientes FR antes del tratamiento. La expresión de la citoquina *IL1B* es significativamente mayor en los pacientes PR incluso despúes del tratamiento (p=0,038).

En el análisis funcional de ontologías genómicas se identificó una sobre-representación estadísticamente significativa de 150 funciones biológicas en el campo de cancerización antes del tratamiento (p-valor<0.005), las cuales se agruparon en cuatro grandes grupos biológicos: "generación de especies oxígeno reactivas";

“mecanismos de reparación del DNA”; “procesos de división celular” y “lípidos”. De igual modo se identificaron 66 procesos asociados al tratamiento con fotoliasa, que se agruparon en: “comunicación celular y señalización”, “adhesión celular” y “desarrollo tisular”.

Por otro lado, las lesiones con una respuesta rápida al tratamiento presentaban una sobre-representación de 24 funciones, la mayoría de ellas asociadas a respuesta celular y homeostasis; y las lesiones con una respuesta parcial presentavan 28 funciones biológicas sobre-representadas relacionadas con procesos inflamatorios, procesos metabólicos, producción de citoquinas y apoptosis.

DOI: 10.1111/exd.12177
www.wileyonlinelibrary.com/journal/EXD

Letter to the Editor

Role of *CPI-17* in restoring skin homoeostasis in cutaneous field of cancerization: effects of topical application of a film-forming medical device containing photolyase and UV filters

Joan Anton Puig-Butillé[1,2], Josep Malvehy[2,3], Miriam Potrony[1,2], Carles Trullas[4], Francisco Garcia-García[5,6], Joaquin Dopazo[5,6,7] and Susana Puig[2,3]

[1]Melanoma Unit, Biochemistry and Molecular Genetics Service, Hospital Clinic of Barcelona, IDIBAPS, Barcelona, Spain; [2]CIBER de Enfermedades Raras, Instituto de Salud Carlos III, Barcelona, Spain; [3]Melanoma Unit, Dermatology Department, Hospital Clinic of Barcelona, IDIBAPS, Barcelona, Spain; [4]ISDIN, Barcelona, Spain; [5]Department of Bioinformatics, Centro de Investigación Príncipe Felipe, Valencia, Spain; [6]Functional Genomics Node, National Institute of Bioinformatics, CIPF, Valencia, Spain; [7]CIBER de Enfermedades Raras (CIBERER), Valencia, Spain
Correspondence: Susana Puig, MD, Consultant, Melanoma Unit, Dermatology Department, Hospital Clinic Barcelona, Villarroel 170. 08036 Barcelona, Spain, Tel.: +34-93-2275400 ext 2893, Fax: +34-93-2275438, e-mails: susipuig@gmail.com; spuig@clinic.ub.es

Abstract: Cutaneous field of cancerization (CFC) is caused in part by the carcinogenic effect of the cyclobutane pyrimidine dimers CPD and 6-4 photoproducts (6-4PPs). Photoreactivation is carried out by photolyases which specifically recognize and repair both photoproducts. The study evaluates the molecular effects of topical application of a film-forming medical device containing photolyase and UV filters on the precancerous field in AK from seven patients. Skin improvement after treatment was confirmed in all patients by histopathological and molecular assessment. A gene set analysis showed that skin recovery was associated with biological processes involved in tissue homoeostasis and cell maintenance. The CFC response was associated with over-expression of the *CPI-17* gene, and a dependence on the initial expression level was observed ($P = 0.001$). Low *CPI-17* levels were directly associated with pro-inflammatory genes such as *TNF* ($P = 0.012$) and *IL-1B* ($P = 0.07$). Our results suggest a role for *CPI-17* in restoring skin homoeostasis in CFC lesions.

Key words: actinic keratoses – *CPI-17* – cutaneous field of cancerization – expression array – *PPP1R14A*

Accepted for publication 20 May 2013

Background

Cutaneous field of cancerization (CFC) is associated with genomic alterations due to the carcinogenic effect of sun exposure (1) and comprises actinic keratoses (AKs) and squamous cell carcinomas (SCCs) (2). UV radiation, particularly UVB, promotes the production of cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs) which subsequently interfere with biological processes that are critical for cell viability (3). The nucleotide excision repair (NER) system is employed by mammal cells to remove UV-induced DNA damage (4). However, whereas 6-4PPs are efficiently recognized and removed by the NER system, CPDs recognition and removal is poor (5,6). Many organisms have an additional repair mechanism named photoreactivation, which is carried out by photolyases which specifically recognize and repair either CPDs or 6-4PPs (7). The potential of DNA photolyases in skin cancer prevention has been increasingly recognized. Beneficial effects after transferring a CPD photolyase into mammals have been obtained in transgenic mice (8). Transgenic expression of photolyases showed a 40% increase in CPD lesion repair, improved resistance against UV-induced effects suppressing the formation of skin carcinomas. Furthermore, topical application of liposome formulations with CPD photolyases onto human skin provides protection against UVB-induced damage (9).

Questions addressed

We explored the molecular effects of topical application of Eryfotona® AK-NMSC (Eryf-AK; ISDIN, Barcelona, Spain), a film-forming medical device containing Repairsomes® (photolyase in liposomes and UV filters), in patients with CFC.

Experimental design

For experimental design and procedures see Data S1.

Results

Three of seven patients with CFC (AK Pretreatment biopsies) presented a complete histological clearance, one patient presented histological clearance in more than 80% of the sample, and three additional cases presented partial histological improvement associated with inflammation. Based upon the histopathology

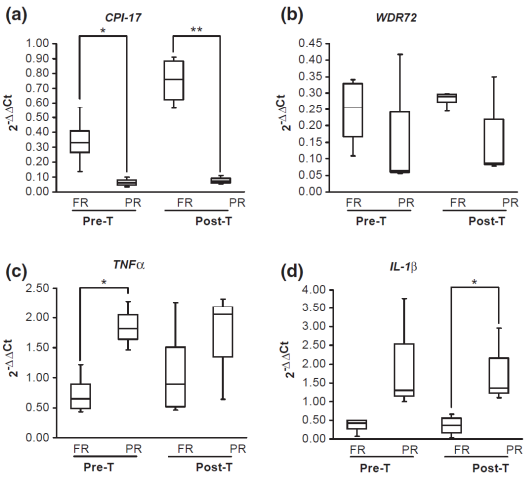


Figure 1. Values of relative quantification of selected genes and markers by subtype of patients (fast responders versus partial responders) and time of treatment (prior pretreatment or posttreatment): (a) Values of relative quantification of *CPI-17* ($2^{-\Delta\Delta Ct}$). **P*-values <0.05, ***P*-values <0.001. (b) Values of relative quantification of *WDR72* ($2^{-\Delta\Delta Ct}$). *P*-values pretreatment lesion = 0.631; *P*-value posttreatment lesion = 0.211. (c) Values of relative quantification of *TNFα* ($2^{-\Delta\Delta Ct}$). **P*-values <0.05; *P*-value posttreatment lesion = 0.447. (d) Values of relative quantification of *IL-1B* ($2^{-\Delta\Delta Ct}$). **P*-values <0.05; *P*-value pretreatment lesion = 0.070.

assessment after Eryf-AK treatment, the subjects were classified as fast responders (FR) versus slow-partial responders (PR) (Table S1).

The differential gene expression analysis of CFC pretreatment versus posttreatment assessment failed to detect deregulated genes after correction for multiple testing. However, over-expressions of *CPI-17* gene (2.8-fold increase, $P = 0.039$) and *WDR72* gene (1.9-fold increase, $P = 0.040$) were detected in FR subgroup. *CPI-17* expression differences between FR and PR subgroups were confirmed by RT-PCR ($P = 0.001$) (Figure 1a). In contrast, no significant differences ($P = 0.211$) were observed for *WDR72* (Figure 1b). Initial *CPI-17* levels were higher in FR than PR patients ($P = 0.045$; Figure 1a).

Pathological conditions such as inflammation modulate *CPI-17* expression (10). Thus, expression of *TNF* and *IL-1B* cytokines were evaluated. FR showed lower expression levels of *TNF* ($P = 0.012$) and *IL-1B* ($P = 0.07$) after Eryf-AK (Figure 1c,d). Posttreatment PR lesions still showed a high *IL-1B* expression level ($P = 0.038$).

Gene set analysis identified 150 biological functions ($P < 0.005$; Table S2) associated with CFC, which were classified in four major biological clusters: 'generation of reactive oxygen species', 'mechanisms involved in DNA repair', 'cell division process' and 'lipids'.

Sixty six processes were associated with Eryf-AK treatment ($P < 0.005$, Table S3), which were grouped as 'cell communication and signalling', 'cell adhesion' and 'tissue development'.

Gene set analysis according to treatment response identified 24 GOs over-expressed in FR subgroup mostly involved in cell response and homoeostasis and 28 GO's associated with PR lesions which were related to inflammation and cytokine production, apoptosis and lipid metabolic processes (Table 1).

Conclusions

We identified biological processes associated with CFC such as ROS production and DNA damage repair processes that may be induced in part by CPDs and/or lipid metabolism. Lipid content changes are important in AK and BCC (11). After treatment, we found over-expression of fundamental processes related to tissue reconstitution (cell communication, signalling and adhesion).

Based on treatment response, treated PR biopsies showed an over-expression of apoptotic process, lipid metabolism, cytokine production and inflammation which are directly related to AK. Inflammation is important for AK maintenance which is abolished by the topical use of diclofenac combined with hyaluronic acid through a selective inhibition of COX2 (12–14). The histopathological evaluation showed the presence of AK in at least 20% of the biopsy specimen from PR patients. In treated FR subgroup, we observed an improvement in cell homoeostasis and adhesion

Table 1. Gene ontology terms detected by gene set analysis in posttreatment biopsies by response subgroup

Response to treatment[1]	Gene Ontology terms (ID and *P*-value)[2]
Fast responders (FR)	Cell-cell signalling (0007267, $P = 3.02E-06$); Embryonic development (0009790, $P = 1.15E-05$); Cell development (0048468, $P = 1.46E-05$); Secretion (0046903, $P = 2.10E-05$); Regulation of secretion (0051046, $P = 1.22E-04$); Muscle contraction (0006936, $P = 1.87E-04$); Chemical homoeostasis (0048878, $P = 2.44E-04$); Anterior/posterior pattern formation (0009952, $P = 3.05E-04$); Cellular homoeostasis (0019725, $P = 4.93E-04$); Embryonic development ending in birth or egg hatching (0009792, $P = 6.73E-04$); Chordate embryonic development (0043009, $P = 1.8E-03$); Homoeostatic process (0042592, $P = 1.30E-03$); Ion homoeostasis (0050801, $P = 1.53E-03$), Secretion by cell (0032940, $P = 1,53E-03$); Response to steroid hormone stimulus (0048545, $P = 2.00E-03$); Cell fate commitment (0045165, $P = 2.19E-03$); Response to hypoxia (0001666, $P = 2.22E-03$); Cellular chemical homoeostasis (0055082, $P = 2.23E-03$); Regulation of blood vessel size (0050880, $P = 2.76E-03$); Smooth muscle contraction (0006939, $P = 3.26E-03$); Response to inorganic substance (0010035, $P = 4.09E-03$); Response to hormone stimulus (0009725, $P = 4.37E-03$); Neurological system process (0050877, $P = 3.08E-02$); Cell morphogenesis (0000902, $P = 1.17E+00$)
Partial responders (PR)	Organic acid metabolic process (0006082, $P = 3.35E-18$); Carboxylic acid metabolic process (0019752, $P = 3.35E-18$); Lipid metabolic process (0006629 $P = 1.28E-13$); Cellular lipid metabolic process (0044255, $P = 8.13E-13$); Lipid biosynthetic process (0008610, $P = 2.09E-09$); Regulation of immune response (0050776, $P = 1.09E-08$); Steroid metabolic process (0008202, $P = 7.70E-08$); Regulation of interleukin-6 production (0032675, $P = 2.07E-07$); Regulation of cytokine production (0001817, $P = 2.07E-07$); Positive regulation of immune response (0050778, $P = 2,81E-06$); Carbohydrate metabolic process (0005975, $P = 3.67E-05$); Leucocyte migration (0050900, $P = 4.62E-05$); Neutral lipid metabolic process (0006638, $P = 1.65E-04$); Glycerol ether metabolic process (0006662, $P = 1.65E-04$); Regulation of lipid metabolic process (0019216, $P = 1.80E-04$); Positive regulation of cytokine production (0001819, $P = 1.83E-04$); Response to drug (0042493, $P = 3.12E-04$); cellular carbohydrate metabolic process (0044262, $P = 3.61E-04$); Locomotory behaviour (0007626, $P = 3.91E-04$); Lipid storage (0019915, $P = 8.60E-04$); Response to organic substance (0010033, $P = 2.62E-03$); Interleukin-12 production (0032615, $P = 2.95E-03$); Apoptosis (0006915, $P = 2.96E-03$); Negative regulation of cytokine production (0001818, $P = 3.01E-03$); Programmed cell death (0012501, $P = 3.68E-03$); Response to molecule of bacterial origin (0002237, $P = 3.96E-03$); Regulation of programmed cell death (0043067, $P = 4.06E-03$); Amine metabolic process (0009308, $P = 4.74E-03$)

[1]Patients were classified as fast responders and partial responders based on the histopathology assessment after Eryf-AK treatment (see Table S1).
[2]The ID numbers and the *P*-values are indicated for each GO.

which correlates with the improvement in histopathological measures (Puig et al. 2012; submitted for publication).

CPI-17 over-expression was associated with normal phenotype recovery. *CPI-17* is one of the major Ser/Thr phosphatase isoforms, and its activation suppresses the MYPT1-PP1δ activity resulting in muscle contraction (10). *CIP-17* expression is detected in multiple cell types (15–17) involved in several processes (16,18). MYTP1 inhibition results in more prominent focal adhesions and absence of cell migration (19). *CPI-17* is directly associated with focal adhesion kinases (20) and located at focal adhesions in fibroblasts and keratinocytes (21). MYPT1-PP1δ complex can also regulate the dephosphorylation of retinoblastoma protein (pRb) (22) which shows a deregulated activation in AK. We observed that inflammation modulates *CPI-17* expression in CFC. Thus, processes such as DNA damage or ROS production may cause *CPI-17* down-deregulation, which could lead to uncontrolled MYPT1-PP1δ activity. Deregulated phosphatase activity in CFC may affect cell motility, cell adhesion and cell cycle control mediated by pRb.

In conclusion, 1-month Eryf-AK treatment improved the field of cancerization and restored normal phenotype in at least a subset of samples, through *CPI-17* up-regulation.

Acknowledgements

S.P and J.M designed the research study. S.P collected skin biopsies and obtained the clinical data. J.A.P.B performed the whole-genome expression arrays. M.P performed the RT-PCR. J.A.P.B, F.G.G and J.D analysed and interpreted the results; J.A.P.B wrote the paper. C.T reviewed the manuscript. The present project was partially funded by a grant from ISDIN. The research at the Melanoma Unit in Barcelona is partially funded by Grants from Fondo de Investigaciones Sanitarias (09/01393), Spain; by the *CIBER de Enfermedades Raras* of the Instituto de Salud Carlos III, Spain; by the AGAUR 2009 SGR 1337 of the Catalan Government, Spain. This work is also partly supported by grants BIO2008-04212 from the Spanish Ministry of Science and Innovation (MICINN) and PROMETEO/2010/001 from the GVA-FEDER.

Conflict of interests

The authors state no conflict of interests. The sponsors had no role in the design and conduct of the study and interpretation of data.

References

1 Park W S, Lee H K, Lee J Y *et al.* Hum Pathol 1996: **27**: 1180–1184.
2 Cockerell C J. J Am Acad Dermatol 2000: **42**: 11–17.
3 Vink A A, Roza L. J Photochem Photobiol, B 2001: **65**: 101–104.
4 Hoeijmakers J H. Nature 2001: **411**: 366–374.
5 Bohr V A, Smith C A, Okumoto D S *et al.* Cell 1985: **40**: 359–369.
6 Mitchell D L. Photochem Photobiol 1988: **48**: 51–57.
7 Essen L O, Klar T. Cell Mol Life Sci 2006: **63**: 1266–1277.
8 Jans J, Schul W, Sert Y G *et al.* Curr Biol 2005: **15**: 105–115.
9 Stege H, Roza L, Vink A A *et al.* Proc Natl Acad Sci U S A 2000: **97**: 1790–1795.
10 Eto M. J Biol Chem 2009: **284**: 35273–35277.
11 Vural P, Canbaz M, Sekcuki D *et al.* Int J Dermatol 1999: **38**: 439–442.
12 Maltusch A, Rowert-Huber J, Matthies C *et al.* J Dtsch Dermatol Ges 2011: **9**: 1011–1017.
13 Fecker L F, Stockfleth E, Braun F K *et al.* J Invest Dermatol 2010: **130**: 2098–2109.
14 Rodust P M, Fecker L F, Stockfleth E *et al.* Exp Dermatol 2012: **21**: 520–525.
15 Eto M, Senba S, Morita F *et al.* FEBS Lett 1997: **410**: 356–360.
16 Eto M, Bock R, Brautigan D L *et al.* Neuron 2002: **36**: 1145–1158.
17 Woodsome T P, Eto M, Everett A *et al.* J Physiol 2001: **535**: 553–564.
18 Jin H, Sperka T, Herrlich P *et al.* Nature 2006: **442**: 576–579.
19 Xia D, Stull J T, Kamm K E. Exp Cell Res 2005: **304**: 506–517.
20 Fresu M, Bianchi M, Parsons J T *et al.* Biochem J 2001: **358**: 407–414.
21 Villa-Moruzzi E, Tognarini M, Cecchini G *et al.* Cell Adhes Commun 1998: **5**: 297–305.
22 Kiss A, Lontay B, Becsi B *et al.* Cell Signal 2008: **20**: 2059–2070.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Study design.

Table S1. Patient demographics and classification based on response to treatment.

Table S2. GO's biological processes overrepresented in CFC prior to Eryfotona AK-NMSC treatment.

Table S3. GO's biological processes overrepresented in lesional skin post Eryfotona AK-NMSC treatment.

DATA S1:

Study Design

A pilot, prospective, controlled, interventional clinical study was performed to evaluate the effect of Eryfotona® AK-NMSC (Eryf-AK), a film-forming medical device containing Repairsomes® (DNA-repairing enzyme photolyase in liposomes and UV filters), in the treatment of the cutaneous field of cancerization (CFC).

Inclusion criteria was patients with an area larger than 3.6x3.6 cm containing multiple lesions in a sun-exposed skin area which were classified as actinic keratosis (AK) based on *in vivo* reflectance confocal microscopy assessment and later confirmed by punch biopsies.

Eleven patients with multiple scalp AK and two patients with xeroderma pigmentosum, with an area larger than 3.6x3.6 cm affected by CFC in a sun-exposed skin area, were included. Written informed consent was obtained from all patients after having read and understood the information approved by the ethics committee. The study was approved by the institutional research board and was conducted according to the Declaration of Helsinki Principles. Thirteen patients (mean age 72 years) were screened; one patient refused the treatment after the first evaluation and before the first biopsy (ery 009). Two patients refused the second biopsy due to concomitant personal issues (ery 006 and ery 011). In another three patients RNA extraction failed in one of the samples before or after treatment (ery007, ery012 and ery013).

Seven patients (two of them affected by xeroderma pigmentosum) completed the study. The test product was applied twice a day in the treatment evaluation area for four weeks, in the morning and 4-6 hours later.

Samples

The area assessed for CFC was divided in four 0.8x0.8 cm subareas. A 3-mm punch biopsy was obtained from area 1 for histopathological evaluation and another 3-mm punch biopsy was obtained from area 2 before starting treatment, and preserved for RNA extraction. After four weeks of Eryf-AK application, two additional punch biopsies were obtained from area 3 for histopathology exam and from area 4 for additional RNA extraction.

RNA extraction

Punch biopsies were embedded in RNAlater (Quiagen, US) and stored at -80ºC. Total RNA isolation was conducted using Trizol (Invitrogen Life Technologies, Carlsbad, CA) extraction-based method as indicated in the manufacturer's protocol, followed by purification in commercial columns (Quiagen, Valencia, CA). Briefly, disruption and homogenization of skin samples was performed using Polytron System PT1200E (Kinematica, Switzerland) homogenized and lysed in Trizol. After chloroform addition, RNA was isolated from the aqueous phase. RNA was precipitated with isopropanol, washed with 70% ethanol, and redissolved in RNase-free buffer. Total isolated RNA was further purified using the RNeasy kit (Qiagen, Valencia, CA). RNA concentration was

measured using a NanoDrop Spectrophotometer (Thermo Scientific) and integrity of the RNA was verified with a Bioanalyzer 2100 (Agilent, US). The RNA integrity number was higher than 7.9 for all samples.

Expression array

Analysis of global gene expression was performed using the Whole Human Genome (4x44k) Oligo Microarray kit (G4112F, Agilent, US). The microarray contains probes from over 19.596 specific human genes and transcripts with public domain annotations (RefSeq, Goldenpath Ensembl Unigene Human Genome and GenBank databases). Overall, 50 ng of RNA were labeled using Low input Quickamp Labeling kit (Agilent, US). In all samples, 10 commercial control probes were added to standardize the results (RNA Spike-in kit, one color, Agilent, US). The arrays were scanned using the DNA Microarray Scanner G2565CA (Agilent, US); Feature Extraction Software (Agilent, US) was used to perform the quality control process and extract the information.

TaqMan real-time reverse transcriptase polymerase chain reaction

To confirm the microarray results, the expression of four genes was evaluated by TaqMan real-time reverse transcriptase polymerase chain reaction (RT-PCR). cDNA was reverse-transcribed from total RNA using TaqMan PCR Core Reagment kit (Roche Applied Science, Penzbergf, Germany). RT-PCR was performed using TaqMan Universal PCR master Mix (Roche Applied Science, Penzbergf, Germany). The reaction was performed in an ABI 7900HT sequence detection instrument (Applied Biosystems, CA, US). TaqMan gene-specific primers and probes for selected genes (*CPI-17, WDR72, TNF* and *IL-1B*) were purchased from Applied Biosystems. The *GADPH* gene was used to normalize each sample. RNA from normal skin was used as a calibrator.

Statistical analyses

For array raw data normalization purposes, the Agilent Processed Signal was standardized across arrays using quantile normalization. Principal Component Analysis (PCA) method was used to explore the data generated by the expression arrays to assess if XP patients have a different expression that could be identified. The analyses did not identify "components" associated exclusively with the expression profile from the XP patients. Differential gene expression analysis was carried out using the Linear Models for Microarray Data (limma) package from Bioconductor (http://www.bioconductor.org). Multiple testing adjustments of P-value estimates were performed according to Benjamini and Hochberg. Gene set analysis was carried out for the Gene Ontology (GO) terms and using a logistic regression model in Babelomics (http://babelomics.bioinfo.cipf.es/). GO annotation for the genes in the microarray was taken from Ensembl 56 release (http://www.ensembl.org). Quantitative PCR of selected genes was evaluated using the relative quantification method of ΔΔCt. Expression values were evaluated by t-tests for means equality using the SPSS 17.0 software. P-values less than or equal to 0.05 were considered to be statistically significant.

Table S1: Patient demographics and classification based on response to treatment.

XP: patient affected by xeroderma pigmentosum; M: male; F: female; AK: actinic keratosis. T0: time at patient inclusion; TF: assessment after 4-week treatment with Eryfotona AK-NMSC. [A] Classification based on the response after 4-week treatment (histology at TF)

Patient	Age (y.o.)	Gender	XP	Site of lesion	Histology T0	Histology TF	Responders[A]
ery001	92	M	No	scalp	AK	Epidermal atrophia with focal disarranged basal layer	Fast
ery002	62	M	No	scalp	AK	AK and lympho-eosinophilic infiltrate	Slow or partial
ery003	76	M	No	scalp	AK	Focal changes of AK in the context of photodamaged skin	Fast
ary004	29	F	Yes	forearm	AK	Skin with basal pigmentation and melanophages	Fast
ery005	28	M	Yes	forearm	AK	Normal skin	Fast
ery008	79	M	No	scalp	AK	AK and lympho-eosinophilic infiltrate	Slow or partial
ery010	67	M	No	scalp	AK	AK	Slow or partial

Table S2: GO's biological processes overrepresented in CFC prior to Eryfotona AK-NMSC treatment.

150 GO's were detected in the gene set analysis (levels from 3 to 9, p-value <0.005)).[A] Number of deregulated genes in the analysis. [B] Number of genes included in the Gene Ontology group.

ID	Function	Number of genes [A]	Total number of genes [B]	Adjusted p value
GO:0045184	establishment of protein localization	940	1040	1.37E-06
GO:0015031	protein transport	933	1031	1.42E-06
GO:0007049	cell cycle	850	954	7.20E-12
GO:0006629	lipid metabolic process	873	949	3.80E-10
GO:0046907	intracellular transport	828	902	8.60E-05
GO:0044265	cellular macromolecule catabolic process	790	880	6.48E-04
GO:0044255	cellular lipid metabolic process	724	784	5.39E-10
GO:0006082	organic acid metabolic process	620	679	7.26E-14
GO:0055114	oxidation reduction	628	677	8.81E-17
GO:0005975	carbohydrate metabolic process	624	676	1.87E-03
GO:0019752	carboxylic acid metabolic process	616	674	4.35E-13
GO:0034613	cellular protein localization	605	659	9.35E-05
GO:0022402	cell cycle process	580	649	2.65E-11
GO:0006259	DNA metabolic process	584	648	1.13E-09
GO:0051276	chromosome organization	525	637	1.63E-05
GO:0006412	translation	506	631	1.81E-05
GO:0033554	cellular response to stress	562	630	1.65E-04
GO:0006886	intracellular protein transport	582	630	3.00E-05
GO:0006396	RNA processing	586	595	1.07E-03
GO:0006066	alcohol metabolic process	490	523	9.25E-05
GO:0009308	amine metabolic process	468	497	3.83E-03
GO:0046483	heterocycle metabolic process	472	484	4.67E-03
GO:0000278	mitotic cell cycle	433	478	1.29E-12
GO:0007608	sensory perception of smell	130	455	1.90E-04

GO:0006519	cellular amino acid and derivative metabolic process	393	447	1.80E-06
GO:0006974	response to DNA damage stimulus	382	429	1.66E-08
GO:0006091	generation of precursor metabolites and energy	361	403	5.39E-11
GO:0000279	M phase	341	394	1.25E-15
GO:0008610	lipid biosynthetic process	369	393	1.92E-06
GO:0034984	cellular response to DNA damage stimulus	353	389	9.99E-05
GO:0006281	DNA repair	307	335	1.67E-04
GO:0051726	regulation of cell cycle	284	322	3.82E-05
GO:0006520	cellular amino acid metabolic process	275	303	1.05E-05
GO:0051301	cell division	255	287	3.11E-16
GO:0000087	M phase of mitotic cell cycle	242	275	1.53E-14
GO:0006333	chromatin assembly or disassembly	206	268	2.12E-04
GO:0007067	Mitosis	231	265	8.28E-15
GO:0051186	cofactor metabolic process	236	261	8.53E-18
GO:0006323	DNA packaging	192	256	1.28E-08
GO:0008202	steroid metabolic process	228	244	2.80E-04
GO:0006260	DNA replication	219	238	6.25E-15
GO:0006631	fatty acid metabolic process	227	236	5.52E-06
GO:0055085	transmembrane transport	195	222	1.90E-06
GO:0006334	nucleosome assembly	161	222	1.44E-07
GO:0006644	phospholipid metabolic process	201	220	2.84E-03
GO:0016042	lipid catabolic process	179	211	6.94E-04
GO:0006457	protein folding	186	201	2.78E-04
GO:0015980	energy derivation by oxidation of organic compounds	174	197	4.18E-04
GO:0006725	cellular aromatic compound metabolic process	178	193	1.87E-04
GO:0046486	glycerolipid metabolic process	161	169	4.83E-03
GO:0007346	regulation of mitotic cell cycle	140	166	1.10E-03

GO:0006119	oxidative phosphorylation	113	134	1.22E-07
GO:0022900	electron transport chain	110	132	2.43E-11
GO:0007005	mitochondrion organization	115	129	1.33E-05
GO:0006310	DNA recombination	113	125	2.02E-05
GO:0042254	ribosome biogenesis	124	124	3.07E-04
GO:0006694	steroid biosynthetic process	115	124	4.73E-05
GO:0045333	cellular respiration	104	122	9.73E-10
GO:0046395	carboxylic acid catabolic process	120	121	4.61E-06
GO:0006766	vitamin metabolic process	106	120	4.35E-06
GO:0051321	meiotic cell cycle	98	112	9.24E-04
GO:0007126	meiosis	96	110	5.96E-04
GO:0006518	peptide metabolic process	94	106	3.41E-03
GO:0008654	phospholipid biosynthetic process	103	106	1.06E-03
GO:0016125	sterol metabolic process	102	105	1.52E-03
GO:0006633	fatty acid biosynthetic process	96	104	5.97E-04
GO:0016072	rRNA metabolic process	96	96	2.10E-03
GO:0042157	lipoprotein metabolic process	85	93	3.84E-03
GO:0015992	proton transport	81	93	2.81E-03
GO:0006364	rRNA processing	92	92	4.04E-03
GO:0018958	phenol metabolic process	82	88	1.22E-03
GO:0051438	regulation of ubiquitin-protein ligase activity	76	82	1.10E-04
GO:0000075	cell cycle checkpoint	69	81	8.87E-06
GO:0022904	respiratory electron transport chain	67	81	4.71E-06
GO:0008033	tRNA processing	79	80	1.37E-04
GO:0006839	mitochondrial transport	79	79	5.31E-06
GO:0009116	nucleoside metabolic process	66	78	4.90E-03
GO:0065002	intracellular protein transmembrane transport	78	78	1.02E-03
GO:0051351	positive regulation of ligase activity	72	77	1.22E-04
GO:0051443	positive regulation of ubiquitin-protein ligase	69	74	2.73E-04

	activity			
GO:0007059	chromosome segregation	69	73	6.50E-08
GO:0051437	positive regulation of ubiquitin-protein ligase activity during mitotic cell cycle	67	72	3.36E-04
GO:0051444	negative regulation of ubiquitin-protein ligase activity	66	71	5.42E-04
GO:0042773	ATP synthesis coupled electron transport	57	71	1.23E-06
GO:0051436	negative regulation of ubiquitin-protein ligase activity during mitotic cell cycle	65	69	3.24E-04
GO:0043043	peptide biosynthetic process	62	67	1.76E-04
GO:0006261	DNA-dependent DNA replication	60	67	3.92E-06
GO:0050708	regulation of protein secretion	57	65	4.95E-03
GO:0045454	cell redox homeostasis	63	65	7.86E-05
GO:0006289	nucleotide-excision repair	52	63	2.25E-04
GO:0042158	lipoprotein biosynthetic process	58	61	1.75E-03
GO:0006308	DNA catabolic process	51	61	5.15E-05
GO:0042775	mitochondrial ATP synthesis coupled electron transport	53	61	2.37E-07
GO:0006302	double-strand break repair	56	59	5.52E-06
GO:0006354	RNA elongation	51	57	3.48E-03
GO:0006497	protein amino acid lipidation	54	56	7.15E-04
GO:0019395	fatty acid oxidation	52	52	1.91E-06
GO:0009451	RNA modification	50	50	1.54E-03
GO:0007051	spindle organization	39	50	3.11E-03
GO:0007093	mitotic cell cycle checkpoint	40	48	3.21E-04
GO:0009239	enterobactin biosynthetic process	41	48	1.29E-05
GO:0006220	pyrimidine nucleotide metabolic process	41	42	2.88E-04
GO:0016126	sterol biosynthetic process	42	42	1.33E-04
GO:0009062	fatty acid catabolic process	39	41	3.99E-05

GO:0009060	aerobic respiration	38	40	3.04E-06
GO:0006071	glycerol metabolic process	37	37	4.58E-03
GO:0006505	GPI anchor metabolic process	36	36	2.61E-03
GO:0006084	acetyl-CoA metabolic process	30	33	1.22E-03
GO:0006695	cholesterol biosynthetic process	32	33	7.92E-05
GO:0000819	sister chromatid segregation	30	33	1.03E-04
GO:0007006	mitochondrial membrane organization	32	32	3.44E-04
GO:0000070	mitotic sister chromatid segregation	29	32	1.60E-04
GO:0006626	protein targeting to mitochondrion	26	29	1.25E-06
GO:0009262	deoxyribonucleotide metabolic process	27	28	2.02E-04
GO:0006284	base-excision repair	27	27	3.66E-03
GO:0030262	apoptotic nuclear changes	21	25	4.96E-03
GO:0006270	DNA replication initiation	22	25	6.82E-06
GO:0007031	peroxisome organization	23	23	1.12E-04
GO:0050709	negative regulation of protein secretion	14	22	3.18E-03
GO:0007098	centrosome cycle	20	22	4.22E-04
GO:0008299	isoprenoid biosynthetic process	18	21	2.96E-06
GO:0048535	lymph node development	9	20	2.75E-03
GO:0065005	protein-lipid complex assembly	13	19	1.21E-03
GO:0000038	very-long-chain fatty acid metabolic process	16	18	2.25E-03
GO:0006309	DNA fragmentation involved in apoptosis	13	18	2.49E-03
GO:0000724	double-strand break repair via homologous recombination	18	18	1.21E-07
GO:0006297	nucleotide-excision repair, DNA gap filling	16	17	4.71E-04
GO:0006637	acyl-CoA metabolic process	17	17	3.24E-04
GO:0008272	sulfate transport	15	16	3.22E-03
GO:0008209	androgen metabolic process	10	16	1.50E-03

GO:0019363	pyridine nucleotide biosynthetic process	15	15	3.84E-04
GO:0009264	deoxyribonucleotide catabolic process	14	14	2.86E-03
GO:0034384	high-density lipoprotein particle clearance	8	14	1.74E-03
GO:0006625	protein targeting to peroxisome	13	13	4.39E-03
GO:0007625	grooming behavior	9	12	3.95E-03
GO:0042375	quinone cofactor metabolic process	12	12	6.01E-04
GO:0051298	centrosome duplication	9	12	1.51E-04
GO:0033108	mitochondrial respiratory chain complex assembly	11	11	1.87E-03
GO:0031649	heat generation	7	10	4.44E-03
GO:0006312	mitotic recombination	9	9	1.83E-03
GO:0007007	inner mitochondrial membrane organization	9	9	7.21E-04
GO:0009263	deoxyribonucleotide biosynthetic process	7	8	9.61E-05
GO:0022616	DNA strand elongation	7	7	2.90E-03
GO:0060192	negative regulation of lipase activity	7	7	1.21E-04
GO:0034379	very-low-density lipoprotein particle assembly	7	7	9.44E-05
GO:0042769	DNA damage response, detection of DNA damage	6	6	4.36E-03
GO:0000089	mitotic metaphase	5	6	3.33E-03
GO:0015802	basic amino acid transport	5	6	1.37E-03
GO:0006271	DNA strand elongation during DNA replication	5	5	5.30E-04
GO:0051005	negative regulation of lipoprotein lipase activity	5	5	5.19E-04

Table S3: GO's biological processes overrepresented in lesional skin post Eryfotona AK-NMSC treatment.

66 GO's were identified in the gene set analysis (levels from 3 to 9, p-value <0.005). [A] Number of deregulated genes in the analysis. [B] Number of genes included in the Gene Ontology group.

ID	Function	Number of genes [A]	Total number of genes [B]	Adjusted p value
GO:0007399	nervous system development	965	1136	2.09E-04
GO:0010646	regulation of cell communication	957	1070	5.03E-05
GO:0007155	cell adhesion	888	953	3.77E-05
GO:0048468	cell development	782	921	7.65E-04
GO:0009966	regulation of signal transduction	824	912	1.54E-05
GO:0006468	protein amino acid phosphorylation	893	893	2.54E-05
GO:0009888	tissue development	699	808	5.10E-05
GO:0009887	organ morphogenesis	711	800	3.26E-04
GO:0006928	cellular component movement	588	666	5.96E-04
GO:0009790	embryonic development	537	619	5.63E-04
GO:0010629	negative regulation of gene expression	439	499	4.62E-03
GO:0048870	cell motility	424	484	1.67E-03
GO:0016481	negative regulation of transcription	398	458	3.37E-03
GO:0007167	enzyme linked receptor protein signaling pathway	426	432	1.51E-04
GO:0016477	cell migration	350	405	1.58E-03
GO:0001501	skeletal system development	374	394	1.65E-03
GO:0048871	multicellular organismal homeostasis	291	339	1.31E-03
GO:0048598	embryonic morphogenesis	287	334	7.41E-04
GO:0007265	Ras protein signal transduction	309	316	9.15E-05
GO:0030029	actin filament-based process	273	299	4.18E-03
GO:0001944	vasculature development	277	294	1.86E-04
GO:0001568	blood vessel development	272	288	1.67E-04
GO:0007169	transmembrane receptor protein tyrosine kinase signaling pathway	269	276	2.18E-03
GO:0051056	regulation of small GTPase mediated signal transduction	270	270	8.21E-06
GO:0009409	response to cold	217	266	3.02E-03
GO:0006816	calcium ion transport	226	248	4.20E-03

GO:0008015	blood circulation	225	247	1.05E-03
GO:0042309	homoiothermy	198	244	2.18E-03
GO:0050826	response to freezing	197	243	2.24E-03
GO:0007507	heart development	202	230	4.65E-04
GO:0046578	regulation of Ras protein signal transduction	221	221	4.33E-05
GO:0048729	tissue morphogenesis	168	193	9.58E-04
GO:0031589	cell-substrate adhesion	152	168	7.48E-06
GO:0006814	sodium ion transport	145	155	1.28E-03
GO:0007178	transmembrane receptor protein serine/threonine kinase signaling pathway	142	145	2.66E-04
GO:0009187	cyclic nucleotide metabolic process	121	141	8.94E-04
GO:0030155	regulation of cell adhesion	123	140	1.28E-03
GO:0007160	cell-matrix adhesion	125	138	3.53E-04
GO:0060173	limb development	98	113	1.70E-04
GO:0030198	extracellular matrix organization	96	110	1.97E-06
GO:0035108	limb morphogenesis	95	109	2.12E-04
GO:0030326	embryonic limb morphogenesis	86	96	8.22E-04
GO:0007179	transforming growth factor beta receptor signaling pathway	83	89	1.30E-03
GO:0002573	myeloid leukocyte differentiation	59	77	1.03E-03
GO:0003007	heart morphogenesis	73	77	1.24E-03
GO:0048771	tissue remodeling	69	76	4.11E-04
GO:0045785	positive regulation of cell adhesion	57	68	7.96E-04
GO:0030509	BMP signaling pathway	60	61	1.31E-03
GO:0002455	humoral immune response mediated by circulating immunoglobulin	35	44	3.32E-03
GO:0046849	bone remodeling	33	36	3.05E-03
GO:0007602	phototransduction	32	35	1.21E-03
GO:0010811	positive regulation of cell-substrate adhesion	31	32	9.77E-04
GO:0045216	cell-cell junction organization	30	30	3.98E-03
GO:0030199	collagen fibril organization	28	28	2.37E-04
GO:0030048	actin filament-based movement	21	26	3.32E-04
GO:0034103	regulation of tissue remodeling	20	20	4.12E-03
GO:0035116	embryonic hindlimb morphogenesis	17	18	2.64E-03
GO:0030901	midbrain development	15	17	3.79E-03
GO:0043462	regulation of ATPase activity	11	15	5.77E-04

GO:0032964	collagen biosynthetic process	14	15	1.18E-04
GO:0060070	Wnt receptor signaling pathway through beta-catenin	13	15	4.07E-03
GO:0014910	regulation of smooth muscle cell migration	12	12	3.79E-03
GO:0007168	receptor guanylyl cyclase signaling pathway	6	6	5.99E-05
GO:0043171	peptide catabolic process	6	6	1.50E-04
GO:0001957	intramembranous ossification	5	5	3.23E-03
GO:0010815	bradykinin catabolic process	2	2	2.30E-04

DISCUSIÓN

I. VÍAS MOLECULARES Y GENES ASOCIADOS A LA SUSCEPTIBILIDAD AL CÁNCER CUTÁNEO

1. LA DESREGULACIÓN DEL TRANSCRIPTOMA COMO EVENTO INICIAL EN CÁNCER CUTÁNEO.

2. DIFERENCIAS EPIDEMIOLÓGICAS DEL CÁNCER CUTÁNEO: DIFERENCIAS MOLECULARES.

3. GEN *CDKN2A*, VÍA DE NOTCH, RIESGO A MELANOMA Y LESIONES DE RIESGO.

4. EL GEN *MC1R* Y ESTRÉS OXIDATIVO: MÁS ALLÁ DE LA SUSCEPTIBLIDAD A CÁNCER CUTÁNEO.

5. LA VARIANTE p.R163Q DEL GEN *MC1R* Y RIESGO A CÁNCER CUTÁNEO ASOCIADO A LA RADIACIÓN UV CRÓNICA.

II. VÍAS MOLECULARES Y GENES ALTERADOS EN CÁNCER CUTÁNEO

1. IDENTIFICACIÓN DE GENES IMPLICADOS EN LA CARCINOGÉNESIS DEL CÁNCER CUTÁNEO INDEPENDIENTE DE LA RADIACIÓN UV.

2. IDENTIFICACIÓN DE GENES IMPLICADOS EN CÁNCER CUTÁNEO FOTOINDUCIDO.

III. ESTRATÉGIAS DE ANÁLISIS DE LA EXPRESIÓN GENÓMICA: ARRAYS DE EXPRESIÓN.

I. VÍAS MOLECULARES Y GENES ASOCIADOS A LA SUSCEPTIBILIDAD AL CÁNCER CUTÀNEO.

1. LA DESREGULACIÓN DEL TRANSCRIPTOMA COMO EVENTO INICIAL EN CÁNCER CUTÁNEO.

Previo a la realización de esta tesis, se había postulado que células fenotípicamente normales portadoras de una mutación "single-hit" en un gen supresor de tumores presentan un transcriptoma desregulado. La firma molecular asociada a una mutación germinal conferiría unas características pre-malignas a las células en cultivo, por lo que dicha firma molecular podría considerarse un evento inicial en el desarrollo de un cáncer. Bellacosa A et al. observaron que las células epiteliales de mama y ovario de portadores de mutaciones germinales en el gen *BRCA1* o *BRCA2* presentan desregulados múltiples genes implicados en cáncer de mama o ovario [104]. Herbert B.S et al. observaron el mismo fenómeno en las células epiteliales morfológicamente normales de individuos portadores de mutaciones en el gen *P53* (Síndrome de Li-Fraumeni)[105].

Los resultados de estos estudios indican que para un determinado tipo de célula, la firma molecular está formada en parte por genes directamente implicados en la susceptibilidad y desarrollo del cáncer. Por lo que la identificación de estas alteraciones iniciales pueden permitir identificar dianas moleculares críticas para el desarrollo del tumor.

En el trabajo I hemos demostrado que las células de un tejido susceptible a desarrollar cáncer; como el tejido cutáneo de los portadores de mutaciones o variantes en los genes *MC1R* o *CDKN2A* también presentan un transcriptoma constitutivamente desregulado. Y además, las firmas observadas en células sanas se mantienen durante el proceso carcinogénico, observándose también en las formas de cáncer cutáneo (melanoma y carcinomas escamosos).

Las células cutáneas de portadores de mutación en *CDKN2A* presentan principalmente una desregulación de genes relacionados con componentes celulares, metabolismo y respuesta inmune. Estos resultados reflejan el papel importante de la inmunidad innata y las vías moleculares de la respuesta inmunológica en los estadios iniciales del desarrollo

del cáncer cutáneo y especialmente del melanoma. Estudios previos observaron un incremento del riesgo a desarrollar melanoma asociado a polimorfismos del receptor de la Interleuquina 6 (*IL-6R*)(rs6684439, rs4845618, rs4845622 y rs8192284) [106] y al polimorfismo IL-10--1082 AA localizado en el promotor de la Interleuquina 10 (*IL10*) [107]. Yang XR. et al, analizaron 152 genes implicados en reparación del ADN, apoptosis y respuesta inmune, y detectaron un incremento del riesgo a desarrollar melanoma en los portadores del polimorfismo rs2069882 en el gen de la Interleuquina 9 (*IL9*) [108]. De forma interesante, observaron que este polimorfismo incrementaba el riesgo en las familias portadoras de mutación en el gen *CDKN2A;* lo que sugiere que genes implicados en procesos inmunológicos podrían actuar de factores modificadores del riesgo en individuos portadores de mutaciones germinales en *CDKN2A*.

Por otra parte, las células con el gen *CDKN2A* mutado presentan una desregulación constitutiva de genes implicados en la melanogénesis y diferenciación epidérmica. Por ejemplo, se observó una infra-expresión del gen de diferenciación del melanocito *TYPR1* y una sobre-expresion del gen *MFI2*, el cual tiene un papel en la proliferación celular del melanoma [109], y del gen *EEA1* el cual es un marcador de endosomas de formas tempranas [110]. Los genes *EEA1* y *MFI2* cooperan regulando la fusión y el tráfico de los endosomas [111]. Estos endosomas tempranos son precursores directos de los melanosomas, los orgánulos especializados en la biosíntesis de la melanina y su posterior almacenamiento. Estos resultados sugieren que, la desorganización de las estructuras melanosomales y cambios en la producción de pigmento favorecen la transformación maligna de los melanocitos. El hecho de que varias proteínas melanosomales funcionen *in-vivo* como dianas de la respuesta autoinmune podría sugerir que los genes implicados en el desarrollo del melanosoma tienen un papel en la carcinogenesis debido a su relación con la respuesta inmune más que a su función en los procesos relacionados con la pigmentación [112, 113]. En la mayoría de los tumores, el proceso de diferenciación celular discurre en sentido contrario al de la carcinogénesis mientras que en el melanoma, la diferenciación celular, plasmada en la producción de melanina, transcurre de la mano del proceso de carcinogénesis asociándose a un peor pronóstico del tumor [114].

Por otro lado, se ha detectado una firma de expresión génica asociada a la presencia de variantes no funcionales en el gen *MC1R*, que está formada por genes implicados en procesos fisiológicos claves como son la diferenciación celular, adhesión celular y progresión del ciclo celular. Dichos procesos están directamente relacionados con la etiología del cáncer de piel. Además se han detectado la desregulación de otros procesos claves discutidos en más detalle en el apartado 4 ("EL GEN MC1R Y ESTRÉS OXIDATIVO: MÁS ALLÁ DE LA SUSCEPTIBILIDAD A CÁNCER CUTÁNEO").

Por tanto, en base a los resultados del trabajo I, parece razonable considerar que en células fenotípicamente sanas pero con susceptibilidad a desarrollar cáncer, puede observarse un perfil de expresión desregulado similar al que puede observarse en melanoma y carcinomas escamosos, representando una fase inicial del inicio y desarrollo del cáncer cutáneo. El estudio de los genes que forman estos perfiles puede ser de gran utilidad para la identificación de biomarcadores de riesgo a cáncer cutáneo y/o de genes modificadores del riesgo en portadores de mutaciones o variantes no funcionales en los genes *CDKN2A* ó *MC1R*, respectivamente.

2. DIFERENCIAS EPIDEMIOLÓGICAS DEL CÁNCER CUTÁNEO: DIFERENCIAS MOLECULARES.

La epidemiología del cáncer de piel es compleja debido a la naturaleza multigénica de la enfermedad. Aunque el melanoma y el cáncer cutáneo no melanoma comparten unos factores de riesgo claramente asociados a la radiación UV (daño actínico y lentigos solares) o relacionados con la respuesta a la radiación (fototipo, color de pelo y color de ojos claros); la incidencia de melanoma y cáncer de piel no melanoma observada muestra diferencias considerables. Esa complejidad se observa también a nivel molecular. Mientras que variantes del gen *MC1R* incrementan el riesgo a desarrollar melanoma y cáncer cutáneo no melanoma [65, 67-70], la presencia de alteraciones germinales en el gen *CDKN2A* incrementa particularmente el riesgo a melanoma[76, 82, 83]; aunque el gen tiene un papel importante durante la progresión de todos los tipos de tumores [89, 90, 93, 100, 101].

Estos datos indican que ambas entidades presentan un patrón molecular único el cual debe estar relacionado con procesos propios de cada tipo celular.

En el trabajo I se identificó 485 genes que estaban alterados en el mismo sentido (sobreexpresado o infraexpresado) tanto en los co-cultivos portadores de variantes en *MC1R* como en portadores de mutación en *CDKN2A*, mientras que 136 se encontraban inversamente expresados entre estas dos condiciones genéticas. Un resultado relevante del trabajo I fue identificar que el patrón de expresión asociado al gen *MC1R* no funcional era más similar entre las diferentes formas de cáncer cutáneo (SSC y melanomas) que el patrón asociado al gen *CDKN2A* mutado.

Aunque parte del patrón de expresión asociado a las mutaciones en *CDKN2A* es común en los co-cultivos y las formas de cáncer cutáneo como por ejemplo, la desregulación de los genes de la respuesta al interferón y la immunomodulación, se observa que un subgrupo de genes muestran un patrón de expresión inverso entre SSC y melanomas ó células con la mutación en CDKN2A. La expresión de estos 11 genes es más similar entre el melanoma y los co-cultivos pero no lo es con la expresión en SCC (Tabla 2).

Varios estudios han demostrado como la expresión inversa del gen *CEACAM1*, identificado en este subgrupo, puede favorecer la carcinogénesis de diversas formas neoplásicas de manera contrária. *CEACAM1* se encuentra infra-expresado en carcinoma escamoso oral [115] mientras que se encuentra sobreexpresado en melanomas considerándose incluso un biomarcador clínico[116].

Para comprender mejor estas complejas relaciones sería necesario realizar nuevos estudios que permitieran identificar el papel de los genes identificados en la etiología de los diferentes tipos de cáncer cutáneos y su relación con las diferencias epidemiológicas observadas así como su relación con los genes de susceptibilidad *MC1R* y *CDKN2A*.

Gen	Nombre	Función del gen	Status en MM o cél. *CDKN2A* mutadas	Status en SSC
TBX1	T-box transcription factor	Estimulador de proliferación durante el desarrollo e inhibidor del crecimiento	Infraexpresado	Sobreexpresado
CLDN17	Claudin 17	Mantenimiento de la polaridad de la célula; implicado en la transduccion de señales	Infraexpresado	Sobreexpresado
RFX2	Factor regulador X 2 (factor de transcripción)	Crecimiento celular y modulador de la respuesta de la célula al gen NRAS	Infraexpresado	Sobreexpresado
COL5A3	Colageno, tipo V, alpha 3	Componente del colágeno fibrilar	Infraexpresado	Sobreexpresado
IFIT2	Interferon-induced protein with tetratricopeptide repeats 2	Promotor apoptosis celular	Sobreexpresado	Infraexpresado
CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1	Regulador del la proliferación celular, crecimento tumoral, apoptosis, angiongenesis, migración de las células endoteliales	Sobreexpresado	Infraexpresado
WDR78	WD repeat domain 78	Se desconoce la función	Sobreexpresado	Infraexpresado
LXN	Latexin	Inhibidor de las metalocarboxipeptidasas dependientes de zinc	Sobreexpresado	Infraexpresado
SLC39A8	Xolute carrier family 39 (zinc transporter), member 8	Transportador del Zinc durante procesos inflamatorios	Sobreexpresado	Infraexpresado
ZNF83	Zinc finger protein 8	Se desconoce la función	Sobreexpresado	Infraexpresado
RPS27L	Ribosomal protein S27-like	Posible componente de la subunidad ribosomal 40 S	Sobreexpresado	Infraexpresado
LSM3	LSM3 homolog, U6 small nuclear RNA associated S. cerevisiae	Participa en el "splicing" del pre-mRNA	Sobreexpresado	Infraexpresado
CTSK	Catepsin K	Remodelación ósea; invasión tumoral	Sobreexpresado	Infraexpresado
PMP22	Peripheral myelin protein 22	Componente de la Mielina	Sobreexpresado	Infraexpresado

Tabla 2. Listado de genes con un patrón de expresión inverso entre carcinomas celulares escamosos y melanomas o células con mutación en el gen *CDKN2A*

3. GEN *CDKN2A*, VÍA DE SEÑALIZACIÓN DE NOTCH, RIESGO A MELANOMA Y LESIONES DE RIESGO.

Existen diferentes características fenotípicas asociadas a un mayor riesgo a desarrollar melanoma como son el tipo de pigmentación, densidad de efélides y de léntigos solares, el número de nevus comunes o la presencia de nevus atípicos (Tabla 1). Chang YM et al. analizaron la presencia y características de los nevus de 5.421 casos de melanoma y 6.966 controles y demostraron que el principal factor de riesgo fenotípico de desarrollar melanoma es tener un alto número de nevus melanocíticos [117].

El hecho de que determinados parámetros relacionados con los nevus como su localización, el número total de nevus comunes o de nevus atípicos en un individuo son factores predictivos de que dicho individuo sea portador de una mutación germinal en el gen *CDKN2A* [118] sugiere que dicho gen desarrollaría una función en el proceso de nevogénesis, aunque en la actualidad se desconoce el mecanismo molecular.

En el trabajo I, se ha observado una infra-expresión de la vía de señalización mediada por el gen Notch en las células portadoras de mutación en el gen *CDKN2A*. Aunque no se detectó una desregulacion del propio gen *NOTCH1*, las células mutadas presentaban una infra-expresión de varios genes de la vía, incluyendo el co-activador de Notch1, el gen *MAML1* que controla la capacidad de Notch1 para promover el crecimiento. La vía molecular mediada por Nocth1 es esencial para las interacciones epidérmicas queratinocito-melanocito [119, 120]. Resultados recientes sugieren que esta vía es un enlace entre el control de la diferenciación y la proliferación, y, en consecuencia, de la homeostasis cutánea [121, 122].

El papel de la señalización mediada por Notch 1 en el cáncer cutáneo es complejo y ambivalente puesto que puede considerarse como una vía con efectos oncogénicos así como con efectos supresores de tumor (Tabla 3)

Tipos de cáncer cutáneo y lesiones benignas	Estado de la vía de Notch
Nevus comunes melanocíticos	infraregulada
Nevus displásicos atípicos	sobreregulada
Melanoma	sobreregulada
SCC de zonas protegidas del sol	sobreregulada
SCC de zonas expuestas al sol	infraregulada
BCC (subtipo nodular y superificial)	sobreregulada
BCC (subtiposubtipo basoescamoso y esclerodermiforme)	infraregulada
Carcinoma celular de Merkel	sobreregulada
Sarcoma de Kaposi	sobreregulada
Metástasis cutáneas de tumores neuroendocrinos	infraregulada

Tabla 3. Estado de la vía de Notch en cáncer cutáneo o lesiones benignas

En base a los datos epidemiológicos y moleculares previamente publicados, nuestros resultados sugieren que el papel del gen *CDKN2A* en la nevogénesis es, en parte, mediante su efecto en la vía de señalización de Notch 1. La desregulacion basal de la vía de forma constitutiva, ofrecería una ventaja proliferativa al melanocito, la cual sería responsable, al menos en parte, del mayor número de nevus comunes melanocíticos observado en la piel de portadores de mutación en *CDKN2A*.

4. EL GEN *MC1R* Y ESTRÉS OXIDATIVO: MÁS ALLÁ DE LA SUSCEPTIBLIDAD A CÁNCER CUTÁNEO.

Actualmente, está incrementando el interés por el conocimiento de las posibles funciones del gen *MC1R* independientes de su papel en la pigmentación. En este sentido, en el trabajo I se observa que las células con un receptor no funcional presentan un mayor daño del ADN incluso sin ser expuestas a la RUV, sugiriendo que factores endógenos provocarían dicho daño. Además, en el trabajo II, se ha identificado una variante del gen sin una repercusión en la producción de pigmento como factor de riesgo

a desarrollar tumores asociados a la exposición crónica a la RUV (apartado 5: "LA VARIANTE p.R163Q DEL GEN *MC1R* Y RIESGO A CÁNCER CUTÁNEO ASOCIADO A LA RADIACIÓN UV CRÓNICA").

Las células con un receptor no funcional presentan una sobre-representación de la vía de cíclo celular (hsa04110) y replicación del ADN (hsa03030) así como de las vías de recombinación homóloga (hsa03440) y reparación "Mismatch" (hsa 03430). Por tanto, cabe la posibilidad, de que estas células presenten una mayor tasa de errores de replicación y recombinación del DNA debido a una desregulación del propio ciclo celular facilitando la aparición de mutaciones en genes clave para la carcinogénesis.

Por otro lado, se observa una sobre-representación de la vía de la fosforilación oxidativa (hsa00190), la cual es necesaria para la producción de adenosín trifosfato (ATP). Este proceso, a pesar de ser vital para el metabolismo celular, produce una proporción de especies reactivas del oxigeno (superóxido y peróxido de hidrógeno), lo que lleva a un aumento del estrés oxidativo en la célula mediante la propagación de radicales libres. El estrés oxidativo provoca daño celular e inestabilidad genómica por lo que es considerado un "hallmark" necesario en el proceso carcinogénico [123]. Junto con el estrés oxidativo, se observa una sobre-regulación de la vía de reparación por escisión de bases (hsa03410) la cual repara los daños causados por dichos radicales de oxigeno. Estos resultados indican que parte del daño oxidativo observado en los melanocitos y queratinocitos es independiente de la radiación UV.

En el mismo sentido, Mitra y col. demostraron en modelos murinos, que en ausencia de RUV, las variantes de pelo-rojo junto con la activación del oncogén *B-RAF* son suficientes para inducir melanoma, pero si se inhibe totalmente la síntesis de feomelanina (ratones albinos), se bloquea la inducción del tumor y expresan menor daño oxidativo celular [124].

El estrés oxidativo también contribuye al envejecimiento celular y se ha relacionado con múltiples enfermedades. De forma notable, las células cutáneas con el receptor no funcional presentan una sobre-regulación de vías relacionadas con enfermedades neurodegenerativas (enfermedad de Hugtington (hsa05016); enfermedad de Alzheimer

(hsa 05010) y enfermedad de Parkinson (hsa05012). El gen *MC1R* se expresa en las células del sistema nervioso y su activación disminuye el estrés oxidativo además de tener efectos anti-inflamatorios e inmunomoduladores [125]. Cabe destacar que el incremento del estrés oxidativo es un proceso presente en la etiopatogénia de las tres enfermedades neurodegenerativas identificadas en el estudio [126-128].

La relación entre cáncer cutáneo y enfermedades neurodegenerativas se ha observado, a nivel epidemiológico, entre el riesgo a desarrollar melanoma y Parkinson [129-131]. Los pacientes de Parkinson tienen el doble de riesgo de desarrollar melanoma comparado con la población general; y los pacientes de melanoma el doble de riesgo a desarrollar Parkinson. Recientemente se ha descrito también que los pacientes pelirrojos tienen más riesgo de Parkinson, observándose una tendencia a un mayor riesgo en los portadores del polimorfismo de cabello rojo p.R151C [132], por lo que se ha postulado que dicha relación podría deberse a genes implicados en la síntesis de pigmento [132-134].

En base a los resultados del trabajo II, y fuera del ámbito de la presente tesis, se ha caracterizado el gen *MC1R* en 697 pacientes de Parkinson, 519 pacientes de Alzheimer y 482 controles libres de enfermedades neurodegenerativas. Los estudios caso-control revelan que la variante de color de pelo rojo p.R160W incrementa el riesgo a desarrollar Parkinson (OR=2,26 IC.95%=1,09-4,69; p-valor=0,02; Anexo II) y la variante p.V92M aumenta el riesgo a Alzheimer (OR=1,97, IC.95%=1,09-3,58, p=0,018, Anexo III). Los resultados del trabajo I y los estudios caso-control indican que el gen *MC1R* tiene un papel en el riesgo a desarrollar enfermedades neurodegenerativas.

En su conjunto, estos resultados ponen de manifiesto la implicación del gen *MC1R* en el estrés oxidativo celular, sugiriendo que los individuos portadores de variantes no funcionales presentan unos niveles de estrés oxidativo basal superiores, lo cual incrementa el riesgo a desarrollar cáncer cutáneo y otras enfermedades. Por ello, sería de gran interés investigar estrategias de protección a estas enfermedades mediante mecanismos antioxidantes.

5. LA VARIANTE p.R163Q DEL GEN *MC1R* Y RIESGO A CÁNCER CUTÁNEO ASSOCIADO A LA RADIACIÓN UV CRÓNICA.

La exposición solar crónica se encuentra estrechamente relacionada con un subtipo especial de MM que aparece en zonas crónicamente fotoexpuestas y con elevado daño actínico, el Lentigo Maligno melanoma. Estos tipos de cáncer cutáneo considerados crónicamente fotoinducidos, se caracterizan por desarrollarse en zona altamente fotoexpuestas. Existe poca información de los componentes genéticos germinales que incrementan el riesgo a desarrollar este tipo de cáncer cutáneo. Aunque es ampliamente conocido el aumento del riesgo a cáncer cutáneo debido a variantes del gen *MC1R*, nunca se había evaluado, previa al estudio incluido en esta tesis, la hipótesis de que determinadas variantes puedan estar asociadas a un subtipo específico de melanoma.

En el estudio II se analizó las variantes del gen en base a las características histopatológicas de los tumores de melanoma de 1679 pacientes, permitiendo la inclusión de múltiples melanomas primarios del mismo paciente. Datos previos indican que los diferentes melanomas de un mismo paciente pueden considerarse como eventos independientes de la misma enfermedad [135]. Cabe destacar que su inclusión fue debida a que se disponía de la evaluación histopatológica de todos los tumores desarrollados por el mismo paciente.

En el estudio se observó un mayor número total de variantes y mayor número de variantes color de pelo rojo en los subtipos LMM y SSM, los cuales se asocian con una exposición a la radiación UV. Por otro lado, el estudio pone de manifiesto que ciertas variantes del *MC1R* se pueden encontrar intrínsicamente ligadas a un subtipo etiopatológico específico de MM, puesto que se detectó que la variante p.R163Q incrementa el riesgo a desarrollar melanomas asociados a una fotoexposición crónica (subtipo LMM). Estudios previos detectaron que la variante p.R163Q incrementa el riesgo a desarrollar lesiones asociadas a la radiación UV, tanto lesiones benignas como malignas (Tabla 4). En población asiática, donde p.R163Q es la variante más común, los portadores de dicha variantes presentan mayor riesgo a desarrollar efélides y lentigos solares [136]. Aunque la presencia de lentigos solares es además de un indicador de

fotoenvejecimiento, un fuerte factor predictivo de desarrollar LMM [137] (aumentando el riesgo hasta 16 veces), no se detecta una mayor frecuencia de LMMs en población asiática. Ésto podría deberse a que las poblaciones asiáticas muestran comportamientos de protección frente a la radiación UV mayores que las poblaciones caucásicas [138]. Por otra parte, los portadores de la variante p.R163Q de poblaciones europeas presentan un mayor riesgo a desarrollar cáncer de piel no melanoma [139].

MC1R	Característica	OR; IC 95%	p	Num.	ref.
R163Q	Efélides	2.08; 1.01–4.12	0.046	245	*Ref. 43*
	Lentigos solares	1.75; 1.13–2.75	0.024	245	*Ref. 43*
	Cáncer de piel no melanona (BCC)	1.76; 1.06–2.95	0.030	529* ; 533**	*Ref. 45*
	Cáncer de piel melanoma (LMM)	***2.16; 1.07-4.37***	***0.044***	***1679******	***Estudio II***

Tabla 4. Características fenotípicas asociadas a la variante p.R163Q

Nuestro estudio indica que los portadores de la variante p.R163Q presentan un mayor riesgo a desarrollar MMs en zonas de piel crónicamente expuestas a la radiación UV. Junto con los resultados obtenidos previamente, se puede considerar que la asociación detectada podría deberse en parte a un mayor fotoenvejecimiento y daño inducido por la radiación UV.

De forma interesante, la variante p.R163Q no tiene una repercusión fenotípica puesto que no tiene efecto significativo en la síntesis de eumelanina ([140]; Anexo I); por ello es considerada un pseudo-allelo. En el estudio tampoco se detectó una asociación estadísticamente significativa entre p.R163Q y características asociadas a la pigmentación (fototipo, color de pelo, color de ojos). Estos datos demuestran que la relevancia biológica de esta variante debe de estar relacionada con vías no canónicas en la que particípa el gen. *MC1R* puede activar la vía de señalización de las MAPquinasas

[141] y se ha descrito recientemente que la variante p.R163Q promueve una disminución selectiva de la activación de dicha vía [142].

Los resultados del estudio indican que la variante p.R163Q puede ser, al menos en parte, responsable del riesgo a desarrollar LMM en poblaciones con fenotipos no consideraros de riesgo (fototipos y color de pelo oscuro) mediante su papel en vías no relacionadas con la síntesis de melanina.

II. VÍAS MOLECULARES Y GENES ALTERADOS EN CÁNCER CUTÁNEO

1. IDENTIFICACIÓN DE GENES IMPLICADOS EN LA CARCINOGÉNESIS DEL CÁNCER CUTÁNEO INDEPENDIENTE DE LA RADIACIÓN UV.

El subtipo de melanoma lentiginoso acral (ALM) difiere de las otras formas de melanomas tanto en las alteraciones moleculares que adquiere como a su relación con la RUV. El trabajo IV de la tesis constituye la primera caracterización molecular de una serie de tumores de este subtipo de población mediterránea.

En el estudio no se detectó un aumento de variantes germinales en el gen *MC1R* en los pacientes con ALM respecto a la población control indicando, conjuntamente con los resultados del trabajo II, que este subtipo de MM no se encuentra asociado a la interacción entre la RUV y la pigmentación.

En MM, las mutaciones activadoras en *N-RAS* o *B-RAF* estimulan la proliferación celular mediante la activación constitutiva de la vía de señalización de las MAPquinasas. Curtin et al. identificaron que dichas alteraciones se correlacionan con el grado de daño inducido por la RUV y la localización del tumor primario [94]. Mientras que alteraciones en *B-RAF* se detectan mayoritariamente en tumores de zonas del cuerpo intermitentemente fotoexpuestas, mutaciones en el gen *N-RAS* se observan en melanomas de zonas con daño solar crónico. Sin embargo, estas alteraciones se han descrito también en el subtipo ALM (Tabla 5). En el trabajo de la presente tesis, se han observado mutaciones en el gen *N-RAS* en el 17.6% de los ALM analizados. En cambio, no se detectaron mutaciones en el gen *B-RAF* sugiriendo que otras alteraciones pueden

promover la activación de la proliferación celular. Un mecanismo postulado por Curtin et al. sería que la desregulación de la proliferación pueda deberse a ganancias de los genes *CCND1* o *CDK4*, los cuales se encuentran localizados distalmente en la misma vía de las MAPquinasas, lo que tendría el mismo efecto que alteraciones en *B-RAF* o *N-RAS* [94]. En nuestro estudio, la elevada frecuencia de amplificaciones en el gen *CCND1* (31.25%) y en menor medida en el gen *CDK4* (12.5%) podrían explicar la no detección de mutaciones en el gen *B-RAF*. Estos resultados muestran la necesidad de analizar el estado mutacional de los genes de la vía de las MAPquinasas y de otros genes como *CCND1* y *CDK4* en el mismo grupo de muestras. Pocos estudios han analizado de forma exhaustiva la presencia de estas alteraciones en la misma serie de tumores (Tabla 5).

Una característica molecular de los melanomas no relacionados con la radiación UV es la presencia de múltiples amplificaciones focales [143]. En el análisis de regiones candidatas, detectamos cuatro regiones alteradas de forma recurrente (1p13.2, 5p25, 11q13 y 20q13.3). La distribución de dichas alteraciones no sucede al azar y sugiere la existencia de tres subgrupos moleculares de ALM.

Las alteraciones en vías que estimulan la proliferación celular se consideran los eventos genéticos iniciales en el desarrollo del tumor [160]. Según nuestros resultados, en el desarrollo de ALMs, la desregulación de la proliferación celular podría resultar de alteraciones en los genes *N-RAS*, *AURKA* o *CCND1*, por lo que tumores con una de estas alteraciones tenderían a no presentar ninguna de las otras dos. Cabe destacar que las pérdidas cromosómicas del gen *CDKN2A,* implicado en el control del ciclo celular, es la alteración más frecuente en la serie de tumores estudiados y no se encuentra asociadas a ninguna de las ganancias génicas observadas.

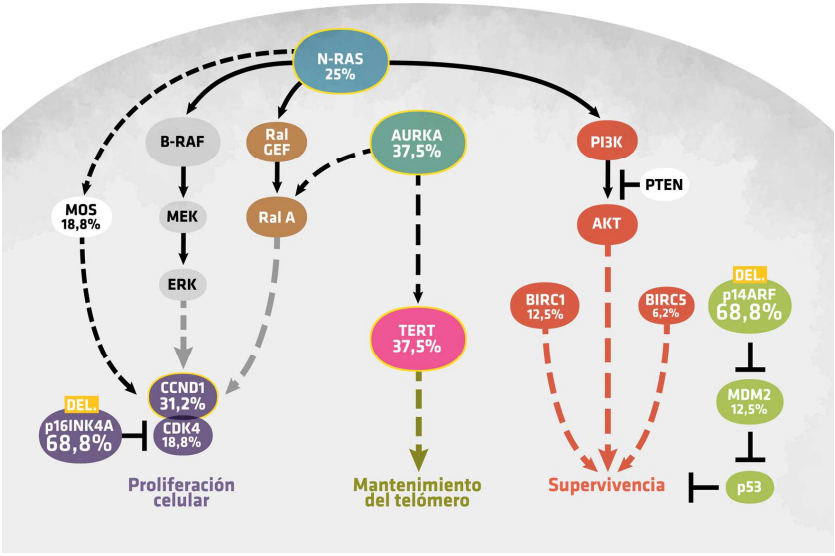
Las alteraciones en el gen *TERT* han sido consideradas como un segundo evento durante el desarrollo del MM [160]. Ganancias de este gen ya se habían descrito previamente tanto en líneas celulares de MM como en tumores [161], lo que promovería la activación de la actividad telomerasa. Sin embargo, la sobre-expresión de la proteína también podría conseguirse mediante otros mecanismos [162]. En células epiteliales de ovario y mama se ha observado que *AURKA* puede estimular la inducción del gen y por tanto de la actividad

telomerasa [162]. Por lo que la actividad telomerasa podría encontrarse inducida en los tumores ALM mediante amplificaciones del propio gen o por alteraciones en el gen *AURKA*.

% mut. *B-RAF*	**% mut. *N-RAS***	**% gan. *CCND1***	**País [a]**	**referencia**
No analizado	No analizado	47% (7/15)[amp]	EUA/ Alemania	[143]
No analizado	No analizado	44% cases (8/18)	EUA	[144]
No analizado	No analizado	22,7% (5/22)[amp]	EUA/ Alemania	[145]
13% (3/23)	No analizado	No analizado	USA/ Japó	[146]
50% (2/4)	25% (1/4)	No analizado	Alemania	[147]
33% (5/15)	0% (0/15)	No analizado	Japón	[148]
10.7% (3/28)[1]	3.6% (1/28)[1]	23.8% (5/21)[1]	Japón	[149]
25% (6/24)[2]	12.5% (3/24)[2]	29% (7/24)	USA/ Japón	[94]
15% (2/13)	No analizado	No analizado	Reino Unido	[150]
9.5% (2/21)	47.4% (9/19)	No analizado	Reino Unido	[97]
No analizado	No analizado	23.5% (4/17)	Suiza	[151]
25% (2/8)	16.7% (1/6)[4]	No analizado	Japón	[152]
16.7% (2/12)	11.1% (1/9)	No analizado	USA/ Japón	[153]
30% (7/23)	4% (1/23)	No analizado	USA	[154]
19.9% (34/171)	**6.25% (8/128)**	**29.3% (29/99)**		**Total de Estudios previos**
0% (0/17)	***17.6% (3/17)***	***31.25% (5/16)***	***España***	***Trabajo IV***

Tabla 5: Estado de los genes *B-RAF*, *N-RAS* y *CCND1* en melanoma lentiginoso acral: *mut:* mutaciones; *gan:* ganancias. a: Origen de las muestras considerando la localización del biobanco o Institución donde se llevó a cabo el estudio. Amp: Amplificación de la region 11q13 detectada por hibridación genómica comparada (CGH). 1: El estudio incluye 13 tumores primarios acrales y 15 metástasis. 2: Tumores de localizaciones acrales clasificados histopatológicamente como melanomas de extensión superficial.

A partir de los resultados de esta tesis del trabajo III, se ha validado el papel de las alteraciones de *CCND1*, *TERT* y *AURKA* en una serie de 58 tumores de ALM (34 tumores ALM invasivos y 24 nevus acrales) mediante la técnica de hibridacion de fluorescencia in situ (FISH). El estudio confirma que los genes *CCND1*, *TERT* y *AURKA* no se encuentran alterados en los nevus acrales, por lo que dichas alteraciones deben estar implicadas en la progresión a formas neoplásicas más diferenciadas (ANEXO IV). Además el estudio revela que el kit comercial de diagnostico de melanoma (Vysis Melanoma FISH Probe Kit, Abbot S.A) mejora su sensibilidad de diagnóstico al incorporar el estado de los genes *TERT* y *AURKA* (el kit contiene una sonda para evaluar el gen *CCND1*).



Alteraciones detectadas en melanomas acrales lentigionosos: se indica la frecuencia de cada alteración y relación entre genes, relaciones directas (líneas continuas) e indirectas (líneas discontínuas).

2. IDENTIFICACIÓN DE GENES IMPLICADOS EN CÁNCER CUTÁNEO FOTOINDUCIDO.

Los mecanismos de reparación del ADN tienen una función crítica en la recuperación del fenotipo celular normal en células dañadas debido a la exposición UV crónica, como las que se encuentran en el campo de cancerización cutáneo. El trabajo IV evaluó los cambios moleculares que se producían en la recuperación de piel dañada mediante la aplicación tópica de un compuesto comercial que contenía fotoliasa. El diseño del estudio

permite identificar genes claves en dicha recuperación y por tanto que deben encontrarse desregulados durante el proceso carcinogénico.

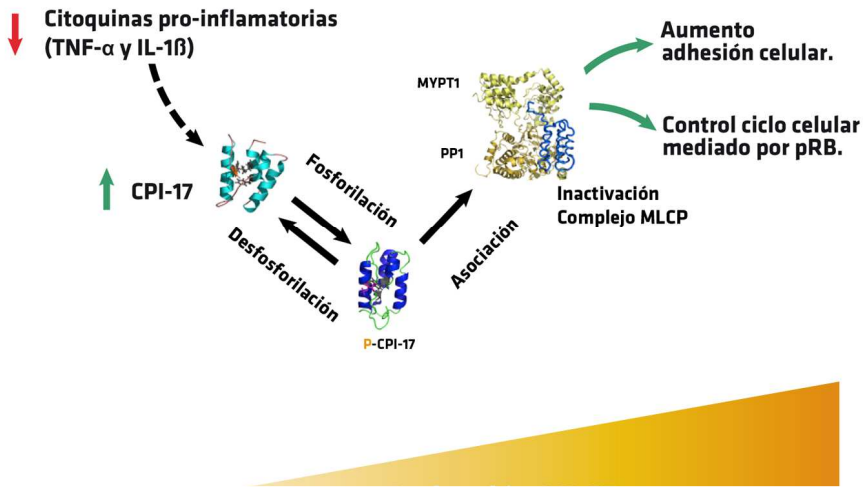
El estudio identificó una sobre-expresión de los genes *CPI-17* y *WDR72* asociado a la recuperación del fenotipo normal. Sin embargo, solo la expresión del gen *CPI-17* fué revalidada mediante RT-PCR.

CPI-17 codifica el inhibidor específico de PP1 (proteina fosfatasa 1) isoforma delta (PP1δ), la cual es una de las principales fosfatasas Serina/Treonina en las células de los mamiferos. El papel de *CPI-17* ha sido ampliamente estudiado en la contracción del músculo liso. La activación de CIP-17 promueve la supresión del complejo MYPT1-PP1δ. CIP-17 reconoce selectivamente la PP1δ asociada a MYPT1 (myosin phosphatase-targeting subunit-1) lo que resulta en una inhibición del MLCP, esta inhibición promueve la fosforilación de la miosina y por tanto, la contracción muscular [163]. La expresión de *CIP-17* se ha detectado en múltiples tipos celulares [164, 165] por lo que esta proteína puede estar implicada en otros procesos biológicos [166, 167]. En las células HeLa, la inhibición de MYTP1 también resulta en una acumulación de la miosina en su forma activa lo que conduce a unas adhesiones focales más destacadas produciendo una abolición total de la migración celular de dichas células [168]. Además, estas células y otros tipos celulares como los fibroblastos o los queratinocitos muestran un enriquecimiento de PP1δ en las adhesiones focales [169]. Estas evidencias indican que el complejo MYPT1-PP1δ puede estar implicado en la regulación de las funciones de las adhesiones focales. De hecho, se ha descrito una asociación de PP1δ con las quinasas de adhesión focal (FAK) [170]. En el trabajo IV encontramos una sobre-representación de funciones biológicas como las adhesiones focales, homeostasis celular o señalización célula-célula en las biopsias de piel de los pacientes con una respuesta rápida al tratamiento, lo que sugiere que *CPI-17* es esencial para recuperar el fenotipo normal en el campo de cancerización de las queratosis actínicas restaurando el control sobre la organización celular, adhesión celular y migración.

La desregulación del ciclo celular es un evento crítico en el proceso carcinogénico cutáneo. Alteraciones en el mecanismo molecular de la activación de la proteína del

retinoblastoma (pRb) como por ejemplo la sobre-expresión de ciclina D1 (*CCND1*) y la pérdida del inhibidor de ciclo celular son procesos que ocurren en las queratosis actínicas [171]. En su estado activo, la pRb es fosforilada y es capaz de actuar como un supresor de tumores inhibiendo la procesión del ciclo celular. Aunque el papel de las proteínas fosfatasas es menos conocido en este proceso, parece razonable pensar que la desfosforilación de la proteína pRB puede regular el complejo MYPT1-PP1δ [172]. Por lo que el restablecimiento del control del ciclo celular en las queratosis actínicas estaría también mediado por la actividad de *CPI-17* la cual podría promover la actividad de pRB. De forma interesante, todos los pacientes con una respuesta rápida al tratamiento, presentaban un nivel superior de expresión de *CPI-17* antes del tratamiento (ANEXO V).

Cambios en los niveles de expresión del gen pueden ser causados por condiciones patológicas como la inflamación [163, 173]. Nuestro trabajo indica que la inflamación modula la expresión de *CPI-17* en las queratosis actínicas. La evaluación de las citoquinas pro-inflamatorias mostró que las lesiones con baja expresión de IL-1B y *TNF* presentaban niveles altos de *CPI-17* antes del tratamiento comparado con los pacientes con una respuesta parcial. Por lo que el perfil antes del tratamiento de altos niveles de *CPI-17* y bajos niveles de *IL-1B* y *TNF* se asocia a una mejor respuesta al tratamiento.



Mecanismo de acción del gen *CIP-17* en el campo de cancerización

En base a los resultados obtenidos, postulamos que la inflamación del tejido cutáneo debida a procesos biológicos como el daño al DNA o estrés oxidativo causaría una infra-regulación de la expresión de *CPI-17* en las queratosis actínicas lo cual conduciría a una actividad incontrolada del complejo MYPT1-PP1δ. Además la activad fosfatasa desregulada podría afectar diferentes procesos biológico como son la motilidad y adhesión celular y el control del ciclo celular mediado por pRB.

Este estudio indica que el gen *CPI-17* tiene un papel destacado en la reconstitución de la homeostasis cutánea y remarca la importancia de las fosfatasas en los procesos carcinogénicos cutáneos.

III. ESTRATÉGIAS DE ANÁLISIS DE LA EXPRESIÓN GÉNICA: ARRAYS DE EXPRESIÓN.

En los trabajos I y IV se han utilizado técnicas de genética molecular convencionales como PCR en tiempo real para analizar la expresión génica de determinados genes. Esta técnica se basa en el estudio de genes de forma aislada sin englobar la interación que tiene lugar en la célula entre múltiples genes. Junto a ésta, se ha utilizado la tecnología de "microarray"desarrollada e implementada en el campo de la genética molecular durante la realización de la tesis.

Las tecnologías de alto rendimiento basadas en la tecnólogia "microarray" han cambiado de forma sustancial la habilidad para estudiar las bases moleculares de las células y/o tejidos sanos o enfermos. Los "arrays" de expresión permiten analizar en una muestra determinada la expresión de miles de tránscritos simultáneamente, generando una enorme cantidad de datos (el array utilizado en los trabajos de esta tesis incluye 19.596 transcritosN). Este hecho hace que sea indispensable el uso de herramientas bioinformáticas para el análisis y posterior interpetación de los resultados. El proceso inicial es la normalización de los datos obtenidos, con el objetivo de reducir la variabilidad experimental entre los diferentes puntos del array y manteniendo la variabilidad biológica [174]. Posteriormente, se realizan los análisis de expresión diferencial mediante estadísticos como los t-test, test no-parametricos y modelos

Bayesanos para obtener una lista de genes diferencialmente expresados entre condiciones experimentales.

Existen algunas limitaciones destacables de esta tecnología, como son la capacidad de detección y cuantificación precisa de transcritos poco abundantes, el ruido biológico que puede aparecer al analizar un número pequeño de muestras o limitaciones metodológicas como los procedimientos de corrección estrictos de los p-valores crudos con el fin de evitar resultados falsos positivos[175]. Por lo que, cambios en condiciones experimentales no siempre dan lugar a variaciones significativas en el nivel de expresión. Un ejemplo de ello son las diferencias entre los trabajos I y IV en cuanto al número de transcritos que alcanzaron un nivel de significación estadística en el análisis de expresión diferencial. Mientras que en el trabajo I se observó un elevado número de tránscritos desregulados; en el trabajo IV no se detectaron genes diferencialmente expresados entre las muestras antes y después del tratamiento, e incluso en el análisis según el tipo de respuesta al tratamiento sólo 2 genes alcanzaron la significación estadística (revalidando mediante RT-PCR el gen *CPI-17).*

Por otro lado, puede darse el caso de que múltiples genes de una vía molecular determinada se encuentren desregulados sin alcanzar de forma individual un valor estadísticamente significativo, pero sí que en conjunto promuevan un efecto biológico mediante la regulación de dicha vía. En base a este este concepto, y dado que los análisis de expresión diferencial son útiles para describir los canvios de expresión génica pero tienen un uso limitado para entender los mecanismos subyacentes que gobiernan la red de interacciones moleculares en un contexto celular, se han desarrollado los llamados análisis de vías ó de enriquecimento funcional. Estos análisis se basan en la clasificación de los genes en base a sus diferencias en la expresión (p-valores crudos) utilizando para cada transcrito anotaciones funcionales. Existen dos recursos principales de información funcional: la ontología genómica la cual clasifica jerarquicamente los genes en base a su función o proceso biológico (*http://www. geneontology.org/*; [176]) o bases de datos focalizadas en las vías moleculares como KEEG (*http://www.genome.ad.jp/kegg/*;[177]) o GenMAPP (*http://www.genmapp.org;* [178]).

El uso de un análisis de enriquecimiento funcional, permitió detectar en el trabajo IV procesos biológicos sobrerepresentados de forma común en las muestras incluídas (lesiones antes del tratamiento y lesiones con una mejora completa ó parcial después del tratamiento) independientemente de no detectar genes diferencialmente expresados y de la heterogeneidad intramuestral observada inicialmente (análisis de componentes principales, ANEXO V). En el trabajo I, dichas herramientas identificaron vías moleculares asociadas tanto al gen *CDKN2A* (vía de señalización de Notch) como al gen *MC1R* (vías asociadas al estrés oxidativo o enfermedades neurodegenerativas entre otras). Este tipo de información no se detecta mediante un análisis de expresión diferencial, lo que pone de manifiesto el potencial de estas técnicas en identificar mecanismos biológicos relacionados con una condición experimental determinada.

Por último, la utilización de los "arrays" de expresión ha generado, durante los últimos años, una enorme cantidad de datos la mayoria de los cuales se encuentran almacenados en repositorios de libre acceso como son la SMD (*http://genome-www.stanford.edu/microarray*; [179]), la GEO (*http://www.ncbi.nlm.nih.gov/geo;* [180]) o la ArrayExpress (*http://www.ebi.ac.uk/arrayexpress*; [181]). La libre utilización de los datos almacenados en estos repositorios supone un gran recurso para analizar eficazmente hipótesis sin un coste económico, así como para revalidar resultados en otros grupos de muestras. Un claro ejemplo de la utilidad de los análisis *in-silico* es el trabajo I en el que se utilizó datos de expresión génica previamente publicados.

CONCLUSIONES

1. La presencia de alteraciones germinales en el gen *CDKN2A* o la presencia de variantes no funcionales en el gen *MC1R* promueven la desregulación del transcriptoma de las células cutáneas, confiriendo unas características optimas para su transformación maligna, por lo que puede considerarse como un evento clave e inicial en la carcinogénesis.

2. Los patrones de expresión causados por mutaciones/variantes en los genes *CDKN2A* y *MC1R* se mantienen durante el proceso de progresión del cáncer cutáneo.

3. Los portadores de variantes no funcionales en el gen *MC1R* presentan un mayor estrés oxidativo basal el cual incrementa el riesgo a desarrollar cáncer cutáneo independientemente del efecto de dichas variantes en las características fenotípicas asociadas a la pigmentación, así como a enfermedades neurodegenerativas.

4. La variante p.R163Q del gen *MC1R* incrementa el riesgo a desarrollar melanomas asociados a la exposición solar crónica mediante mecanismos independientes al proceso de pigmentación.

5. Siendo la activación de la ciclia D1 una de las vías moleculares fundamentales en la activación de la vía MAPquinasas en los melanomas acrales, independientes de la exposición al sol, está presente en solamente un tercio de los tumores.

6. Las alteraciones en el gen *AURKA* y *TERT* promueven la carcinogénesis en los melanomas no asociados a la radiación UV (melanomas acrales) sin alteraciones en el gen *CCND1*.

7. El gen *CPI-17* es clave para el restablecimiento de la homeostasis de la piel en el campo de cancerización mediante su función en el control de las adhesiones focales intracelulares.

REFERENCIAS

1. Buettner PG and Raasch BA. Incidence rates of skin cancer in Townsville, Australia. Int J Cancer. 1998; 78(5):587-593.
2. Lo JS, Snow SN, Reizner GT, Mohs FE, Larson PO and Hruza GJ. Metastatic basal cell carcinoma: report of twelve cases with a review of the literature. J Am Acad Dermatol. 1991; 24(5 Pt 1):715-719.
3. Robinson JK and Dahiya M. Basal cell carcinoma with pulmonary and lymph node metastasis causing death. Arch Dermatol. 2003; 139(5):643-648.
4. von Domarus H and Stevens PJ. Metastatic basal cell carcinoma. Report of five cases and review of 170 cases in the literature. J Am Acad Dermatol. 1984; 10(6):1043-1060.
5. Boyd AS, Shyr Y and King LE, Jr. Basal cell carcinoma in young women: an evaluation of the association of tanning bed use and smoking. J Am Acad Dermatol. 2002; 46(5):706-709.
6. Khanna M, Fortier-Riberdy G, Smoller B and Dinehart S. Reporting tumor thickness for cutaneous squamous cell carcinoma. J Cutan Pathol. 2002; 29(6):321-323.
7. Fitzpatrick TB. The validity and practicality of sun-reactive skin types I through VI. Arch Dermatol. 1988; 124(6):869-871.
8. Gandini S, Sera F, Cattaruzza MS, Pasquini P, Zanetti R, Masini C, Boyle P and Melchi CF. Meta-analysis of risk factors for cutaneous melanoma: III. Family history, actinic damage and phenotypic factors. Eur J Cancer. 2005; 41(14):2040-2059.
9. Garbe C and Leiter U. Melanoma epidemiology and trends. Clin Dermatol. 2009; 27(1):3-9.
10. Chen ST, Geller AC and Tsao H. Update on the Epidemiology of Melanoma. Curr Dermatol Rep. 2013; 2(1):24-34.
11. Jemal A, Saraiya M, Patel P, Cherala SS, Barnholtz-Sloan J, Kim J, Wiggins CL and Wingo PA. Recent trends in cutaneous melanoma incidence and death rates in the United States, 1992-2006. J Am Acad Dermatol. 2013; 65(5 Suppl 1):S17-25 e11-13.
12. Eisemann N, Waldmann A, Geller AC, Weinstock MA, Volkmer B, Greinert R, Breitbart EW and Katalinic A. Non-Melanoma Skin Cancer Incidence and Impact of Skin Cancer Screening on Incidence. J Invest Dermatol. 2013.
13. Lewis B and Rose G. Prevention of coronary heart disease: putting theory into practice. J R Coll Physicians Lond. 1991; 25(1):21-26.
14. Wang SQ, Setlow R, Berwick M, Polsky D, Marghoob AA, Kopf AW and Bart RS. Ultraviolet A and melanoma: a review. J Am Acad Dermatol. 2001; 44(5):837-846.
15. Bataille V, Winnett A, Sasieni P, Newton Bishop JA and Cuzick J. Exposure to the sun and sunbeds and the risk of cutaneous melanoma in the UK: a case-control study. Eur J Cancer. 2004; 40(3):429-435.
16. Green A, Beardmore G, Hart V, Leslie D, Marks R and Staines D. Skin cancer in a Queensland population. J Am Acad Dermatol. 1988; 19(6):1045-1052.
17. de Vries E, Trakatelli M, Kalabalikis D, Ferrandiz L, Ruiz-de-Casas A, Moreno-Ramirez D, Sotiriadis D, Ioannides D, Aquilina S, Apap C, Micallef R, Scerri L, Ulrich M, Pitkanen S, Saksela O, Altsitsiadis E, et al. Known and potential new risk factors for skin cancer in European populations: a multicentre case-control study. Br J Dermatol. 2013; 167 Suppl 2:1-13.
18. Green A. A theory of site distribution of melanomas: Queensland, Australia. Cancer Causes Control. 1992; 3(6):513-516.
19. Alexandrescu DT, Maslin B, Kauffman CL, Ichim TE and Dasanu CA. Malignant melanoma in pigmented skin: does the current interventional model fit a different clinical, histologic, and molecular entity? Dermatol Surg. 2013; 39(9):1291-1303.
20. Traianou A, Ulrich M, Apalla Z, De Vries E, Bakirtzi K, Kalabalikis D, Ferrandiz L, Ruiz-de-Casas A, Moreno-Ramirez D, Sotiriadis D, Ioannides D, Aquilina S, Apap C, Micallef R, Scerri L, Pitkanen S, et al. Risk factors for actinic keratosis in eight European centres: a case-control study. Br J Dermatol. 2013; 167 Suppl 2:36-42.

21. Fu W and Cockerell CJ. The actinic (solar) keratosis: a 21st-century perspective. Arch Dermatol. 2003; 139(1):66-70.
22. Marks R, Rennie G and Selwood TS. Malignant transformation of solar keratoses to squamous cell carcinoma. Lancet. 1988; 1(8589):795-797.
23. Glogau RG. The risk of progression to invasive disease. J Am Acad Dermatol. 2000; 42(1 Pt 2):23-24.
24. Salasche SJ. Epidemiology of actinic keratoses and squamous cell carcinoma. J Am Acad Dermatol. 2000; 42(1 Pt 2):4-7.
25. Mittelbronn MA, Mullins DL, Ramos-Caro FA and Flowers FP. Frequency of pre-existing actinic keratosis in cutaneous squamous cell carcinoma. Int J Dermatol. 1998; 37(9):677-681.
26. Hurwitz RM and Monger LE. Solar keratosis: an evolving squamous cell carcinoma. Benign or malignant? Dermatol Surg. 1995; 21(2):184.
27. Heaphy MR, Jr. and Ackerman AB. The nature of solar keratosis: a critical review in historical perspective. J Am Acad Dermatol. 2000; 43(1 Pt 1):138-150.
28. Person JR. An actinic keratosis is neither malignant nor premalignant: it is an initiated tumor. J Am Acad Dermatol. 2003; 48(4):637-638.
29. Twombly R. New carcinogen list includes estrogen, UV radiation. J Natl Cancer Inst. 2003; 95(3):185-186.
30. Kanavy HE and Gerstenblith MR. Ultraviolet radiation and melanoma. Semin Cutan Med Surg. 2013; 30(4):222-228.
31. Godar DE. Worldwide increasing incidences of cutaneous malignant melanoma. J Skin Cancer. 2013; 2011:858425.
32. Bikle DD. Protective actions of vitamin D in UVB induced skin cancer. Photochem Photobiol Sci. 2012; 11(12):1808-1816.
33. Burnett ME and Wang SQ. Current sunscreen controversies: a critical review. Photodermatol Photoimmunol Photomed. 2013; 27(2):58-67.
34. Berwick M, Armstrong BK, Ben-Porat L, Fine J, Kricker A, Eberle C and Barnhill R. Sun exposure and mortality from melanoma. J Natl Cancer Inst. 2005; 97(3):195-199.
35. Melnikova VO and Ananthaswamy HN. Cellular and molecular events leading to the development of skin cancer. Mutat Res. 2005; 571(1-2):91-106.
36. Fernandez AA, Paniker L, Garcia R and Mitchell DL. Recent advances in sunlight-induced carcinogenesis using the Xiphophorus melanoma model. Comp Biochem Physiol C Toxicol Pharmacol. 2011; 155(1):64-70.
37. Cadet J, Sage E and Douki T. Ultraviolet radiation-mediated damage to cellular DNA. Mutat Res. 2005; 571(1-2):3-17.
38. Moyal DD and Fourtanier AM. Broad-spectrum sunscreens provide better protection from solar ultraviolet-simulated radiation and natural sunlight-induced immunosuppression in human beings. J Am Acad Dermatol. 2008; 58(5 Suppl 2):S149-154.
39. Mouret S, Leccia MT, Bourrain JL, Douki T and Beani JC. Individual photosensitivity of human skin and UVA-induced pyrimidine dimers in DNA. J Invest Dermatol. 2013; 131(7):1539-1546.
40. Afaq F, Adhami VM and Mukhtar H. Photochemoprevention of ultraviolet B signaling and photocarcinogenesis. Mutat Res. 2005; 571(1-2):153-173.
41. Rees JL. The genetics of sun sensitivity in humans. Am J Hum Genet. 2004; 75(5):739-751.
42. Friedberg EC. How nucleotide excision repair protects against cancer. Nat Rev Cancer. 2001; 1(1):22-33.
43. Placzek M, Przybilla B, Kerkmann U, Gaube S and Gilbertz KP. Effect of ultraviolet (UV) A, UVB or ionizing radiation on the cell cycle of human melanoma cells. Br J Dermatol. 2007; 156(5):843-847.
44. Bertram CG, Gaut RM, Barrett JH, Randerson-Moor J, Whitaker L, Turner F, Bataille V, dos Santos Silva I, Swerdlow AJ, Bishop DT and Newton Bishop JA. An assessment of a variant of

the DNA repair gene XRCC3 as a possible nevus or melanoma susceptibility genotype. J Invest Dermatol. 2004; 122(2):429-432.
45. McHugh PJ, Spanswick VJ and Hartley JA. Repair of DNA interstrand crosslinks: molecular mechanisms and clinical relevance. Lancet Oncol. 2001; 2(8):483-490.
46. Zhao C, Snellman E, Jansen CT and Hemminki K. In situ repair of cyclobutane pyrimidine dimers in skin and melanocytic nevi of cutaneous melanoma patients. Int J Cancer. 2002; 98(3):331-334.
47. Pavey S and Gabrielli B. Alpha-melanocyte stimulating hormone potentiates p16/CDKN2A expression in human skin after ultraviolet irradiation. Cancer Res. 2002; 62(3):875-880.
48. Gruis NA and van Doorn R. Melanocortin 1 receptor function: shifting gears from determining skin and nevus phenotype to fetal growth. J Invest Dermatol. 2013; 132(8):1953-1955.
49. Mouret S, Forestier A and Douki T. The specificity of UVA-induced DNA damage in human melanocytes. Photochem Photobiol Sci. 2013; 11(1):155-162.
50. Bohr VA, Smith CA, Okumoto DS and Hanawalt PC. DNA repair in an active gene: removal of pyrimidine dimers from the DHFR gene of CHO cells is much more efficient than in the genome overall. Cell. 1985; 40(2):359-369.
51. Mitchell DL. The relative cytotoxicity of (6-4) photoproducts and cyclobutane dimers in mammalian cells. Photochem Photobiol. 1988; 48(1):51-57.
52. Essen LO and Klar T. Light-driven DNA repair by photolyases. Cell Mol Life Sci. 2006; 63(11):1266-1277.
53. Schul W, Jans J, Rijksen YM, Klemann KH, Eker AP, de Wit J, Nikaido O, Nakajima S, Yasui A, Hoeijmakers JH and van der Horst GT. Enhanced repair of cyclobutane pyrimidine dimers and improved UV resistance in photolyase transgenic mice. EMBO J. 2002; 21(17):4719-4729.
54. Jans J, Schul W, Sert YG, Rijksen Y, Rebel H, Eker AP, Nakajima S, van Steeg H, de Gruijl FR, Yasui A, Hoeijmakers JH and van der Horst GT. Powerful skin cancer protection by a CPD-photolyase transgene. Curr Biol. 2005; 15(2):105-115.
55. Stege H, Roza L, Vink AA, Grewe M, Ruzicka T, Grether-Beck S and Krutmann J. Enzyme plus light therapy to repair DNA damage in ultraviolet-B-irradiated human skin. Proc Natl Acad Sci U S A. 2000; 97(4):1790-1795.
56. Nikolaou V, Stratigos AJ and Tsao H. Hereditary nonmelanoma skin cancer. Semin Cutan Med Surg. 2013; 31(4):204-210.
57. Njauw CN, Kim I, Piris A, Gabree M, Taylor M, Lane AM, DeAngelis MM, Gragoudas E, Duncan LM and Tsao H. Germline BAP1 inactivation is preferentially associated with metastatic ocular melanoma and cutaneous-ocular melanoma families. PLoS One. 2013; 7(4):e35295.
58. Sturm RA. A golden age of human pigmentation genetics. Trends Genet. 2006; 22(9):464-468.
59. Rees JL. Genetics of hair and skin color. Annu Rev Genet. 2003; 37:67-90.
60. Sturm RA and Frudakis TN. Eye colour: portals into pigmentation genes and ancestry. Trends Genet. 2004; 20(8):327-332.
61. Ito S and Wakamatsu K. Quantitative analysis of eumelanin and pheomelanin in humans, mice, and other animals: a comparative review. Pigment Cell Res. 2003; 16(5):523-531.
62. Leonard JH, Marks LH, Chen W, Cook AL, Boyle GM, Smit DJ, Brown DL, Stow JL, Parsons PG and Sturm RA. Screening of human primary melanocytes of defined melanocortin-1 receptor genotype: pigmentation marker, ultrastructural and UV-survival studies. Pigment Cell Res. 2003; 16(3):198-207.
63. Dessinioti C, Antoniou C, Katsambas A and Stratigos AJ. Melanocortin 1 receptor variants: functional role and pigmentary associations. Photochem Photobiol. 2013; 87(5):978-987.
64. Schiaffino MV. Signaling pathways in melanosome biogenesis and pathology. Int J Biochem Cell Biol. 2013; 42(7):1094-1104.
65. Kosiniak-Kamysz A, Pospiech E, Wojas-Pelc A, Marcinska M and Branicki W. Potential association of single nucleotide polymorphisms in pigmentation genes with the development of basal cell carcinoma. J Dermatol. 2012; 39(8):693-698.

66. Nag M and Nandi N. Effect of three organophosphates on respiration in rat brain and liver tissue. Biosci Rep. 1991; 11(1):7-10.
67. Bastiaens MT, ter Huurne JA, Kielich C, Gruis NA, Westendorp RG, Vermeer BJ and Bavinck JN. Melanocortin-1 receptor gene variants determine the risk of nonmelanoma skin cancer independently of fair skin and red hair. Am J Hum Genet. 2001; 68(4):884-894.
68. Andresen PA, Nymoen DA, Kjaerheim K, Leivestad T and Helsing P. Susceptibility to Cutaneous Squamous Cell Carcinoma in Renal Transplant Recipients Associates with Genes Regulating Melanogenesis Independent of their Role in Pigmentation. Biomark Cancer. 2013; 5:41-47.
69. Liboutet M, Portela M, Delestaing G, Vilmer C, Dupin N, Gorin I, Saiag P, Lebbe C, Kerob D, Dubertret L, Grandchamp B, Basset-Seguin N and Soufir N. MC1R and PTCH gene polymorphism in French patients with basal cell carcinomas. J Invest Dermatol. 2006; 126(7):1510-1517.
70. Demenais F, Mohamdi H, Chaudru V, Goldstein AM, Newton Bishop JA, Bishop DT, Kanetsky PA, Hayward NK, Gillanders E, Elder DE, Avril MF, Azizi E, van Belle P, Bergman W, Bianchi-Scarra G, Bressac-de Paillerets B, et al. Association of MC1R variants and host phenotypes with melanoma risk in CDKN2A mutation carriers: a GenoMEL study. J Natl Cancer Inst. 2010; 102(20):1568-1583.
71. Kanetsky PA, Panossian S, Elder DE, Guerry D, Ming ME, Schuchter L and Rebbeck TR. Does MC1R genotype convey information about melanoma risk beyond risk phenotypes? Cancer. 2010; 116(10):2416-2428.
72. Serrano M, Hannon GJ and Beach D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. Nature. 1993; 366(6456):704-707.
73. Serrano M, Lee H, Chin L, Cordon-Cardo C, Beach D and DePinho RA. Role of the INK4a locus in tumor suppression and cell mortality. Cell. 1996; 85(1):27-37.
74. Honda R and Yasuda H. Association of p19(ARF) with Mdm2 inhibits ubiquitin ligase activity of Mdm2 for tumor suppressor p53. EMBO J. 1999; 18(1):22-27.
75. Rizos H, Puig S, Badenas C, Malvehy J, Darmanian AP, Jimenez L, Mila M and Kefford RF. A melanoma-associated germline mutation in exon 1beta inactivates p14ARF. Oncogene. 2001; 20(39):5543-5547.
76. Bishop DT, Demenais F, Goldstein AM, Bergman W, Bishop JN, Bressac-de Paillerets B, Chompret A, Ghiorzo P, Gruis N, Hansson J, Harland M, Hayward N, Holland EA, Mann GJ, Mantelli M, Nancarrow D, et al. Geographical variation in the penetrance of CDKN2A mutations for melanoma. J Natl Cancer Inst. 2002; 94(12):894-903.
77. Ruiz A, Puig S, Malvehy J, Lazaro C, Lynch M, Gimenez-Arnau AM, Puig L, Sanchez-Conejo J, Estivill X and Castel T. CDKN2A mutations in Spanish cutaneous malignant melanoma families and patients with multiple melanomas and other neoplasia. J Med Genet. 1999; 36(6):490-493.
78. Puig S, Malvehy J, Badenas C, Ruiz A, Jimenez D, Cuellar F, Azon A, Gonzalez U, Castel T, Campoy A, Herrero J, Marti R, Brunet-Vidal J and Mila M. Role of the CDKN2A locus in patients with multiple primary melanomas. J Clin Oncol. 2005; 23(13):3043-3051.
79. Kumar R, Smeds J, Berggren P, Straume O, Rozell BL, Akslen LA and Hemminki K. A single nucleotide polymorphism in the 3'untranslated region of the CDKN2A gene is common in sporadic primary melanomas but mutations in the CDKN2B, CDKN2C, CDK4 and p53 genes are rare. Int J Cancer. 2001; 95(6):388-393.
80. Suarez C, Rodrigo JP, Ferlito A, Cabanillas R, Shaha AR and Rinaldo A. Tumours of familial origin in the head and neck. Oral Oncol. 2006; 42(10):965-978.
81. Cabanillas R, Astudillo A, Valle M, de la Rosa J, Alvarez R, Duran NS and Cadinanos J. Novel germline CDKN2A mutation associated with head and neck squamous cell carcinomas and melanomas. Head Neck. 2011; 35(3):E80-84.
82. Stacey SN, Sulem P, Masson G, Gudjonsson SA, Thorleifsson G, Jakobsdottir M, Sigurdsson A, Gudbjartsson DF, Sigurgeirsson B, Benediktsdottir KR, Thorisdottir K, Ragnarsson

R, Scherer D, Hemminki K, Rudnai P, Gurzau E, et al. New common variants affecting susceptibility to basal cell carcinoma. Nat Genet. 2009; 41(8):909-914.
83. Jefferies S, Edwards SM, Hamoudi RA, A'Hern R, Foulkes W, Goldgar D and Eeles R. No germline mutations in CDKN2A (p16) in patients with squamous cell cancer of the head and neck and second primary tumours. Br J Cancer. 2001; 85(9):1383-1386.
84. Nelson MA, Einspahr JG, Alberts DS, Balfour CA, Wymer JA, Welch KL, Salasche SJ, Bangert JL, Grogan TM and Bozzo PO. Analysis of the p53 gene in human precancerous actinic keratosis lesions and squamous cell cancers. Cancer Lett. 1994; 85(1):23-29.
85. Spencer JM, Kahn SM, Jiang W, DeLeo VA and Weinstein IB. Activated ras genes occur in human actinic keratoses, premalignant precursors to squamous cell carcinomas. Arch Dermatol. 1995; 131(7):796-800.
86. Rehman I, Quinn AG, Healy E and Rees JL. High frequency of loss of heterozygosity in actinic keratoses, a usually benign disease. Lancet. 1994; 344(8925):788-789.
87. Rehman I, Takata M, Wu YY and Rees JL. Genetic change in actinic keratoses. Oncogene. 1996; 12(12):2483-2490.
88. Kushida Y, Miki H and Ohmori M. Loss of heterozygosity in actinic keratosis, squamous cell carcinoma and sun-exposed normal-appearing skin in Japanese: difference between Japanese and Caucasians. Cancer Lett. 1999; 140(1-2):169-175.
89. Mortier L, Marchetti P, Delaporte E, Martin de Lassalle E, Thomas P, Piette F, Formstecher P, Polakowska R and Danze PM. Progression of actinic keratosis to squamous cell carcinoma of the skin correlates with deletion of the 9p21 region encoding the p16(INK4a) tumor suppressor. Cancer Lett. 2002; 176(2):205-214.
90. Kanellou P, Zaravinos A, Zioga M, Stratigos A, Baritaki S, Soufla G, Zoras O and Spandidos DA. Genomic instability, mutations and expression analysis of the tumour suppressor genes p14(ARF), p15(INK4b), p16(INK4a) and p53 in actinic keratosis. Cancer Lett. 2008; 264(1):145-161.
91. Gailani MR, Stahle-Backdahl M, Leffell DJ, Glynn M, Zaphiropoulos PG, Pressman C, Unden AB, Dean M, Brash DE, Bale AE and Toftgard R. The role of the human homologue of Drosophila patched in sporadic basal cell carcinomas. Nat Genet. 1996; 14(1):78-81.
92. Aszterbaum M, Beech J and Epstein EH, Jr. Ultraviolet radiation mutagenesis of hedgehog pathway genes in basal cell carcinomas. J Investig Dermatol Symp Proc. 1999; 4(1):41-45.
93. Kim MY, Park HJ, Baek SC, Byun DG and Houh D. Mutations of the p53 and PTCH gene in basal cell carcinomas: UV mutation signature and strand bias. J Dermatol Sci. 2002; 29(1):1-9.
94. Curtin JA, Fridlyand J, Kageshita T, Patel HN, Busam KJ, Kutzner H, Cho KH, Aiba S, Brocker EB, LeBoit PE, Pinkel D and Bastian BC. Distinct sets of genetic alterations in melanoma. N Engl J Med. 2005; 353(20):2135-2147.
95. Millington GW. Mutations of the BRAF gene in human cancer, by Davies et al. (Nature 2002; 417: 949-54). Clin Exp Dermatol. 38(2):222-223.
96. Rodolfo M, Daniotti M and Vallacchi V. Genetic progression of metastatic melanoma. Cancer Lett. 2004; 214(2):133-147.
97. Saldanha G, Potter L, Daforno P and Pringle JH. Cutaneous melanoma subtypes show different BRAF and NRAS mutation frequencies. Clin Cancer Res. 2006; 12(15):4499-4505.
98. Takata M, Goto Y, Ichii N, Yamaura M, Murata H, Koga H, Fujimoto A and Saida T. Constitutive activation of the mitogen-activated protein kinase signaling pathway in acral melanomas. J Invest Dermatol. 2005; 125(2):318-322.
99. Curtin JA, Busam K, Pinkel D and Bastian BC. Somatic activation of KIT in distinct subtypes of melanoma. J Clin Oncol. 2006; 24(26):4340-4346.
100. Bastian BC, LeBoit PE, Hamm H, Brocker EB and Pinkel D. Chromosomal gains and losses in primary cutaneous melanomas detected by comparative genomic hybridization. Cancer Res. 1998; 58(10):2170-2175.

101. Kumar R, Smeds J, Lundh Rozell B and Hemminki K. Loss of heterozygosity at chromosome 9p21 (INK4-p14ARF locus): homozygous deletions and mutations in the p16 and p14ARF genes in sporadic primary melanomas. Melanoma Res. 1999; 9(2):138-147.
102. Puig S, Ruiz A, Lazaro C, Castel T, Lynch M, Palou J, Vilalta A, Weissenbach J, Mascaro JM and Estivill X. Chromosome 9p deletions in cutaneous malignant melanoma tumors: the minimal deleted region involves markers outside the p16 (CDKN2) gene. Am J Hum Genet. 1995; 57(2):395-402.
103. Chaudru V, Laud K, Avril MF, Miniere A, Chompret A, Bressac-de Paillerets B and Demenais F. Melanocortin-1 receptor (MC1R) gene variants and dysplastic nevi modify penetrance of CDKN2A mutations in French melanoma-prone pedigrees. Cancer Epidemiol Biomarkers Prev. 2005; 14(10):2384-2390.
104. Bellacosa A, Godwin AK, Peri S, Devarajan K, Caretti E, Vanderveer L, Bove B, Slater C, Zhou Y, Daly M, Howard S, Campbell KS, Nicolas E, Yeung AT, Clapper ML, Crowell JA, et al. Altered gene expression in morphologically normal epithelial cells from heterozygous carriers of BRCA1 or BRCA2 mutations. Cancer Prev Res (Phila). 2010; 3(1):48-61.
105. Herbert BS, Chanoux RA, Liu Y, Baenziger PH, Goswami CP, McClintick JN, Edenberg HJ, Pennington RE, Lipkin SM and Kopelovich L. A molecular signature of normal breast epithelial and stromal cells from Li-Fraumeni syndrome mutation carriers. Oncotarget. 2012; 1(6):405-422.
106. Gu F, Qureshi AA, Niu T, Kraft P, Guo Q, Hunter DJ and Han J. Interleukin and interleukin receptor gene polymorphisms and susceptibility to melanoma. Melanoma Res. 2008; 18(5):330-335.
107. Howell WM, Turner SJ, Bateman AC and Theaker JM. IL-10 promoter polymorphisms influence tumour development in cutaneous malignant melanoma. Genes Immun. 2001; 2(1):25-31.
108. Yang XR, Pfeiffer RM, Wheeler W, Yeager M, Chanock S, Tucker MA and Goldstein AM. Identification of modifier genes for cutaneous malignant melanoma in melanoma-prone families with and without CDKN2A mutations. Int J Cancer. 2009; 125(12):2912-2917.
109. Guo J, Wen DR, Huang RR, Paul E, Wunsch P, Itakura E and Cochran AJ. Detection of multiple melanoma-associated markers in melanoma cell lines by RT in situ PCR. Exp Mol Pathol. 2003; 74(2):140-147.
110. Raposo G and Marks MS. The dark side of lysosome-related organelles: specialization of the endocytic pathway for melanosome biogenesis. Traffic. 2002; 3(4):237-248.
111. Ramanathan HN and Ye Y. The p97 ATPase associates with EEA1 to regulate the size of early endosomes. Cell Res. 2011; 22(2):346-359.
112. Sakai C, Kawakami Y, Law LW, Furumura M and Hearing VJ, Jr. Melanosomal proteins as melanoma-specific immune targets. Melanoma Res. 1997; 7(2):83-95.
113. Hearing VJ. Biochemical control of melanogenesis and melanosomal organization. J Investig Dermatol Symp Proc. 1999; 4(1):24-28.
114. Jonsson G, Busch C, Knappskog S, Geisler J, Miletic H, Ringner M, Lillehaug JR, Borg A and Lonning PE. Gene expression profiling-based identification of molecular subtypes in stage IV melanomas with different clinical outcome. Clin Cancer Res. 2013; 16(13):3356-3367.
115. Zhou CJ, Qu X, Yang YM, Wang FF, Dong ZQ, Wang CQ, Zhang XY, Liu GX, Wei FC and Sun SZ. CEACAM1 distribution and it's effects on angiogenesis and lymphangiogenesis in oral carcinoma. Oral Oncol. 2009; 45(10):883-886.
116. Beauchemin N and Arabzadeh A. Carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) in cancer progression and metastasis. Cancer Metastasis Rev. 2013; 32(3-4):643-671.
117. Chang YM, Newton-Bishop JA, Bishop DT, Armstrong BK, Bataille V, Bergman W, Berwick M, Bracci PM, Elwood JM, Ernstoff MS, Green AC, Gruis NA, Holly EA, Ingvar C, Kanetsky PA, Karagas MR, et al. A pooled analysis of melanocytic nevus phenotype and the risk of cutaneous melanoma at different latitudes. Int J Cancer. 2009; 124(2):420-428.

118. Bishop JA, Wachsmuth RC, Harland M, Bataille V, Pinney E, Mac KP, Baglietto L, Cuzick J and Bishop DT. Genotype/phenotype and penetrance studies in melanoma families with germline CDKN2A mutations. J Invest Dermatol. 2000; 114(1):28-33.
119. Schouwey K, Delmas V, Larue L, Zimber-Strobl U, Strobl LJ, Radtke F and Beermann F. Notch1 and Notch2 receptors influence progressive hair graying in a dose-dependent manner. Dev Dyn. 2007; 236(1):282-289.
120. Okuyama R, Tagami H and Aiba S. Notch signaling: its role in epidermal homeostasis and in the pathogenesis of skin diseases. J Dermatol Sci. 2008; 49(3):187-194.
121. Xin Y, Lu Q and Li Q. IKK1 control of epidermal differentiation is modulated by notch signaling. Am J Pathol. 2011; 178(4):1568-1577.
122. Lin HY, Kao CH, Lin KM, Kaartinen V and Yang LT. Notch signaling regulates late-stage epidermal differentiation and maintains postnatal hair cycle homeostasis. PLoS One. 2011; 6(1):e15842.
123. Hanahan D and Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011; 144(5):646-674.
124. Mitra D, Luo X, Morgan A, Wang J, Hoang MP, Lo J, Guerrero CR, Lennerz JK, Mihm MC, Wargo JA, Robinson KC, Devi SP, Vanover JC, D'Orazio JA, McMahon M, Bosenberg MW, et al. An ultraviolet-radiation-independent pathway to melanoma carcinogenesis in the red hair/fair skin background. Nature. 2012; 491(7424):449-453.
125. Catania A. Neuroprotective actions of melanocortins: a therapeutic opportunity. Trends Neurosci. 2008; 31(7):353-360.
126. Zhou C, Huang Y and Przedborski S. Oxidative stress in Parkinson's disease: a mechanism of pathogenic and therapeutic significance. Ann N Y Acad Sci. 2008; 1147:93-104.
127. Wang X, Wang W, Li L, Perry G, Lee HG and Zhu X. Oxidative stress and mitochondrial dysfunction in Alzheimer's disease. Biochim Biophys Acta. 2013.
128. Browne SE, Ferrante RJ and Beal MF. Oxidative stress in Huntington's disease. Brain Pathol. 1999; 9(1):147-163.
129. Liu R, Gao X, Lu Y and Chen H. Meta-analysis of the relationship between Parkinson disease and melanoma. Neurology. 2011; 76(23):2002-2009.
130. Olsen JH, Friis S and Frederiksen K. Malignant melanoma and other types of cancer preceding Parkinson disease. Epidemiology. 2006; 17(5):582-587.
131. Olsen JH, Friis S, Frederiksen K, McLaughlin JK, Mellemkjaer L and Moller H. Atypical cancer pattern in patients with Parkinson's disease. Br J Cancer. 2005; 92(1):201-205.
132. Gao X, Simon KC, Han J, Schwarzschild MA and Ascherio A. Genetic determinants of hair color and Parkinson's disease risk. Ann Neurol. 2009; 65(1):76-82.
133. Meng S, Song F, Chen H, Gao X, Amos CI, Lee JE, Wei Q, Qureshi AA and Han J. No association between Parkinson disease alleles and the risk of melanoma. Cancer Epidemiol Biomarkers Prev. 2011; 21(1):243-245.
134. Hassin-Baer S, Laitman Y, Azizi E, Molchadski I, Galore-Haskel G, Barak F, Cohen OS and Friedman E. The leucine rich repeat kinase 2 (LRRK2) G2019S substitution mutation. Association with Parkinson disease, malignant melanoma and prevalence in ethnic groups in Israel. J Neurol. 2009; 256(3):483-487.
135. Orlow I, Tommasi DV, Bloom B, Ostrovnaya I, Cotignola J, Mujumdar U, Busam KJ, Jungbluth AA, Scolyer RA, Thompson JF, Armstrong BK, Berwick M, Thomas NE and Begg CB. Evaluation of the clonal origin of multiple primary melanomas using molecular profiling. J Invest Dermatol. 2009; 129(8):1972-1982.
136. Motokawa T, Kato T, Hashimoto Y and Katagiri T. Effect of Val92Met and Arg163Gln variants of the MC1R gene on freckles and solar lentigines in Japanese. Pigment Cell Res. 2007; 20(2):140-143.
137. Kvaskoff M, Siskind V and Green AC. Risk factors for lentigo maligna melanoma compared with superficial spreading melanoma: a case-control study in Australia. Arch Dermatol. 2011; 148(2):164-170.

138. Cheng S, Lian S, Hao Y, Kang N, Li S, Nie Y and Zhang F. Sun-exposure knowledge and protection behavior in a North Chinese population: a questionnaire-based study. Photodermatol Photoimmunol Photomed. 2010; 26(4):177-181.
139. Scherer D, Bermejo JL, Rudnai P, Gurzau E, Koppova K, Hemminki K and Kumar R. MC1R variants associated susceptibility to basal cell carcinoma of skin: interaction with host factors and XRCC3 polymorphism. Int J Cancer. 2008; 122(8):1787-1793.
140. Wakamatsu K, Kavanagh R, Kadekaro AL, Terzieva S, Sturm RA, Leachman S, Abdel-Malek Z and Ito S. Diversity of pigmentation in cultured human melanocytes is due to differences in the type as well as quantity of melanin. Pigment Cell Res. 2006; 19(2):154-162.
141. Busca R, Abbe P, Mantoux F, Aberdam E, Peyssonnaux C, Eychene A, Ortonne JP and Ballotti R. Ras mediates the cAMP-dependent activation of extracellular signal-regulated kinases (ERKs) in melanocytes. EMBO J. 2000; 19(12):2900-2910.
142. Doyle JR, Fortin JP, Beinborn M and Kopin AS. Selected melanocortin 1 receptor single-nucleotide polymorphisms differentially alter multiple signaling pathways. J Pharmacol Exp Ther. 2012; 342(2):318-326.
143. Bastian BC, Kashani-Sabet M, Hamm H, Godfrey T, Moore DH, 2nd, Brocker EB, LeBoit PE and Pinkel D. Gene amplifications characterize acral melanoma and permit the detection of occult tumor cells in the surrounding skin. Cancer Res. 2000; 60(7):1968-1973.
144. Sauter ER, Yeo UC, von Stemm A, Zhu W, Litwin S, Tichansky DS, Pistritto G, Nesbit M, Pinkel D, Herlyn M and Bastian BC. Cyclin D1 is a candidate oncogene in cutaneous melanoma. Cancer Res. 2002; 62(11):3200-3206.
145. Bastian BC, Olshen AB, LeBoit PE and Pinkel D. Classifying melanocytic tumors based on DNA copy number changes. Am J Pathol. 2003; 163(5):1765-1770.
146. Maldonado JL, Fridlyand J, Patel H, Jain AN, Busam K, Kageshita T, Ono T, Albertson DG, Pinkel D and Bastian BC. Determinants of BRAF mutations in primary melanomas. J Natl Cancer Inst. 2003; 95(24):1878-1890.
147. Reifenberger J, Knobbe CB, Sterzinger AA, Blaschke B, Schulte KW, Ruzicka T and Reifenberger G. Frequent alterations of Ras signaling pathway genes in sporadic malignant melanomas. Int J Cancer. 2004; 109(3):377-384.
148. Sasaki Y, Niu C, Makino R, Kudo C, Sun C, Watanabe H, Matsunaga J, Takahashi K, Tagami H, Aiba S and Horii A. BRAF point mutations in primary melanoma show different prevalences by subtype. J Invest Dermatol. 2004; 123(1):177-183.
149. Liao YH, Hsu SM and Huang PH. ARMS depletion facilitates UV irradiation induced apoptotic cell death in melanoma. Cancer Res. 2007; 67(24):11547-11556.
150. Lang J and MacKie RM. Prevalence of exon 15 BRAF mutations in primary melanoma of the superficial spreading, nodular, acral, and lentigo maligna subtypes. J Invest Dermatol. 2005; 125(3):575-579.
151. Glatz-Krieger K, Pache M, Tapia C, Fuchs A, Savic S, Glatz D, Mihatsch M and Meyer P. Anatomic site-specific patterns of gene copy number gains in skin, mucosal, and uveal melanomas detected by fluorescence in situ hybridization. Virchows Arch. 2006; 449(3):328-333.
152. Takata M, Lin J, Takayanagi S, Suzuki T, Ansai S, Kimura T, Cerroni L and Saida T. Genetic and epigenetic alterations in the differential diagnosis of malignant melanoma and spitzoid lesion. Br J Dermatol. 2007; 156(6):1287-1294.
153. Beadling C, Jacobson-Dunlop E, Hodi FS, Le C, Warrick A, Patterson J, Town A, Harlow A, Cruz F, 3rd, Azar S, Rubin BP, Muller S, West R, Heinrich MC and Corless CL. KIT gene mutations and copy number in melanoma subtypes. Clin Cancer Res. 2008; 14(21):6821-6828.
154. Chernoff KA, Bordone L, Horst B, Simon K, Twadell W, Lee K, Cohen JA, Wang S, Silvers DN, Brunner G and Celebi JT. GAB2 amplifications refine molecular classification of melanoma. Clin Cancer Res. 2009; 15(13):4288-4291.
155. Koynova DK, Jordanova ES, Milev AD, Dijkman R, Kirov KS, Toncheva DI and Gruis NA. Gene-specific fluorescence in-situ hybridization analysis on tissue microarray to refine the region of chromosome 20q amplification in melanoma. Melanoma Res. 2007; 17(1):37-41.

156. Tanaka E, Hashimoto Y, Ito T, Okumura T, Kan T, Watanabe G, Imamura M, Inazawa J and Shimada Y. The clinical significance of Aurora-A/STK15/BTAK expression in human esophageal squamous cell carcinoma. Clin Cancer Res. 2005; 11(5):1827-1834.
157. Zhou H, Kuang J, Zhong L, Kuo WL, Gray JW, Sahin A, Brinkley BR and Sen S. Tumour amplified kinase STK15/BTAK induces centrosome amplification, aneuploidy and transformation. Nat Genet. 1998; 20(2):189-193.
158. Yao JE, Yan M, Guan Z, Pan CB, Xia LP, Li CX, Wang LH, Long ZJ, Zhao Y, Li MW, Zheng FM, Xu J, Lin DJ and Liu Q. Aurora-A down-regulates IkappaBalpha via Akt activation and interacts with insulin-like growth factor-1 induced phosphatidylinositol 3-kinase pathway for cancer cell survival. Mol Cancer. 2009; 8:95.
159. Lim KH, Baines AT, Fiordalisi JJ, Shipitsin M, Feig LA, Cox AD, Der CJ and Counter CM. Activation of RalA is critical for Ras-induced tumorigenesis of human cells. Cancer Cell. 2005; 7(6):533-545.
160. Bennett DC. How to make a melanoma: what do we know of the primary clonal events? Pigment Cell Melanoma Res. 2008; 21(1):27-38.
161. Pirker C, Holzmann K, Spiegl-Kreinecker S, Elbling L, Thallinger C, Pehamberger H, Micksche M and Berger W. Chromosomal imbalances in primary and metastatic melanomas: over-representation of essential telomerase genes. Melanoma Res. 2003; 13(5):483-492.
162. Yang H, Ou CC, Feldman RI, Nicosia SV, Kruk PA and Cheng JQ. Aurora-A kinase regulates telomerase activity through c-Myc in human ovarian and breast epithelial cells. Cancer Res. 2004; 64(2):463-467.
163. Eto M. Regulation of cellular protein phosphatase-1 (PP1) by phosphorylation of the CPI-17 family, C-kinase-activated PP1 inhibitors. J Biol Chem. 2009; 284(51):35273-35277.
164. Eto M, Senba S, Morita F and Yazawa M. Molecular cloning of a novel phosphorylation-dependent inhibitory protein of protein phosphatase-1 (CPI17) in smooth muscle: its specific localization in smooth muscle. FEBS Lett. 1997; 410(2-3):356-360.
165. Woodsome TP, Eto M, Everett A, Brautigan DL and Kitazawa T. Expression of CPI-17 and myosin phosphatase correlates with Ca(2+) sensitivity of protein kinase C-induced contraction in rabbit smooth muscle. J Physiol. 2001; 535(Pt 2):553-564.
166. Eto M, Bock R, Brautigan DL and Linden DJ. Cerebellar long-term synaptic depression requires PKC-mediated activation of CPI-17, a myosin/moesin phosphatase inhibitor. Neuron. 2002; 36(6):1145-1158.
167. Jin H, Sperka T, Herrlich P and Morrison H. Tumorigenic transformation by CPI-17 through inhibition of a merlin phosphatase. Nature. 2006; 442(7102):576-579.
168. Xia D, Stull JT and Kamm KE. Myosin phosphatase targeting subunit 1 affects cell migration by regulating myosin phosphorylation and actin assembly. Exp Cell Res. 2005; 304(2):506-517.
169. Villa-Moruzzi E, Tognarini M, Cecchini G and Marchisio PC. Protein phosphatase 1 delta is associated with focal adhesions. Cell Adhes Commun. 1998; 5(4):297-305.
170. Fresu M, Bianchi M, Parsons JT and Villa-Moruzzi E. Cell-cycle-dependent association of protein phosphatase 1 and focal adhesion kinase. Biochem J. 2001; 358(Pt 2):407-414.
171. Burnworth B, Arendt S, Muffler S, Steinkraus V, Brocker EB, Birek C, Hartschuh W, Jauch A and Boukamp P. The multi-step process of human skin carcinogenesis: a role for p53, cyclin D1, hTERT, p16, and TSP-1. Eur J Cell Biol. 2007; 86(11-12):763-780.
172. Kiss A, Lontay B, Becsi B, Markasz L, Olah E, Gergely P and Erdodi F. Myosin phosphatase interacts with and dephosphorylates the retinoblastoma protein in THP-1 leukemic cells: its inhibition is involved in the attenuation of daunorubicin-induced cell death by calyculin-A. Cell Signal. 2008; 20(11):2059-2070.
173. Ohama T, Hori M, Fujisawa M, Kiyosue M, Hashimoto M, Ikenoue Y, Jinno Y, Miwa H, Matsumoto T, Murata T and Ozaki H. Downregulation of CPI-17 contributes to dysfunctional motility in chronic intestinal inflammation model mice and ulcerative colitis patients. J Gastroenterol. 2008; 43(11):858-865.

174. Hegde P, Qi R, Abernathy K, Gay C, Dharap S, Gaspard R, Hughes JE, Snesrud E, Lee N and Quackenbush J. A concise guide to cDNA microarray analysis. Biotechniques. 2000; 29(3):548-550, 552-544, 556 passim.
175. Hocquette JF. Where are we in genomics? J Physiol Pharmacol. 2005; 56 Suppl 3:37-70.
176. Harris MA, Clark J, Ireland A, Lomax J, Ashburner M, Foulger R, Eilbeck K, Lewis S, Marshall B, Mungall C, Richter J, Rubin GM, Blake JA, Bult C, Dolan M, Drabkin H, et al. The Gene Ontology (GO) database and informatics resource. Nucleic Acids Res. 2004; 32(Database issue):D258-261.
177. Kanehisa M, Goto S, Kawashima S, Okuno Y and Hattori M. The KEGG resource for deciphering the genome. Nucleic Acids Res. 2004; 32(Database issue):D277-280.
178. Dahlquist KD, Salomonis N, Vranizan K, Lawlor SC and Conklin BR. GenMAPP, a new tool for viewing and analyzing microarray data on biological pathways. Nat Genet. 2002; 31(1):19-20.
179. Gollub J, Ball CA, Binkley G, Demeter J, Finkelstein DB, Hebert JM, Hernandez-Boussard T, Jin H, Kaloper M, Matese JC, Schroeder M, Brown PO, Botstein D and Sherlock G. The Stanford Microarray Database: data access and quality assessment tools. Nucleic Acids Res. 2003; 31(1):94-96.
180. Edgar R, Domrachev M and Lash AE. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res. 2002; 30(1):207-210.
181. Baker AB. The fat embolism syndrome, results of a therapeutic regime. Anaesth Intensive Care. 1976; 4(1):53-55.
182. McKhann GM, Knopman DS, Chertkow H, Hyman BT, Jack CR, Jr., Kawas CH, Klunk WE, Koroshetz WJ, Manly JJ, Mayeux R, Mohs RC, Morris JC, Rossor MN, Scheltens P, Carrillo MC, Thies B, et al. The diagnosis of dementia due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. Alzheimers Dement. 2011; 7(3):263-269.

TABLAS COMPLEMENTARIAS Y ESTUDIOS ADICIONALES

ANEXO I. REPERCUSIÓN FUNCIONAL Y FENOTÍPICA DE LAS VARIANTES DEL GEN *MC1R*

La tabla presenta el impacto funcional en la vía de pigmentación y fenotípico de las variantes más recurrentes. Abreviaciones: TM = dominio transmembrana, RE = retículo endoplasmático.

Variante *MC1R*	Efecto funcional	Correlación fenotípica
p.V38M	Localizada en el TM1, ligera disminución del acoplamiento de la proteína y de la estimulación de la vía del AMPc. Pérdida de función debida a una reducción de la expresión en superficie celular, retención de la proteína en el RE.	No analizado
p.S41F	Localizada en el dominio TM1, casi completa disminución del acoplamiento de la proteína y de la estimulación de la vía del AMPc. Pérdida de función debida a una reducción de la expresión en superficie, retención de la proteína en el RE.	No analizado
p.V51A	Localizada en el dominio TM1, casi completa disminución del acoplamiento de la proteína y de la estimulación de la vía del AMPc. Pérdida de función debida a una reducción de la expresión en superficie, retención de la proteína en el RE.	No analizado
p.V60L	Ligera disminución de la capacidad de estimular la producción de AMPc.	Rubio/castaño claro
p.D84E	Baja capacidad para estimular la producción de AMPc. Ligera disminución de la capacidad de unión entre la α-MSH y la proteína	Pelirrojo
p.V92M	Proteína con dos veces menos afinidad por la α-MSH. No alteración en el acoplamiento de la proteína y la estimulación de la vía del AMPc	No asociada al fenotipo pelirrojo
p.M128T	Localizada en el TM3, ausencia casi completa del acoplamiento de la proteína y de la estimulación de la vía del AMPc. Incapacidad para unirse a la α-MSH, correcta transportación hacia la superficie de la membrana celular.	No analizado
p.R142H	No analizado	Pelirrojo
p.R151C	Disminución del acoplamiento de la proteína. Pobre estimulación de la vía del AMPc en respuesta a la α-MSH	Pelirrojo y piel blanca
p.I155T	No analizado	Pelirrojo
p.R160W	Disminución del acoplamiento de la proteína. Pobre estimulación de la vía del AMPc en respuesta a la α-MSH	Pelirrojo y piel blanca
p.R163Q	Pérdida selectiva de la activación de las MAPquinasas inducido por α-MSH [142]	No asociada al fenotipo pelirrojo
p.N281S	Localizada en el TM7, no tiene efecto a nivel funcional	No analizado
p.C289R	Localizada en el TM7, ausencia casi completa del acoplamiento de la proteína y de la estimulación de la vía del AMPc. Incapacidad para unirse a la α-MSH, correcta transportación hacia la superficie de la membrana celular.	No analizado
p.D294H	Baja capacidad para estimular la producción de AMPc. Ligera disminución de la capacidad de unión entre la α-MSH y la proteína	Pelirrojo

ANEXO II. ESTUDIO DEL GEN *MC1R* Y RIESGO A DESARROLLAR LA ENFERMEDAD DE PARKINSON

Versión del artículo enviado para su publicación a la revista Annals of Neurology

Title: The *MC1R* red hair variant (p.R160W) increases the risk for Parkinson's disease

Running head: MC1R p.R160W and Parkinson's disease risk.

Gemma Tell-Marti, PhD Student[1], Joan Anton Puig-Butille, PhD Student[2,3], Miriam Potrony, PhD Student[1], Celia Badenas, PhD[3], Josep Malvehy, MD, PhD[1,3], Ruben Fernandez-Santiago, PhD[4] , Mario Ezquerra, PhD[4], María J Martí, PhD[4], Susana Puig, MD, PhD[1,3,5]

1. Dermatology Department, Melanoma Unit, Hospital Clinic & IDIBAPS (Institut d'Investigacions Biomèdiques August Pi i Sunyer). Barcelona, Spain.

2. Biochemical and Molecular Genetics Service, Melanoma Unit, Hospital Clinic & IDIBAPS (Institut d'Investigacions Biomèdiques August Pi i Sunyer)

3. Centro Investigación Biomédica en Enfermedades Raras (CIBERER), ISCIII. Barcelona, Spain.

4. Laboratory of Neurodegenerative Disorders, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS)- Hospital Clínic de Barcelona-Centro de Investigación sobre Enfermedades Neurodegenerativas (CIBERNED), ISCIII Barcelona, Spain.

5. Medicine Department, Universitat de Barcelona, Barcelona, Spain.

Corresponding author:
Susana Puig, MD, PhD
Melanoma Unit, Dermatology Department, Hospital Clinic Barcelona
Villarroel 170. 08036, Barcelona. Spain
susipuig@gmail.com; spuig@clinic.ub.es
Tel.: +34 93 2275400 ext 2422 Fax: +34 93 2275438

Characters in title: 78
Characters in running head: 41
Abstract word count: 248
Body of manuscript word count: 2,421
Number of figures: 0
Number of tables: 3

Abstract

Objective: A bidirectional association between Parkinson's disease (PD) and cutaneous melanoma (CM) has been reported. We examined whether the melanocortin 1 receptor (*MC1R*) gene, a risk factor for CM, was associated with PD risk in Spanish population.

Methods: We included 708 unrelated Spanish PD patients and 486 controls with no neurodegenerative disorder. A case-control study was performed to examine the association between *MC1R* polymorphisms and PD risk. Determination of variants within the coding region was carried out by PCR amplification and direct sequencing. Logistic regression model was used to estimate odds ratios (OR) and the best model of inheritance was chosen using Akaike and Bayesian information criteria.

Results: We found that a non-functional *MC1R* variant (p.R160W) was more frequent in PD patients, specially in late-onset Parkinson's disease (LOPD) patients who developed PD over 40 years of age and are mostly sporadic (OR, 2.26 (1.09-4.69), 95% C.I., P = 0.02), whereas the non-melanoma associated variant p.V60L was less frequent than in controls (P = 0.03).

Interpretation: The association between the melanoma associated variant (p.R160W) and PD suggests that the epidemiological link observed between CM and PD, could be, at least in part, explained by loss of function of *MC1R*, underlying an increase in oxidative damage. Interestingly this variant is strongly associated with red hair colour phenotype, reinforcing that an overrepresentation of red hair individuals had been showed within PD patients. Furthermore our findings highlight the role of *MC1R* in brain biology and add new information to the current knowledge of PD aetiology.

Introduction

An inverse correlation between the risk of developing cancer and Parkinson's disease (PD) has been suggested in several epidemiological studies[1, 2] showing a reduced risk of most kinds of cancer among individuals with PD[1]. However, this inverse relationship has not been observed in the incidence of cutaneous melanoma (CM) in PD patients until a recent study showed that the occurrence of melanoma (OR (95% CI)) was 3.61(1.49, 8.77) times higher among PD patients whereas the occurrence of PD was 1.44 (1.06-1.96) times higher among CM patients[3].

Initially some evidence proposed that this association could be related to the role of the treatments most commonly used in these diseases (levodopa therapy or chemotherapeutic treatments) rather than with the disease itself. However, results reported in other studies refuted these factors as putative causes of the association[4,5, 6].

One hypothesis is that the co-occurrence of PD and CM could be explained due to shared environmental[7,8] or genetic risk factors leading to common pathogenic molecular pathways[9,10]. Because a higher incidence has been reported of CM and PD in Caucasians than in black populations [11, 12] and an increased risk of developing PD has been observed in red hair colour individuals[13] the pigmentation pathway may be related to the risk of developing both diseases.

The melanocortin 1 receptor (*MC1R*) gene, which is a master regulator of skin pigmentation[14], encodes for a G protein-coupled seven transmembrane receptor for melanocortin peptides (α-MSH, ACTH) and mediates its effects mainly by activating a cAMP-dependent signalling pathway[15]. Additionally, in melanocytes, *MC1R* expression has been observed in several types of nervous system cells suggesting that it may be a key regulator in brain cell functions and survival [16, 17]

To date, six non-synonymous *MC1R* polymorphisms have been associated with the 'red hair colour' (RHC) phenotype that is characterized by red hair, fair skin, lack of tanning ability and high UV-radiation sensitivity. Epidemiological studies have reported that several *MC1R* Red hair 'R' variants (p.D84E, p.R142H, p.R151C, p.R160W, p.I155T, p.D294H) or no red hair 'r' variants (p.R163Q) increase the risk of developing CM with ORs (95% CI) ranging from 1.42 (1.09-1.85) for p.R163Q to 2.45 (1.32-4.55) for p.I155T 18. In this study, we analyzed whether MC1R variants associated with increased risk for CM, also modulate the risk for PD.

Subjects and Methods

The study included 708 unrelated Spanish PD patients (mean age: 57.02±12.29 years; female 40.1% and male 59.9%) and 486 age-and gender-matched controls free from any neurodegenerative disorder (mean age: 59.94±13.16 years; female 44.7% and male 55.3%), all of them from a hospital-based series.

All PD patients were evaluated and clinically diagnosed by neurologists experienced in the diagnosis of PD and age of onset (AOO) was estimated as the age when motor or behavioral symptoms were first noticed.

The Subjects of this study were treated according to the World Medical Association Declaration of Helsinki; ethical principles for medical research involving human subjects 19. The study was approved by the Institutional Review Board (IRB) of Hospital Clinic of Barcelona, written informed consent was obtained from all the study subjects.

MC1R molecular screening

DNA was isolated from blood samples using the Wizard® Genomic DNA Purification Kit. Determination of variants within the coding region was carried out by PCR amplification and direct sequencing using 50-100 ng of total DNA. Samples were amplified using primers previously described 20. PCR conditions were: initial denaturizing step at 95ºC for 5 min, followed by 35 cycles (94ºC for 1 min, 55°C for 1 min, 72ºC for 3 min), and a final extension at 72ºC for 10 min and maintaining at 4ºC. PCR products were purified using Multiscreen Filter plates (Millipore) and automatically sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) and an ABI3100 automatic sequencer (Applied Biosystems). Specific internal MC1R primers were designed to analyze the entire coding sequence (TM-F: AACCTGCACTCACCCATGTA and TM-R: TTTAAGGCCAAAGCCCTGGT). Sequences were analyzed using SeqPilot 4.0.1 software (JSI Medical Systems)

Statistical Analysis

MC1R non-synonymous variants were classified as red hair colour 'R' or non-red hair colour 'r' according to previously reported criteria in melanoma studies[18].

Therefore, *MC1R* variants classified as 'R' were p.D84E, p.R142H, p.R151C, p.I155T, p.R160W, p.D294H and rare frameshift or stop codon. All other non-synonymous *MC1R* variants were classified as 'r'. Synonymous variants were considered as wild-type *MC1R* alleles. For the purpose of this study, only non-synonymous amino acid changes with an observed frequency of at least 1% were analysed. Carriers of non-synonymous variants with a frequency < 1% were excluded from the analyses.

Public databases such as dbSNP (http://www.ncbi.nlm.nih.gov/), MelGene DB (http://www.melgene.org/) and Ensmbl genome browser (http://www.ensembl.org/) were used to determine whether the detected non-synonymous variants have been previously described. *In-silico* analysis of each rare non-synonymous variant was carried out using software Polyphen2 (http://genetics.bwh.harvard.edu/pph2/)[21].

Genetic data were analysed using SNPStats software[22.] Allelic and genotypic frequencies of MC1R variants as well as Hardy-Weinberg equilibrium were determined. Multiple logistic regression models (codominant, dominant, recessive, overdominant and log additive) were performed for odds ratios (OR), 95% confidence intervals (95% CI) and P-value. Both Akaike information criterion and Bayesian information criterion were used to choose the model of inheritance that fit the data best. All the reported ORs and 95% CI were adjusted for age and gender. All P-values less than 0.05 were accepted as statistically significant. All tests were two sided.

All red hair colour variants 'R' and non-red hair colour variants 'r' were grouped in two categorical variables. Correlation between number of non-synonymous *MC1R* variants or type of *MC1R* variants (R or r) and Parkinson's disease were calculated by cross tabulations and Pearson's $\chi 2$ using IBM SPSS Statistics 18 (IBM, Armonk, NY, U.S.A.).

PD patients were stratified by the age of onset, early-onset Parkinson's disease (EOPD) ranges from 20 to 40 years and late-onset Parkinson's disease (LOPD) over 40 years. The same association analyses were carried out in these two subsets.

Results

Genotyping of the *MC1R* gene resulted in a total of 51% PD patients and 45.6% controls being wild-type; 39.7% of cases and 44.8% of controls carried one non-synonymous *MC1R* variant, 9.3% of cases and 9.2% of controls carried two non-synonymous *MC1R* variants, and only 0.2% of controls carried three non-synonymous *MC1R* variants. Thus, we identified a total of 38 *MC1R* variants (12 synonymous and 26 non-synonymous) most being detected in a small number of patients or restricted to one, reported as rare MC1R variants (Table 1). Interestingly, rare *MC1R* variants were more frequently observed in PD patients (5.1%) than in the control group (3.3%) (Table 1). Of these, fourteen MC1R variants are novel/ not previously described. Among synonymous variants, the p.T314T variant showed the highest frequency of occurrence in cases (8.5%). We also found eight recurrent non-synonymous variants which showed a frequency of the minor allele at least of 1% in PD patients: p.V60L (12%), p.V92M (6%), p.R151C (3%), p.R160W (2%), p.R163Q (2%), p.R142H (1%), p.I155T (1%) and p.D294H (1%).

The p.R142H variant was excluded from the analyses because it was not in Hardy-Weinberg equilibrium within control groups. Considering that functional analyses for rare *MC1R* variants have not been performed and their effects in the cell are unknown, carriers of rare *MC1R* variants were excluded from the analyses. Thus, the analyses of the *MC1R* variants for seven recurrent variants (p.V60L, p.V92M, p.R151C, p.I155T, p.R160W, p.R163Q and p.D294H) revealed the following results (Table 2). Association analyses showed one significant association for p.V60L variant and two trends for p.R160W and p.D294H which did not reach statistical significance. The p.V60L variant in the *MC1R* gene was associated with decreased risk of developing PD (OR, 0.75 (0.59-0.94), 95% C.I., P=0.015). A trend was detected between the p.D294H variant and decreased risk of developing PD (OR, 0.53 (0.26-1.07), 95% C.I., P=0.07). In contrast, another trend between the p.R160W variant and increased risk of PD (OR, 1.80 (0.93-3.46), 95% C.I., P=0.07) was detected.

We stratified our cohort of PD patients based on the age of onset and assessed the risk associated with *MC1R* variants in late-onset Parkinson's disease (LOPD) patients (88.8%) who developed Parkinson's disease aged over 40 years and is mostly sporadic (N=620) compared with age-and sex-matched controls (N=440). The frequency of *MC1R* variant carriers showed a statistically significant difference ($p<0.037$) between control population (56%) and Parkinson patients (46.5%). We found an association between the p.V60L variant and decreased risk of LOPD (OR 0.76 (0.59-0.98), 95% C.I., P = 0.03) (Table 3). In contrast, the protective trend of the p.D294H observed in the first analysis was not revalidated in LOPD (OR, 0.58 (0.27-1.24), 95% C.I., P= 0.16). Finally, the increased risk detected in p.R160W variant and PD reached a statistically significant value in the LOPD group (OR, 2.26 (1.09-4.69), 95% C.I., P = 0.02).

When the analysis was focused on the number of *MC1R* variants or type of *MC1R* variants (R or r), association with PD was not detected in neither of the analyses. Differences among cases and controls were related to specific variants and not to the total number or type of *MC1R* variants. A gene dosage effect was not observed in our PD set because a correlation between number of variants and an increased risk of developing PD was not detected (data not shown).

Discussion

Epidemiological data indicate a bidirectional association between CM and PD. There is an almost four fold increase in occurrence of CM among PD patients and nearly a two fold increase of PD among CM patients have been reported[3]. Furthermore, evaluation of pigment related phenotypical features showed an overrepresentation of red hair individuals within PD patients compared to control population [13]. The *MC1R* gene, which is the master regulator of pigmentation, is also expressed in brain cells (neurons of the periaqueductal gray matter, astrocytes and schwann cells) in which it may have a role in the anti-inflammatory brain response [17]. In this study we have assessed the role of the *MC1R* as a putative risk factor for PD in the Mediterranean population. The study was conducted on a large cohort of PD patients (mainly sporadic) since the vast majority of PD cases are late-onset and the age of onset is the major known risk factor for the disease.

We found that p.V60L and p.R60W are inversely associated with the late onset form of PD in the Mediterranean population. We detected a more than two fold increased risk of PD associated with the p.R160W variant. This variant has been classified as a loss-of-function RHC variant resulting in an overproduction of pheomelanin which is associated with red hair phenotype (fair skin and red hair colour). The RHC *MC1R* variants, including p.R160W, have been shown to increase the risk of CM [18]. In addition, we detected that the variant p.V60L was more frequent in controls than PD cases. p.V60L which is the most frequent variant observed in a healthy Mediterranean population [15], is not associated with red hair phenotype [23] or increased risk of CM [18]. Thus, our data indicates that role of *MC1R* in PD susceptibility may be restricted to those variants which clearly promote a loss-of function of the *MC1R*. A previous study focused exclusively on the p.R151C RHC variant, suggests that this RHC variant would also increase the risk to develop PD in a North-American population [13]. *MC1R* variants have different distributions among populations 15. The allelic frequency of the p.R151C variant is lower in our population compared to the North-American population. Thus, inter-population differences may interfere in the ability to detect a statistically significant association between other RHC variants and PD in our cohort. Notably, the highest attributable CM risk to MC1R has been associated with both p.R151C and p.R160W and both have been strongly associated with the RHC phenotype [18].

Our findings suggest that the epidemiological link observed between CM and PD, could be, at least in part, explained by non functional *MC1R* alleles. *MC1R* functional studies in melanocytes showed that the p.R160W variant is associated with reduced cell surface protein expression and diminished cAMP production [24], in contrast to the p.V60L variant which did not show these alterations [25]. Previous evidence suggest that stimulation of *MC1R* by α-MSH is associated with reduced oxidative stress [26]. A greater oxidative DNA and lipid damage has been found in skin cells from RHC variants carriers, supporting the hypothesis that the loss-of-function of MC1R produces an increase of oxidative damage [27]. An elevated oxidative stress has been previously associated to the degeneration process in PD brains [28]. In an experimental model, Puig-Butille et al. recently described that the *MC1R* red hair polymorphism alters the transcriptome related with neurodegenerative diseases and specifically PD, in concordance with the results presented herein ("Capturing the biological impact of *CDKN2A* and *MC1R* genes as an early pedisposing event in

melanoma and non melanoma skin cancer." (accepted manuscript in Oncotarget Journal).

In conclusion, we report the largest cohort of LOPD in which the *MC1R* gene has been evaluated at the molecular level and the p.R160W variant has been found associated with an increased risk of PD. Although, this data suggests a role of *MC1R* in the etiology of LOPD, functional studies in brain cell should be carried out to elucidate the molecular mechanisms underlying the risk effect observed for the p.R160W variants. Finally, future studies in other populations ought to further clarify the link of MC1R variants and PD.

Potential conflict of interest

Nothing to report

Acknowledgements

We thank the National Bank of DNA from the University of Salamanca (Spain) for kindly providing DNAs from control healthy subjects. The research at the Melanoma Unit in Barcelona is partially funded by Grants from Fondo de Investigaciones Sanitarias (12/00840) Spain, co-funded by "Fondo Europeo de Desarrollo Regional (FEDER). Unión Europea. Una manera de hacer Europa"; by the AGAUR 2009 SGR 1337 of the Catalan Government, Spain; The work was carried out at the Esther Koplowitz Centre. Barcelona, Spain.

Author Contributions

G.T.M. performed all the experimental work, analysis of sequences and statistical analysis of data. M.P. contributed in the analysis of sequences. JA.P.B designed the project, supervised statistical work and wrote the manuscript with G.T.M. C.B. designed specific internal MC1R primers and supervised the sequence analysis. R.F.S, M.E and M.J.M included patients, provided the DNA samples evaluated in the study (PD patients and controls) and contributed in data interpretation. S.P. and J.M. designed the project, obtained funding and took responsibility for the integrity of the data and the accuracy of the data analysis, supervised the whole project and the manuscript edition. All the co-authors reviewed and commented on the manuscript.

References

1.Bajaj A, Driver JA, Schernhammer ES. Parkinson's disease and cancer risk: a systematic review and meta-analysis. Cancer Causes Control. 2010 May;21(5):697-707.

2.Strongosky AJ, Farrer M, Wszolek ZK. Are Parkinson disease patients protected from some but not all cancers? Neurology. 2008 Nov 11;71(20):1650; author reply -1.

3.Liu R, Gao X, Lu Y, Chen H. Meta-analysis of the relationship between Parkinson disease and melanoma. Neurology. 2011 Jun 7;76(23):2002-9.

4.Fiala KH, Whetteckey J, Manyam BV. Malignant melanoma and levodopa in Parkinson's disease: causality or coincidence? Parkinsonism Relat Disord. 2003 Aug;9(6):321-7.

5.Baade PD, Herrero Hernandez E, Freedman DM, Smithers BM, Fritschi L. No role for melanoma treatment in the association between melanoma and amyotrophic lateral sclerosis or Parkinson's disease. Neuroepidemiology. 2010;35(4):303-4.

6.Olsen JH, Tangerud K, Wermuth L, Frederiksen K, Friis S. Treatment with levodopa and risk for malignant melanoma. Mov Disord. 2007 Jul 15;22(9):1252-7.

7.Rugbjerg K, Harris MA, Shen H, Marion SA, Tsui JK, Teschke K. Pesticide exposure and risk of Parkinson's disease--a population-based case-control study evaluating the potential for recall bias. Scand J Work Environ Health. 2011 Sep;37(5):427-36.

8.Dennis LK, Lynch CF, Sandler DP, Alavanja MC. Pesticide use and cutaneous melanoma in pesticide applicators in the agricultural heath study. Environ Health Perspect. 2010 Jun;118(6):812-7.

9.Gao X, Simon KC, Han J, Schwarzschild MA, Ascherio A. Family history of melanoma and Parkinson disease risk. Neurology. 2009 Oct 20;73(16):1286-91.

10.Paisan-Ruiz C, Houlden H. Common pathogenic pathways in melanoma and Parkinson disease. Neurology. 2010 Nov 2;75(18):1653-5.

11.Dwyer T, Blizzard L, Ashbolt R, Plumb J, Berwick M, Stankovich JM. Cutaneous melanin density of Caucasians measured by spectrophotometry and risk of malignant melanoma, basal cell carcinoma, and squamous cell carcinoma of the skin. Am J Epidemiol. 2002 Apr 1;155(7):614-21.

12.Wright Willis A, Evanoff BA, Lian M, Criswell SR, Racette BA. Geographic and ethnic variation in Parkinson disease: a population-based study of US Medicare beneficiaries. Neuroepidemiology. 2010;34(3):143-51.

13.Gao X, Simon KC, Han J, Schwarzschild MA, Ascherio A. Genetic determinants of hair color and Parkinson's disease risk. Ann Neurol. 2009 Jan;65(1):76-82.

14.Perez Oliva AB, Fernendez LP, Detorre C, et al. Identification and functional analysis of novel variants of the human melanocortin 1 receptor found in melanoma patients. Hum Mutat. 2009 May;30(5):811-22.

15.Gerstenblith MR, Goldstein AM, Fargnoli MC, Peris K, Landi MT. Comprehensive evaluation of allele frequency differences of MC1R variants across populations. Hum Mutat. 2007 May;28(5):495-505.

16.Christiansen JH, Coles EG, Wilkinson DG. Molecular control of neural crest formation, migration and differentiation. Curr Opin Cell Biol. 2000 Dec;12(6):719-24.

17.Catania A. Neuroprotective actions of melanocortins: a therapeutic opportunity. Trends Neurosci. 2008 Jul;31(7):353-60.

18.Raimondi S, Sera F, Gandini S, et al. MC1R variants, melanoma and red hair color phenotype: a meta-analysis. Int J Cancer. 2008 Jun 15;122(12):2753-60.

19.World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects. J Postgrad Med. 2002 Jul-Sep;48(3):206-8.

20.Chaudru V, Laud K, Avril MF, et al. Melanocortin-1 receptor (MC1R) gene variants and dysplastic nevi modify penetrance of CDKN2A mutations in French melanoma-prone pedigrees. Cancer Epidemiol Biomarkers Prev. 2005 Oct;14(10):2384-90.

21.Adzhubei IA, Schmidt S, Peshkin L, et al. A method and server for predicting damaging missense mutations. Nat Methods. 2010 Apr;7(4):248-9.

22.Sole X, Guino E, Valls J, Iniesta R, Moreno V. SNPStats: a web tool for the analysis of association studies. Bioinformatics. 2006 Aug 1;22(15):1928-9.

23.Bastiaens MT, ter Huurne JA, Kielich C, et al. Melanocortin-1 receptor gene variants determine the risk of nonmelanoma skin cancer independently of fair skin and red hair. Am J Hum Genet. 2001 Apr;68(4):884-94.

24.Garcia-Borron JC, Sanchez-Laorden BL, Jimenez-Cervantes C. Melanocortin-1 receptor structure and functional regulation. Pigment Cell Res. 2005 Dec;18(6):393-410.

25.Dessinioti C, Antoniou C, Katsambas A, Stratigos AJ. Melanocortin 1 receptor variants: functional role and pigmentary associations. Photochem Photobiol. 2011 Sep-Oct;87(5):978-87.

26.Abdel-Malek ZA, Knittel J, Kadekaro AL, Swope VB, Starner R. The melanocortin 1 receptor and the UV response of human melanocytes--a shift in paradigm. Photochem Photobiol. 2008 Mar-Apr;84(2):501-8.

27.Mitra D, Luo X, Morgan A, et al. An ultraviolet-radiation-independent pathway to melanoma carcinogenesis in the red hair/fair skin background. Nature. 2012 Nov 15;491(7424):449-53.

28.Zhou C, Huang Y, Przedborski S. Oxidative stress in Parkinson's disease: a mechanism of pathogenic and therapeutic significance. Ann N Y Acad Sci. 2008 Dec;1147:93-104.

Table Legends

Table 1: Rare genetic variants detected in the *MC1R* gene

Variants in bold have not been reported before. Score Polyphen2: predicts possible impact of an amino acid substitution on the structure and function of a human protein (scores close to 0.0, indicate a benign mutation; score close to 1.0, damaging mutation).

Rare *MC1R* variants		Spain		Polyphen
		Parkinson	Controls	Score 2
Synonymous				
A103A	(c.309C>T)het	_	1	_
I138I	**(c.414C>T)het**	_	1	_
A139A	**(c.417C>T)het**	1	_	_
A161A	**(c.483G>A)het**	3	_	_
I168I	**(c.504C>T)het**	_	1	_
Y183Y	**(c.549C>T)het**	_	1	_
R229R	**(c.687C>T)het**	1	_	_
Q233Q	(c.699G>A)het	12	4	_
Y298Y	**(c.894C>T)het**	1	_	_
F300F	(c.900C>T)het	_	1	_
S316S	(c.948C>T)het	1	1	_
Nonsynonymous				
N56D	**(c.166A>G)het**	1	_	1.000
R67W	**(c.199C>T)het**	1	_	1.000
L80V	**(c.238C>G)het**	_	1	1.000
S83P	(c.246T>C)het	1	_	0.999
T95M	(c.284C>T)het	_	1	0.889
V122M	(c.364G>A)het	6	_	0.126
V140M	**(c.418G>A)het**	1	_	0.39
R142C	(c.424 C>T)het	1	_	1.000
Y152*	(c.456C>A)het	1	_	1.000
T272M	(c.815C>T)het	1	_	0.974
V193M	**(c.577G>A)het**	_	1	0.894
R213W	(c.637C>T)het	2	_	0.019
R223W	**(c.677C>T)het**	1	_	0.019
A218T	(c.652G>A)het	1	_	0.935
P230L	(c.689C>T)het	_	1	0.004
P268R	(c.803C>G)het	_	1	1.000
C273W	**(c.819C>G)het**	_	1	1.000

Table 2: Results of analysis of association

a Allele described as 1 (wild type allele) or 2 (variant allele).

AA change	Controls (N=482)					PD patients (N=697)						
	[a]Allele frequency		Genotype frequency			Allele frequency		Genotype frequency			CONTROL vs Parkinson disease	
	1	2	1-1	1-2	2-2	1	2	1-1	1-2	2-2	OR (95% CI)	*P-value*
p.V60L	0.84	0.16	0.72	0.25	0.03	0.88	0.12	0.77	0.21	0.02	**0.75 (0.59-0.94)**	**0.01**
p.V92M	0.94	0.06	0.89	0.11	0	0.94	0.06	0.89	0.11	0	1.04 (0.73-1.50)	0.82
p.R163Q	0.98	0.02	0.96	0.04	0	0.98	0.02	0.97	0.03	0	0.82 (0.44-1.56)	0.55
p.R151C	0.98	0.02	0.958	0.04	0.002	0.97	0.03	0.95	0.05	0	1.16 (0.68-1.99)	0.58
p.I155T	0.99	0.01	0.98	0.02	0	0.99	0.01	0.98	0.02	0	0.89 (0.41-1.92)	0.76
p.R160W	0.99	0.01	0.97	0.03	0	0.98	0.02	0.95	0.05	0	**1.80 (0.93-3.46)**	**0.07**
p.D294H	0.98	0.02	0.96	0.04	0	0.99	0.01	0.98	0.02	0	**0.53 (0.26-1.07)**	**0.07**

Table 3: Results of secondary analysis of association (late-onset Parkinson's disease patients)

a Allele described as 1 (wild type allele) or 2 (variant allele).

AA change	Controls LOPD (> 40 YEARS) (N=440)					PD LOPD (> 40 YEARS) (N=620)						
	[a]Allele frequency		Genotype frequency			Allele frequency		Genotype frequency			CONTROL vs LOPD	
	1	2	1-1	1-2	2-2	1	2	1-1	1-2	2-2	OR (95% CI)	*P-value*
p.V60L	0.85	0.15	0.72	0.25	0.03	0.89	0.11	0.78	0.20	0.02	**0.76 (0.59-0.98)**	**0.03**
p.V92M	0.94	0.06	0.884	0.114	0.002	0.94	0.06	0.887	0.112	0.001	0.94 (0.65-1.37)	0.75
p.R163Q	0.98	0.02	0.96	0.04	0	0.99	0.01	0.97	0.03	0	0.66 (0.33-1.32)	0.25
p.R151C	0.98	0.02	0.955	0.043	0.002	0.98	0.02	0.95	0.05	0	1.08 (0.61-1.89)	0.79
p.I155T	0.99	0.01	0.98	0.02	0	0.99	0.01	0.98	0.02	0	0.87 (0.38-1.97)	0.74
p.R160W	0.99	0.01	0.98	0.02	0	0.98	0.02	0.95	0.05	0	**2.26 (1.09-4.69)**	**0.02**
p.D294H	0.98	0.02	0.97	0.03	0	0.99	0.01	0.98	0.02	0	0.58 (0.27-1.24)	0.16

ANEXO III. ESTUDIO DEL GEN *MC1R* Y RIESGO A DESARROLLAR LA ENFERMEDAD DE ALZHEIMER

Versión del artículo enviado para su publicación a la revista Neurobiology of aging

Title: A common variant in the *MC1R* gene (p.V92M) is associated with Alzheimer's disease risk

Gemma Tell-Marti[a], Joan Anton Puig-Butille [b,c], Miriam Potrony[a], Celia Badenas[b,c] Anna Antonell[d], Raquel Sanchez-Valle[d], José L Molinuevo[d], Alberto Lleó[e,f], Daniel Alcolea[e,f], Juan Fortea[e,f], Ruben Fernandez-Santiag[og], Jordi Clarimón[e,f] , Albert Lladó[d], Susana Puig[a,c,h]

a.Dermatology Department, Melanoma Unit, Hospital Clinic & IDIBAPS (Institut d'Investigacions Biomèdiques August Pi i Sunyer). Barcelona, Spain.

b.Biochemical and Molecular Genetics Service, Melanoma Unit, Hospital Clinic & IDIBAPS (Institut d'Investigacions Biomèdiques August Pi i Sunyer)

c.Centro Investigación Biomédica en Enfermedades Raras (CIBERER), ISCIII. Barcelona, Spain.

d. Alzheimer's Disease and Other Cognitive Disorders Unit, Neurology Service, Hospital Clínic. Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS). Barcelona, Spain.

e. Memory Unit, Neurology Department, Hospital de Sant Pau (Sant Pau Biomedical research Institute), Universitat Autònoma de Barcelona, Barcelona, Spain.

f. CIBERNED, Center of networker Biomedical Research into Neurodegenerative Diseases, Madrid, Spain.

g.Laboratory of Neurodegenerative Disorders, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS)- Hospital Clínic de Barcelona-Centro de Investigación sobre Enfermedades Neurodegenerativas (CIBERNED), ISCIII Barcelona, Spain.

h.Medicine Department, Universitat de Barcelona, Barcelona, Spain.

Corresponding author:
Susana Puig, MD
Melanoma Unit, Dermatology Department, Hospital Clinic Barcelona
Villarroel 170. 08036, Barcelona. Spain
susipuig@gmail.com
Tel.: +34 93 2275400 ext 2422 Fax: +34 93 2275438

Abstract

Despite the recent identification of some novel risk genes for Alzheimer's disease (AD), the genetic etiology of late onset Alzheimer's disease (LOAD) remains largely unknown. The inclusion of these novel risk genes to the risk attributable to the APOE gene accounts for roughly half of the total genetic variance in LOAD. The evidence indicates that undiscovered genetic factors may contribute to AD susceptibility. In the present study, the *MC1R* gene was evaluated in 519 Spanish LOAD patients and in 162 controls. We observed that a common *MC1R* variant not related to pigmentation traits confers a nearly two fold increased risk of developing LOAD (P=0.018), especially in those patients whose genetic risk could not be not explained by APOE genotype. We did not find an association between p.V92M and age of onset of AD. Further studies are needed to elucidate the role of *MC1R* in brain cells through the different *MC1R* pathways.

Keywords: late onset Alzheimer's disease (LOAD); Melanocortin 1 receptor (*MC1R*) gene; p.V92M; common variant; Risk; Cerebrospinal fluid (CSF) biomarkers.

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized clinically by memory and cognitive dysfunction and represents the most common form of dementia in the elderly (Gilbert, 2013). The prevalence of the disease increases after the age of 65 years and the disease onset is usually after the age of 70 years (Jonsson et al., 2013). However, Familial AD (FAD) patients carry autosomal dominant mutations in high-risk Alzheimer susceptibility genes (*APP*, *PSEN1*, and *PSEN2*) present an early age of onset (<65 years). These genes do not play an important role in either Sporadic AD cases or late onset cases (> 65 years) indicating that other genomic variants may be involved in the common forms of the disease. To date, the ε4 allele polymorphism in the Apolipoprotein E (*APOE*) gene has been well established as a risk factor for developing late onset Alzheimer's disease (LOAD) (Bettens et al., 2013). The disease-attributable risk in LOAD patients related to the ε4 allele in *APOE* is less than 50% (Ashford and Mortimer, 2002). Recently, new low-risk variants associated with LOAD have been identified in genome-wide association studies (GWAS) (Harold et al., 2009, Lambert et al., 2009, Seshadri et al., 2010, Hollingworth et al., 2011, Naj et al., 2011) which account for a small proportion of risk. The inclusion of these novel risk genes to the risk attributable to the APOE gene accounts for roughly half of the total genetic variance (Bertram, 2011) indicating that additional undiscovered genetic factors may contribute to the AD susceptibility.

The main pathological hallmarks of AD are extracellular amyloid plaques, intracellular neurofibrillary tangles, and loss of neurons and synapses resulting in brain atrophy (Castellani et al., 2008). Moreover, an elevated level of oxidative damage products has been observed in areas of degeneration in AD brains suggesting that oxidative stress and consequent protein oxidation may be a potential mechanism of neuronal death in AD (Roberts, 1990). The accumulation of intracellular damage determined by reactive oxygen species (ROS) might produce the progressive loss of control over biological homeostasis and the functional impairment typical of damaged brain cells in AD (Cencioni et al., 2013).

Melanocortin 1 receptor (MC1R) gene encodes for a G protein-coupled seven transmembrane receptor for melanocortin peptides (α-MSH, ACTH) and mediates its effects mainly by activating a cAMP-dependent signaling pathway (Gerstenblith et al., 2007). MC1R expression is observed in several types of neuronal cells suggesting that it may be a key regulator in brain cell functions and survival (Catania, 2008). The MC1R activation has anti-inflammatory and immunomodulatory effects in brain cells (Catania, 2008) and promotes pigmentation synthesis in melanocytes (Dessinioti et al., 2011). It has been established that several MC1R polymorphisms constitute a risk factor to develop skin cancer (melanoma and non melanoma skin cancer) (Bastiaens et al., 2001, Raimondi et al., 2008), in part, by promoting an increased oxidative stress in skin cells (Kadekaro et al., 2012). Notably, co-occurrence of Parkinson's disease (PD) and cutaneous melanoma (CM) has been reported in epidemiological studies (Olsen et al., 2006, Liu et al., 2011) and previous evidence indicates that MC1R is involved in the bidirectional link between both diseases (Gao et al., 2009). Thus, we hypothesized that certain MC1R variants may increase the oxidative damage and/or deregulate inflammatory processes in brain cells, which consequently, increase the susceptibility to develop other neurodegenerative disorders beyond PD. In the present study we analyzed the role of the MC1R gene as a putative genetic risk

factor in LOAD patients and we observed that a common MC1R variant not related with pigmentation traits confers a risk to develop LOAD in Spanish population.

Material and Methods

The study includes 519 unrelated LOAD patients, 153 (29.5%) men and 366 (70.5%) women from two hospital-based series from the same geographical area: Alzheimer's Disease and Other Cognitive Disorders Unit at Clinic Hospital of Barcelona and from the Memory Unit at Sant Pau Hospital of Barcelona. Controls were from Alzheimer's Disease and Other Cognitive Disorders Unit at Clinic Hospital of Barcelona, the National Bank of DNA from the University of Salamanca and Memory Unit at Sant Pau Hospital of Barcelona.

In all cases the age of onset was after 65 year (mean age=76.48 ±5.50 years), although in 36 of patients their exact age of onset is not available. All AD patients were diagnosed using the NINCSDS-ADRDA criteria (McKhann et al., 1984). Furthermore, 69/519 had a profile of CSF biomarkers typical of AD (Aβ42/p-tau ratio < 6.43) (Welge et al., 2009). Overall, 162 controls free from any neurodegenerative disorders, 59 (36.4%) men and 103 (63.5%) women with a mean age=73.81±5.87 were included in the study.

All individuals included in the study gave their written informed consent according to the declaration of Helsinki. The ethical committee of clinical investigation at the Hospital Clinic of Barcelona approved the study.

APOE genotype analysis

DNA was isolated from blood samples using the Wizard® Genomic DNA Purification Kit (Promega, Fitchburg, Wisconsin, USA). APOE genotype was determined through the analysis of rs429358 and rs7412 using TaqMan (Applied Biosystems) genotyping technologies.

CSF biomarkers determination

69 subjects underwent a spinal tap, during the morning. The samples were centrifuged and stored in polypropylene tubes at −80ºC within 2h. Levels of Aβ42, t-tau, and p-tau were measured by experienced laboratory personnel using commercial sandwich ELISA kits (Innogenetics, Gent, Belgium) (Antonell et al., 2011).We are participants of the QC program and Aβ42, t-tau and p-tau levels obtained in our lab for the Alzheimer's Association QC samples were within mean ±2 SD.

MC1R molecular screening

Determination of variants within the coding region was carried out by PCR amplification and direct sequencing using 50-100 ng of total DNA. Samples were amplified using previously described primers (Chaudru et al., 2005). PCR conditions were: initial denaturizing step at 95ºC for 5 min, followed by 35 cycles (94ºC for 1 min, 55°C for 1 min, 72ºC for 3 min), and a final extension at 72ºC for 10 min and maintaining at 4ºC. PCR products were purified using Multiscreen Filter plates (Millipore) and automatically sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) and an ABI3100 automatic sequencer (Applied Biosystems). Specific internal MC1R primers were designed to analyze the entire coding sequence (TM-F: AACCTGCACTCACCCATGTA and TM-R: TTTAAGGCCAAAGCCCTGGT). Sequences were analyzed using SeqPilot 4.0.1 software (JSI Medical Systems). The entire coding region was sequenced in 110

LOAD patients and in all controls. In 409 LOAD patients, the MC1R gene was exclusively sequenced using TM-R primer. This strategy allows us to detect all MC1R variants except for the presence of p.D294H and p.T314T variants.

Statistical Analysis

The analysis was focused on those non-synonymous variants observed in at least 1% of cases. Synonymous variants were considered as wild-type MC1R alleles. Public databases such as dbSNP (http://www.ncbi.nlm.nih.gov/), MelGene DB (http://www.melgene.org/) and Ensmbl genome browser (http://www.ensembl.org/) were used to determine whether the detected non-synonymous variants have been previously described. *In-silico* analysis of each rare non-synonymous variant was carried out using software Polyphen2 (http://genetics.bwh.harvard.edu/pph2/) (Adzhubei et al., 2010).

Allelic and genotypic frequencies of MC1R and APOE variants were evaluated using SNPStats software (Sole et al., 2006). Multiple logistic regression models (codominant, dominant, recessive, overdominant and log additive) were performed for odds ratios (OR), 95% confidence intervals (95% CI) and P-value. Both Akaike information criterion and Bayesian information criterion were used to choose the model of inheritance that fit the data best (Log additive model was chosen in all the analyses). Analyses of MC1R variants were adjusted for age, gender and APOE genotype.

The Student t test was used to evaluate whether MC1R and APOE genotypes modifies age of onset (AOO) within the Spanish LOAD group. In the subset of patients with available CSF biomarker data, the putative modifying effect of MC1R variants on these biomarkers was evaluated by the Student t test. Finally, MC1R non-synonymous variants were classified according to reported criteria in melanoma studies such as red hair color 'R' or non-red hair color 'r' (Raimondi et al., 2008). MC1R variants classified as 'R' were p.D84E, p.R142H, p.R151C, p.I155T, p.R160W, p.D294H and rare frameshift or stop codon. All other non-synonymous MC1R variants were classified as 'r'. All 'R' and 'r' variants were grouped in two categorical variables. The risk to develop LOAD by number or type of MC1R variants (R or r) was evaluated by Pearson's $\chi 2$ using IBM SPSS Statistics 18 (IBM, Armonk, NY, U.S.A.). All tests were two sided and p-values of less than 0.05 were accepted as statistically significant.

Results

MC1R genotyping was carried out in 519 LOAD patients and in 162 control subjects. Non significant difference in the gender distribution was detected between patients and controls (p= 0.096). Overall, 45.7% (237/519) of LOAD patients and 53.1% (86/162) of controls did not present MC1R variants. The frequency of one non-synonymous MC1R variant was 46.1% (239/519) in cases and 39.5% (64/162) in controls. Two non-synonymous MC1R variants were detected in 8.3% (43/519) of cases and 7.4% (12/162) of controls and no individual included in the study carried more than 2 non-synonymous MC1R variants.

The study identified 22 MC1R variants (four synonymous and 18 non-synonymous) Three rare variants (p.V156E, p.V174E and p.L309L) had not been previously identified (Table 1). Five recurrent non-synonymous variants showed a frequency ≥1% in LOAD patients: p.V60L (16%), p.V92M (7%), p.R151C (3%), p.R160W (2%) and p.R163Q (2%). All variants were in Hardy-Weinberg equilibrium within both the control population and LOAD patients (allelic and genotypic frequencies are

listed in Table 2). The p.D294H variant was excluded from the analyses because it was only assessed in 110 LOAD patients. Although this is a limitation in our study, a non significant difference in the frequency of variant D294H was observed between 110 LOAD patients (minor allele frequency was 3%) and 162 controls (minor allele frequency was 2%)

The APOE genotype was obtained for all cases and controls. The frequency of heterozygous APOE ε4 carriers was 41% (213/519) in LOAD patients and 20% (32/162) in controls. The APOE ε4/ε4 genotype was only detected in 5% (24/519) of LOAD patients. The carriers of at least one APOE ε4 allele had a higher than three-fold increased risk of developing LOAD (OR: 3.37, 95% CI: 2.22-5.12, P <0.0001)

We evaluated the 5 most common MC1R variants detected in the study with the risk of developing LOAD (Table 2). We detected that variant p.V92M was significantly associated with an increased risk of developing LOAD (OR: 1.97, 95% CI: 1.09-3.58, P= 0.018). The analysis focused on the number or type (R or r) of MC1R variants did not detect any association with LOAD risk (data not shown). Thus, the risk of developing LOAD was associated to a concrete MC1R variant rather than a number or type of variant (data not shown).

We re-evaluated the association between p.V92M MC1R variant and LOAD risk within the subset of 69 patients with typical AD CSF profile (Table 3) and we found it remained statistically significant and, we even evidenced a higher OR (OR: 3.12 95% CI: 1.32-7.38, P= 0.01). We did not detect statistically significant differences in the different CSF biomarkers levels between p.V92M carriers and non carriers (data not shown).

In order to identify whether the p.V92M association with LOAD risk was modulated by the presence of APOE ε4 allele, we evaluated the interaction between both alleles within a complete set of LOAD patients (Table 4). The frequency of variant p.V92M in the MC1R gene was lower in carriers of at least one APOE ε4 allele compared to non-carriers (11.0% and 16.7%, respectively). Although this result did not reach a statistically significant value (p= 0.063), it suggests an inverse correlation between both alleles.

Finally, we observed a significantly lower AOO associated with APOE ε4 allele (P= <0.0001), in contrast, no significant effect on AOO was observed for p.V92M MC1R variant (Table 5).

Discussion

Late onset Alzheimer disease (LOAD) form accounts for more than 90% of AD cases (Bosco et al., 2013). To date, the APOE gene is the major genetic factor in LOAD susceptibility (Li and Grupe, 2007) while other genetic factors related with LOAD susceptibility remain largely unknown. Thus, identification of novel genetic factors may be crucial to detect individuals with an inherited AD risk.

In this study, we report a novel association between the p.V92M variant in the MC1R gene and the risk of developing LOAD. This gene is highly polymorphic, and the p.V92M and other variants are common in the Caucasian population. During the last few years, several genome-wide association studies (GWAS) has been conducted to identify common LOAD risk variants (Harold et al., 2009, Lambert et al., 2009, Seshadri et al., 2010, Hollingworth et al., 2011, Naj et al., 2011, Lambert et al., 2013) and none of these studies reported an association between MC1R and the disease. This could be caused by methodological issues (coverage level of MC1R in SNP-array platforms or statistical conservative correction procedures) or by the molecular or clinical heterogeneity of patients included. Interestingly, we observed an inverse tendency between the APOE genotype and p.V92M suggesting that the

presence of the MC1R variant could contribute to AD susceptibility especially in those patients whose genetic risk could be not attributable to the APOE genotype. Further studies restricted to the MC1R variants and APOE alleles should be conducted using previous GWAS data to elucidate such an inverse correlation.

CSF studies measuring Aβ1-42 and tau protein levels in AD patients with confirmed pathology have demonstrated that abnormal levels of both biomarkers constitute a specific signature of the underlying AD-pathology (senile plaques and neurofibrillary tangles respectively). Furthermore, multiple studies have shown that CSF AD biomarkers have an acceptable diagnostic sensitivity and specificity (Molinuevo et al., 2013). Thus, we included a subset of patients with typical CSF AD biomarkers, demonstrating evidence of AD pathophysiological process and increased probability of AD etiology as a cause of symptomatology of the patient according to NIA-AA criteria (McKhann et al., 2011). This fact is relevant, because different studies have demonstrated neuropathological changes sometimes do not correlate with clinical diagnosis (Balasa et al., 2011). Therefore, the role of the p.V92M MC1R variant in the AD risk was re-analyzed in these well characterized AD patients. Notably, in spite of the sample size reduction, the association remains statistically significant and the LOAD risk in p.V92M carriers increased more than three fold. Thereby, the risk of p.V92M is more evident within more accurately diagnosed AD patients, reaching a magnitude similar to that reported for the APOE ε4 allele or the p.R47H mutation in TREM2 gene (Benitez et al., 2013, Ruiz et al., 2013).

In this study, decreasing of age of onset was restricted to the APOE ε4 allele. Thus, the pV92M variant should be considered as those prior variants that increase the risk of developing AD but do not modulate AOO (Thambisetty et al., 2013).

The MC1R gene encodes a membrane receptor which is expressed in neurons of the periaqueductal gray matter, astrocytes and schwann cells activated by melanocortin peptides (Garcia-Borron et al., 2005, Catania, 2008). This receptor may have an important role in the anti-inflammatory brain response (Catania, 2008) and in female specific mediation mechanisms of analgesia (Liem et al., 2005). MC1R is also expressed in melanocytes, a cell type with a common embryonic origin with brain cells (Christiansen et al., 2000), which determine hair and skin colour (Valverde et al., 1995) and certain variants increase the risk for skin cancer (melanoma and non melanoma skin cancer) (Bastiaens et al., 2001, Raimondi et al., 2008). Functional studies of MC1R variants conducted in melanocytes, reveal that certain variants reduce cell surface protein expression and diminished capacity to stimulate cAMP, resulting in red hair color phenotype (Dessinioti et al., 2011). Interestingly, a MC1R variant related to red hair color phenotype modulates the risk to develop Parkinson diseases (PD) in North American (Gao et al., 2009). These findings explain in part, the previously epidemiological evidence describing a bidirectional link between PD and cutaneous melanoma (CM) (Liu et al., 2011). In contrast to PD, an increased incidence of CM among AD patients and overrepresentation of individuals with natural red hair within AD patients compared to control population has not been reported. It can be explained because the p.V92M variant does not confer a risk to develop CM (Raimondi et al., 2008) and by the fact that variant p.V92M promotes a decrease in the affinity of the receptor for its ligand α-MSH but showed normal cell surface expression and normal capacity to stimulate cAMP consequently, it does not impact on the phenotype (Garcia-Borron et al., 2005, Dessinioti et al., 2011). Notably, a functional deficiency of α-MSH in the brain cells of LOAD patients had been previously reported, suggesting that α-MSH may be critical in the development of LOAD (Anderson et al.,1986) (Rainero et al., 1988).

To date, functional evaluation of MC1R variants in other cell types such as the nervous system cells is limited. However, there is evidence that certain variants may also impact on physiological conditions beyond skin and hair pigmentationn, such as risk of depression disorders (Wu et al., 2011), pain response (Liem et al.,

2005) and anesthetic requirement (Liem et al., 2004). Interestingly, a case-control study indicates that variant p.V92M is associated with the response of desipramine treatment in depression disorder (Wu et al., 2011).

In conclusion, the present study provides evidence that p.V92M MC1R variant increases the risk to develop LOAD. Although, the molecular mechanisms underlying the increased risk of LOAD associated to p.V92M variant are not known, this variant may have biological relevance through non-pigmentation pathways involved in inflammatory or immunomodulatory processes. Further functional studies in brain cells are needed to elucidate the role of this receptor through the different MC1R pathways.

Furthermore, the study highlights the importance of the MC1R gene in neurodegenerative disorders beyond its role in skin cancer susceptibility.

Acknowledgements

We thank the National Bank of DNA from the University of Salamanca (Spain) for kindly providing DNAs from control healthy subjects. The research at the Melanoma Unit in Barcelona is partially funded by Grants from Fondo de Investigaciones Sanitarias (12/00840) Spain co-funded by "Fondo Europeo de Desarrollo Regional (FEDER). Unión Europea. Una manera de hacer Europa", by the AGAUR 2009 SGR 1337 of the Catalan Government, Spain. The work was carried out at the Esther Koplowitz Centre. Barcelona, Spain. This work was partially supported by grant to Dr José Luis Molinuevo (CONSOLIDER-Ingenio program 2010: CSD2010-00045).

Conflict of interest

The authors declare no conflict of interest

References

Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, Kondrashov AS, Sunyaev SR (A method and server for predicting damaging missense mutations. Nat Methods 7:248-249.2010).

Antonell A, Fortea J, Rami L, Bosch B, Balasa M, Sanchez-Valle R, Iranzo A, Molinuevo JL, Llado A (Different profiles of Alzheimer's disease cerebrospinal fluid biomarkers in controls and subjects with subjective memory complaints. J Neural Transm 118:259-262.2011).

Ashford JW, Mortimer JA (Non-familial Alzheimer's disease is mainly due to genetic factors. J Alzheimers Dis 4:169-177.2002).

Balasa M, Gelpi E, Antonell A, Rey MJ, Sanchez-Valle R, Molinuevo JL, Llado A (Clinical features and APOE genotype of pathologically proven early-onset Alzheimer disease. Neurology 76:1720-1725.2011).

Bastiaens MT, ter Huurne JA, Kielich C, Gruis NA, Westendorp RG, Vermeer BJ, Bavinck JN (Melanocortin-1 receptor gene variants determine the risk of nonmelanoma skin cancer independently of fair skin and red hair. Am J Hum Genet 68:884-894.2001).

Benitez BA, Cooper B, Pastor P, Jin SC, Lorenzo E, Cervantes S, Cruchaga C (TREM2 is associated with the risk of Alzheimer's disease in Spanish population. Neurobiol Aging 34:1711 e1715-1717.2013).

Bertram L (Alzheimer's genetics in the GWAS era: a continuing story of 'replications and refutations'. Curr Neurol Neurosci Rep 11:246-253.2011).

Bettens K, Sleegers K, Van Broeckhoven C (Genetic insights in Alzheimer's disease. Lancet Neurol 12:92-104.2013).

Bosco P, Ferri R, Salluzzo MG, Castellano S, Signorelli M, Nicoletti F, Nuovo SD, Drago F, Caraci F (Role of the Transforming-Growth-Factor-beta1 Gene in Late-Onset Alzheimer's Disease: Implications for the Treatment. Curr Genomics 14:147-156.2013).

Castellani RJ, Lee HG, Zhu X, Perry G, Smith MA (Alzheimer disease pathology as a host response. J Neuropathol Exp Neurol 67:523-531.2008).

Catania A (Neuroprotective actions of melanocortins: a therapeutic opportunity. Trends Neurosci 31:353-360.2008).

Cencioni C, Spallotta F, Martelli F, Valente S, Mai A, Zeiher AM, Gaetano C (Oxidative stress and epigenetic regulation in ageing and age-related diseases. Int J Mol Sci 14:17643-17663.2013).

Chaudru V, Laud K, Avril MF, Miniere A, Chompret A, Bressac-de Paillerets B, Demenais F (Melanocortin-1 receptor (MC1R) gene variants and dysplastic nevi modify penetrance of CDKN2A mutations in French melanoma-prone pedigrees. Cancer Epidemiol Biomarkers Prev 14:2384-2390.2005).

Christiansen JH, Coles EG, Wilkinson DG (Molecular control of neural crest formation, migration and differentiation. Curr Opin Cell Biol 12:719-724.2000).

Dessinioti C, Antoniou C, Katsambas A, Stratigos AJ (Melanocortin 1 receptor variants: functional role and pigmentary associations. Photochem Photobiol 87:978-987.2011).

Gao X, Simon KC, Han J, Schwarzschild MA, Ascherio A (Genetic determinants of hair color and Parkinson's disease risk. Ann Neurol 65:76-82.2009).

Garcia-Borron JC, Sanchez-Laorden BL, Jimenez-Cervantes C (Melanocortin-1 receptor structure and functional regulation. Pigment Cell Res 18:393-410.2005).

Gerstenblith MR, Goldstein AM, Fargnoli MC, Peris K, Landi MT (Comprehensive evaluation of allele frequency differences of MC1R variants across populations. Hum Mutat 28:495-505.2007).

Gilbert BJ (The role of amyloid beta in the pathogenesis of Alzheimer's disease. J Clin Pathol 66:362-366.2013).

Harold D, Abraham R, Hollingworth P, Sims R, Gerrish A, (…) Nothen MM, Moebus S, Jockel KH, Klopp N, Wichmann HE, Carrasquillo MM, Pankratz VS, Younkin SG, Holmans PA, O'Donovan M, Owen MJ, Williams J (Genome-wide association study identifies variants at CLU and PICALM associated with Alzheimer's disease. Nat Genet 41:1088-1093.2009).

Hollingworth P, Harold D, Sims R, Gerrish A, Lambert JC, Carrasquillo MM, Abraham R, Hamshere ML, Pahwa JS, Moskvina V, Dowzell K, Jones N, Stretton A, Thomas C, Richards A, Ivanov D, Widdowson C, Chapman J, Lovestone S, Powell J, Proitsi P, Lupton MK, Brayne C, Rubinsztein DC, Gill M, Lawlor B, Lynch A, Brown KS, Passmore PA, Craig D, McGuinness B, Todd S, Holmes C, Mann D, Smith AD, Beaumont H, Warden D, Wilcock G, Love S, Kehoe PG, Hooper NM, Vardy ER, Hardy J, Mead S, Fox NC, Rossor M, Collinge J, Maier W (…) , Mancuso M, Panza F, Solfrizzi V, Nacmias B, Sorbi S, Bossu P, Piccardi P, Arosio B, Annoni G, Seripa D, Pilotto A, Scarpini E, Galimberti D, Brice A, Hannequin D, Licastro F, Jones L, Holmans PA, Jonsson T, Riemenschneider M, Morgan K, Younkin SG, Owen MJ, O'Donovan M, Amouyel P, Williams J (Common variants at ABCA7, MS4A6A/MS4A4E, EPHA1, CD33 and CD2AP are associated with Alzheimer's disease. Nat Genet 43:429-435.2011).

Jonsson T, Stefansson H, Steinberg S, Jonsdottir I, Jonsson PV, Snaedal J, Bjornsson S, Huttenlocher J, Levey AI, Lah JJ, Rujescu D, Hampel H, Giegling I, Andreassen OA, Engedal K, Ulstein I, Djurovic S, Ibrahim-Verbaas C, Hofman A, Ikram MA, van Duijn CM, Thorsteinsdottir U, Kong A, Stefansson K (Variant of TREM2 associated with the risk of Alzheimer's disease. N Engl J Med 368:107-116.2013).

Kadekaro AL, Chen J, Yang J, Chen S, Jameson J, Swope VB, Cheng T, Kadakia M, Abdel-Malek Z (Alpha-melanocyte-stimulating hormone suppresses oxidative stress through a p53-mediated signaling pathway in human melanocytes. Mol Cancer Res 10:778-786.2012).

Lambert JC, Heath S, Even G, Campion D, Sleegers K, Hiltunen M, Combarros O, Zelenika D, Bullido MJ, Tavernier B, Letenneur L, Bettens K, Berr C, Pasquier F, Fievet N, Barberger-Gateau P, Engelborghs S, De Deyn P, Mateo I, Franck A, Helisalmi S, Porcellini E, Hanon O, de Pancorbo MM, Lendon C, Dufouil C, Jaillard C, Leveillard T, Alvarez V, Bosco P, Mancuso M, Panza F, Nacmias B, Bossu P, Piccardi P, Annoni G, Seripa D, Galimberti D, Hannequin D, Licastro F, Soininen H, Ritchie K, Blanche H, Dartigues JF, Tzourio C, Gut I, Van Broeckhoven

C, Alperovitch A, Lathrop M, Amouyel P (Genome-wide association study identifies variants at CLU and CR1 associated with Alzheimer's disease. Nat Genet 41:1094-1099.2009).

Lambert JC, Ibrahim-Verbaas CA, Harold D, Naj AC, Sims R, Bellenguez C, Jun G, Destefano AL, Bis JC, Beecham GW, Grenier-Boley B, Russo G, Thornton-Wells TA, Jones N, Smith AV, Chouraki V, Thomas C, Ikram MA, Zelenika D, Vardarajan BN, Kamatani Y, Lin CF, Gerrish A, Schmidt H, Kunkle B, Dunstan ML, Ruiz A, Bihoreau MT, Choi SH, Reitz C, Pasquier F, Hollingworth P, Ramirez A, Hanon O, Fitzpatrick AL, Buxbaum JD, Campion D, Crane PK, Baldwin C, Becker T, Gudnason V, Cruchaga C, Craig D, Amin N, Berr C, Lopez OL, De Jager PL, Deramecourt V, Johnston JA, Evans D, Lovestone S, Letenneur L, Moron FJ, Rubinsztein DC, Eiriksdottir G, Sleegers K, Goate AM, Fievet N, Huentelman MJ, Gill M, Brown K, Kamboh MI, Keller L, Barberger-Gateau P, McGuinness B, Larson EB, Green R, Myers AJ, Dufouil C, Todd S, Wallon D, Love S, Rogaeva E, Gallacher J, St George-Hyslop P, Clarimon J, Lleo A, Bayer A, Tsuang DW, Yu L, Tsolaki M, Bossu P, Spalletta G, Proitsi P, Collinge J, Sorbi S, Sanchez-Garcia F, Fox NC, Hardy J, Naranjo MC, Bosco P, Clarke R, Brayne C, Galimberti D, Mancuso M, Matthews F, Moebus S, Mecocci P, Del Zompo M, Maier W, Hampel H, Pilotto A, Bullido M, Panza F, Caffarra P, Nacmias B, Gilbert JR, Mayhaus M, Lannfelt L, Hakonarson H, Pichler S, Carrasquillo MM, Ingelsson M, Beekly D, Alvarez V, Zou F, Valladares O, Younkin SG, Coto E, Hamilton-Nelson KL, Gu W, Razquin C, Pastor P, Mateo I, Owen MJ, Faber KM, Jonsson PV, Combarros O, O'Donovan MC, Cantwell LB, Soininen H, Blacker D, Mead S, Mosley TH, Jr., Bennett DA, Harris TB, Fratiglioni L, Holmes C, de Bruijn RF, Passmore P, Montine TJ, Bettens K, Rotter JI, Brice A, Morgan K, Foroud TM, Kukull WA, Hannequin D, Powell JF, Nalls MA, Ritchie K, Lunetta KL, Kauwe JS, Boerwinkle E, Riemenschneider M, Boada M, Hiltunen M, Martin ER, Schmidt R, Rujescu D, Wang LS, Dartigues JF, Mayeux R, Tzourio C, Hofman A, Nothen MM, Graff C, Psaty BM, Jones L, Haines JL, Holmans PA, Lathrop M, Pericak-Vance MA, Launer LJ, Farrer LA, van Duijn CM, Van Broeckhoven C, Moskvina V, Seshadri S, Williams J, Schellenberg GD, Amouyel P (Meta-analysis of 74,046 individuals identifies 11 new susceptibility loci for Alzheimer's disease. Nat Genet.2013).

Li Y, Grupe A (Genetics of late-onset Alzheimer's disease: progress and prospect. Pharmacogenomics 8:1747-1755.2007).

Liem EB, Joiner TV, Tsueda K, Sessler DI (Increased sensitivity to thermal pain and reduced subcutaneous lidocaine efficacy in redheads. Anesthesiology 102:509-514.2005).

Liem EB, Lin CM, Suleman MI, Doufas AG, Gregg RG, Veauthier JM, Loyd G, Sessler DI (Anesthetic requirement is increased in redheads. Anesthesiology 101:279-283.2004).

Liu R, Gao X, Lu Y, Chen H (Meta-analysis of the relationship between Parkinson disease and melanoma. Neurology 76:2002-2009.2011).

McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM (Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. Neurology 34:939-944.1984).

McKhann GM, Knopman DS, Chertkow H, Hyman BT, Jack CR, Jr., Kawas CH, Klunk WE, Koroshetz WJ, Manly JJ, Mayeux R, Mohs RC, Morris JC, Rossor MN, Scheltens P, Carrillo MC, Thies B, Weintraub S, Phelps CH (The diagnosis of dementia due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. Alzheimers Dement 7:263-269.2011).

Molinuevo JL, Gispert JD, Dubois B, Heneka MT, Lleo A, Engelborghs S, Pujol J, de Souza LC, Alcolea D, Jessen F, Sarazin M, Lamari F, Balasa M, Antonell A, Rami L (The AD-CSF-index discriminates Alzheimer's disease patients from healthy controls: a validation study. J Alzheimers Dis 36:67-77.2013).

Naj AC, Jun G, Beecham GW, Wang LS, Vardarajan BN, Buros J, Gallins PJ, Buxbaum JD, Jarvik GP, Crane PK, Larson EB, Bird TD, Boeve BF, Graff-Radford NR, De Jager PL, Evans D, Schneider JA, Carrasquillo MM, Ertekin-Taner N, Younkin SG, Cruchaga C, Kauwe JS, Nowotny P, Kramer P, Hardy J, Huentelman MJ, Myers AJ, Barmada MM, Demirci FY, Baldwin CT, Green RC, Rogaeva E, St George-Hyslop P, Arnold SE, Barber R, Beach T, Bigio EH, Bowen JD, Boxer A, Burke JR, Cairns NJ, Carlson CS, Carney RM, Carroll SL, Chui HC, Clark DG, Corneveaux J, Cotman CW, Cummings JL, DeCarli C, DeKosky ST, Diaz-Arrastia R, Dick M, Dickson DW, Ellis WG, Faber KM, Fallon KB, Farlow MR, Ferris S, Frosch MP, Galasko DR, Ganguli M, Gearing M, Geschwind DH, Ghetti B, Gilbert JR, Gilman S, Giordani B, Glass JD, Growdon JH, Hamilton RL, Harrell LE, Head E, Honig LS, Hulette CM, Hyman BT, Jicha GA, Jin LW, Johnson N, Karlawish J, Karydas A, Kaye JA, Kim R, Koo EH, Kowall NW, Lah JJ, Levey AI, Lieberman AP, Lopez OL,

Mack WJ, Marson DC, Martiniuk F, Mash DC, Masliah E, McCormick WC, McCurry SM, McDavid AN, McKee AC, Mesulam M, Miller BL, Miller CA, Miller JW, Parisi JE, Perl DP, Peskind E, Petersen RC, Poon WW, Quinn JF, Rajbhandary RA, Raskind M, Reisberg B, Ringman JM, Roberson ED, Rosenberg RN, Sano M, Schneider LS, Seeley W, Shelanski ML, Slifer MA, Smith CD, Sonnen JA, Spina S, Stern RA, Tanzi RE, Trojanowski JQ, Troncoso JC, Van Deerlin VM, Vinters HV, Vonsattel JP, Weintraub S, Welsh-Bohmer KA, Williamson J, Woltjer RL, Cantwell LB, Dombroski BA, Beekly D, Lunetta KL, Martin ER, Kamboh MI, Saykin AJ, Reiman EM, Bennett DA, Morris JC, Montine TJ, Goate AM, Blacker D, Tsuang DW, Hakonarson H, Kukull WA, Foroud TM, Haines JL, Mayeux R, Pericak-Vance MA, Farrer LA, Schellenberg GD (Common variants at MS4A4/MS4A6E, CD2AP, CD33 and EPHA1 are associated with late-onset Alzheimer's disease. Nat Genet 43:436-441.2011).

Olsen JH, Friis S, Frederiksen K (Malignant melanoma and other types of cancer preceding Parkinson disease. Epidemiology 17:582-587.2006).

Raimondi S, Sera F, Gandini S, Iodice S, Caini S, Maisonneuve P, Fargnoli MC (MC1R variants, melanoma and red hair color phenotype: a meta-analysis. Int J Cancer 122:2753-2760.2008).

Rainero I, May C, Kaye JA, Friedland RP, Rapoport SI (CSF alpha-MSH in dementia of the Alzheimer type. Neurology 38:1281-1284.1988).

Roberts PJ (Development, characterization, and subcellular location of DNAse activity in HL-60 cells and monocytes. Blood 75:976-983.1990).

Ruiz A, Dols-Icardo O, Bullido MJ, Pastor P, Rodriguez-Rodriguez E, Lopez de Munain A, de Pancorbo MM, Perez-Tur J, Alvarez V, Antonell A, Lopez-Arrieta J, Hernandez I, Tarraga L, Boada M, Lleo A, Blesa R, Frank-Garcia A, Sastre I, Razquin C, Ortega-Cubero S, Lorenzo E, Sanchez-Juan P, Combarros O, Moreno F, Gorostidi A, Elcoroaristizabal X, Baquero M, Coto E, Sanchez-Valle R, Clarimon J (Assessing the role of the TREM2 p.R47H variant as a risk factor for Alzheimer's disease and frontotemporal dementia. Neurobiol Aging 35:444 e441-444.2013).

Seshadri S, Fitzpatrick AL, Ikram MA, DeStefano AL, Gudnason V, Boada M, Bis JC, Smith AV, Carassquillo MM, Lambert JC, Harold D, Schrijvers EM, Ramirez-Lorca R, Debette S, Longstreth WT, Jr., Janssens AC, Pankratz VS, Dartigues JF, Hollingworth P, Aspelund T, Hernandez I, Beiser A, Kuller LH, Koudstaal PJ, Dickson DW, Tzourio C, Abraham R, Antunez C, Du Y, Rotter JI, Aulchenko YS, Harris TB, Petersen RC, Berr C, Owen MJ, Lopez-Arrieta J, Varadarajan BN, Becker JT, Rivadeneira F, Nalls MA, Graff-Radford NR, Campion D, Auerbach S, Rice K, Hofman A, Jonsson PV, Schmidt H, Lathrop M, Mosley TH, Au R, Psaty BM, Uitterlinden AG, Farrer LA, Lumley T, Ruiz A, Williams J, Amouyel P, Younkin SG, Wolf PA, Launer LJ, Lopez OL, van Duijn CM, Breteler MM (Genome-wide analysis of genetic loci associated with Alzheimer disease. JAMA 303:1832-1840.2010).

Sole X, Guino E, Valls J, Iniesta R, Moreno V (SNPStats: a web tool for the analysis of association studies. Bioinformatics 22:1928-1929.2006).

Thambisetty M, An Y, Tanaka T (Alzheimer's disease risk genes and the age-at-onset phenotype. Neurobiol Aging 34:2696 e2691-2695.2013).

Valverde P, Healy E, Jackson I, Rees JL, Thody AJ (Variants of the melanocyte-stimulating hormone receptor gene are associated with red hair and fair skin in humans. Nat Genet 11:328-330.1995).

Welge V, Fiege O, Lewczuk P, Mollenhauer B, Esselmann H, Klafki HW, Wolf S, Trenkwalder C, Otto M, Kornhuber J, Wiltfang J, Bibl M (Combined CSF tau, p-tau181 and amyloid-beta 38/40/42 for diagnosing Alzheimer's disease. J Neural Transm 116:203-212.2009).

Wu GS, Luo HR, Dong C, Mastronardi C, Licinio J, Wong ML (Sequence polymorphisms of MC1R gene and their association with depression and antidepressant response. Psychiatr Genet 21:14-18.2011).

ANEXO IV. ARTÍCULO

TERT and *AURKA* gene copy number gains enhance the detection of acral lentiginous melanomas by fluorescence in situ hybridization".

Versión del artículo aceptado para su publicación en la revista *The Journal of Molecular Diagnostics (J Mol Diagn. 2013 Dec 27. pii: S1525-1578(13)00254-7. doi: 10.1016/j.jmoldx.2013.10.009. [Epub ahead of print])*

TITLE PAGE

TERT and _AURKA_ gene copy number gains enhance the detection of acral lentiginous melanomas by fluorescence in situ hybridization

Alba Diaz,* Joan Anton Puig-Butille,[†,¶] Alexandra Valera,* Concha Muñoz,* Dolors Costa,* Adriana Garcia-Herrera,* Cristina Carrera,[‡,¶] Francesc Sole,[§] Josep Malvehy,[‡,¶] Susana Puig,[‡,¶] and Llucia Alos*

Departments of Pathology,* Biochemical and Molecular Genetics,[†] and Dermatology, [‡] Hospital Clínic, IDIBAPS, University of Barcelona, Spain; Institut de Recerca contra la Leucèmia Josep Carreras,[§] Hospital Germans Trias i Pujol, Universitat Autònoma de Barcelona, Badalona, Spain; Centro Investigación Biomédica en Enfermedades Raras (CIBERER), [¶] Instituto de Salud Carlos III (ISCIII), Barcelona, Spain.

Number of text pages: 9; *number of tables*: 4; *number of figures*: 4.

Short running head: Gene copy number gains in acral melanomas

Grant numbers and sources of support:

This study was supported by a grant from Hospital Clínic de Barcelona (Ajut a la recerca "Josep Font") and partially supported by grants from the Instituto de Salud Carlos III, Ministerio de Sanidad y Consumo, Spain (PI 11/02010); Red Temática de Investigación Cooperativa en Cáncer (RTICC, FEDER) (RD12/0036/0044), Fundación Española de Hematología y Hemoterapia; INBIOMED HEMA-001/2006 and Obra Social La Caixa-Fundació Carreras.

Correspondence and reprints to:

Llucia Alos MD

Department of Pathology, Hospital Clínic

Villarroel 170, Barcelona. Spain

Phone +34 93 227 54 50

Fax +34 93 227 57 17

Email: lalos@clinic.ub.es

ABSTRACT

The study of specific chromosomal loci through fluorescence in situ hybridization (FISH) has proven useful in the differential diagnosis of melanocytic tumours. However, different sensitivity rates have been reached probably due to the molecular heterogeneity of melanomas. Acral lentiginous melanomas are characterized as showing copy number gains of small genomic regions, such as *CCND1*, *TERT* and *AURKA* genes. In a series of 58 acral melanocytic lesions we explored the value of the four-colour FISH probe, together with *MYC*, and assessed the potential diagnostic usefulness of newly developed probes targeting *TERT* and *AURKA*. Moreover we tested the CCND1, TERT and AURKA protein expression by immunohistochemistry. The four-colour FISH probe detected 85.3% of melanomas while *TERT* and *AURKA* copy number gains, 29.4%. When combining the results of all probes, a sensitivity of 97% (CI, 82.9% to 99.8%) was found. No *MYC* copy number gains were detected. No nevi showed aberrations. Immunohistochemistry showed a higher percentage of CCND1, TERT and AURKA positive cells in melanomas, compared to nevi ($P \leq 0.001$). A significant correlation between gene copy number gain and protein expression was found for *CCND1* ($P=0.015$). Our results indicate that the addition of specific FISH probes to the current probe could improve its sensitivity for the diagnosis of acral melanomas. Further studies are needed in a larger number of cases to validate these results.

Introduction

The histopathological evaluation is still the gold standard in differentiating benign from malignant melanocytic lesions. However, its subjectivity and limitations are widely accepted, as a number of cases cannot be easily classified as benign or malignant based only on histopathological evaluation.[178] In recent years investigations on melanocytic tumors have been focused on identifying gene alterations that could help to differentiate benign from malignant melanocytic tumors. Comparative genomic hybridization (CGH) studies have demonstrated that benign and malignant melanocytic tumors differ dramatically in the presence of numerous chromosomal aberrations.[179] Based on these results, a commercially available four-colour fluorescence in situ hybridization (FISH) probe targeting 6p25 (RREB1), 6q23 (MYB), 11q13 (CCND1) and centromere 6 (CEP6) was recently developed to assist in differentiating several problematic melanocytic lesions.[180] This FISH probe has been tested in a variety of clinicopathological settings and has proven useful in increasing sensitivity and specificity in the differential diagnosis between benign nevi and melanomas. However, different results have been found, mainly depending on the melanocytic lesion subtype.[181-185] In fact, there is increasing evidence that melanomas are heterogeneous at the molecular level and specific tumour subtypes may harbor distinct genomic alterations.[97] In this sense, a broader set incorporating 8q24 (MYC) and 9p21 (CDKN2A) probes has been recently proposed to enhance the detection of melanomas, including the spitzoid types.[186]

Acral lentiginous melanoma (ALM), one of the four major histopathological melanoma subtypes recognized by the current WHO classification,[187] has distinctive clinicopathological and molecular characteristics. It frequently harbours multiple and distinctive chromosomal aberrations, mainly gene copy number gains of small genomic regions such as CCND1, TERT and AURKA genes.[97, 103, 179, 188-190] Its low incidence in Caucasians might render difficult its deeply clinicopathological and molecular characterization. In fact, few cases of ALMs and acral nevi (AN) have been studied with the new multiprobe FISH set to date.[183, 186, 191, 192] Furthermore, from the daily practice standpoint, acral pigmented lesions may display site-related atypical features, which can represent potential pitfalls in differentiating benign from malignant tumors.[193]

TERT, at 5p15.33, encodes for the catalytic subunit of telomerase reverse transcriptase which stabilizes telomeric length. Its up-regulation enhances cellular proliferation and plays a critical role in oncogenesis.[194] AURKA, at 20q13, encodes for aurora kinase A, a cell cycle-regulated kinase which is mainly involved in centrosome function and spindle

assembly during chromosome segregation. Therefore, its disregulation leads to genetic instability and aneuploidy.[195]

In this study we include a series of 58 acral melanocytic lesions, comprising both benign and malignant tumors, and we explore the usefulness of the currently available four-colour FISH probe, together with MYC gene status, for differential diagnosis purposes. Furthermore, we evaluate the utility of additional FISH probes targeting TERT and AURKA genes to enhance the detection of ALM. Moreover we test CCND1, TERT and AURKA protein expression by immunohistochemistry.

Materials and Methods

A total of 58 formalin-fixed paraffin-embedded samples of acral melanocytic tumors was collected from 2002 to 2008 from the archives of the Pathology Department at the Hospital Clínic of Barcelona. They included 34 invasive acral lentiginous melanomas (ALM) and 24 acral nevi (AN). Unequivocal diagnosis of ALM or AN was confirmed blindly by two dermatopathologists (A.D., L.A.). All patients were seen and followed up in the Melanoma Unit, Department of Dermatology, of the same hospital and clinical data were retrieved from the review of the clinical files. The study was approved by the Institutional Ethics Review Board and written informed consent was obtained from each participant in accordance with the Declaration of Helsinki.

Elaboration of FISH probes targeting TERT and AURKA

Non-commercial probes targeting telomerase reverse transcriptase (TERT) at 5p15.33 and aurora kinase A (AURKA) at 20q13 were elaborated using bacterial artificial chromosomes (BAC). BAC clones were obtained from the Children's Hospital Oakland Research Institute collection and selected using the genome browser from Centre de Regulació Genòmica, Barcelona, Spain (http://davinci.crg.es/cgi-gbrowse/gbrowse/hg18/, registration is required; date of last accession: 24/05/2012). Then DNA was labeled using a nick translation kit (Abbott Molecular) with Spectrum Green or Red (Abbott Molecular). For AURKA, a centromeric probe was added (CEP20, labelled with Spectrum Orange, Abbott Molecular). For TERT, another BAC-probe located on 5q15 (MCTP1) was developed to be used as chromosome control. Table 1 shows the selected BACs, the gene locus and the labeling-spectrum. Finally, all probes were hybridized to normal metaphase chromosomes in order to verify their location and to normal skin controls obtaining the normal pattern of hybridization (2 signals on average for each probe).

FISH analysis

All cases were analyzed with the commercially available four-colour probe set targeting the ras responsive element binding protein 1 (RREB1) on 6p25, V-myb myeloblastosis viral oncogene homolog (MYB) on 6q23, cyclin D1 (CCND1) on 11q13 and the chromosome 6 centromeric region (Abbott Molecular, Des Plaines, IL, USA). Additionally, cases were hybridized with the commercially available probes LSI Cyclin D1/CEP 11 (Vysis; Abbott Molecular, Des Plaines, IL) and MYC/CEN-8 FISH Probe Mix (Dako; Agilent Technologies, Santa Clara, CA, USA), which contain both fluorescently labeled gene and centromere probes, and with the non-commercial probes targeting TERT and AURKA. From each FFPE sample block, 4 μm thick sections were mounted onto positively charged slides (SuperFrost Plus, ThermoShandon, Pittsburgh, PA) and they were deparaffinized and dehydrated. After that, pre-treatment and pepsin digestion (3 min) were carried out followed by dehydration in 70%, 85%, and 96 % ethanol for 2 minutes each. The slides were then incubated in a hybridizer (Hybridizer S2450, Dako, Denmark) for denaturation at 90ºC for 5 minutes and hybridization at 37ºC for approximately 18 hours. Sections were placed in washing buffer at room temperature for 2–10 min to remove the coverslips and then immersed in 65◦C stringency buffer for 10 min, dehydrated, dried and counterstained with DAPI.

Enumeration of FISH signals

FISH evaluation was carried out using an epifluorescence microscope (Nikon Eclipse 50i, Nikon instruments Inc, Melville, NY) equipped with appropriate single band-pass filter sets (Abbott Molecular). First of all, each case was examined at low-power magnification to select those areas with abnormal copy number. Then at least 20 adjacent nuclei from three different areas were enumerated (total of 60 cells) under high-power magnification (400x). Overlapping nuclei as well as nuclei with less than two signals were not listed. Moreover, cases in which less than 60 nuclei could be evaluated or in which nuclei did not show signals for all probes were excluded. For the multicolour commercial probe set, a case was considered as having a positive FISH result if any of the following criteria were met: (a) gain in 6p25 (RREB1) relative to CEP6 in >55% of cells or (b) gain in 6p25 (RREB1) in >29%, (c) gain in 11q13 (CCND1) in >38% and (d) loss in 6q23 (MYB) relative to CEP6 in >40%, according to the previously determined cut-offs. For the MYC/CEN-8 probe a case was considered positive if ≥ 50% of the enumerated melanoma cells harboured copy number gains of MYC, as previously described.[196] For the LSI Cyclin D1/CEP 11 and the non-commercial probes, a tumor was considered positive (with copy

number gains) if the ratio between gene copy number and centromere copy number was ≥ 1.5. A ratio above 2 was considered amplification, as previously defined. [197] In 19 ALMs the in situ component was also evaluated.

Immunohistochemistry

All cases were stained for CCND1 (clone SP4, dilution 1:100, Abcam, Cambridge, UK), TERT (clone Y182, dilution 1:100, Abcam) and AURKA (dilution 1:300, Abcam) with the automated system Bond Max (Leica Microsystems, Wetzlar, Germany), using the Bond Polymer Refine Red Detection system (Leica Microsystems). Appropriate positive and negative controls were used for each antibody.

The percentage of positive cells was recorded semiquantitatively in each case for cytoplasmic (TERT and AURKA) or nuclear (CCND1, TERT and AURKA) staining.

Statistical analysis

All statistical analyses were performed using PAS W Statistics 18 (SPAA Inc., Chicago, IL). Associations between variables were performed with use of the exact Fisher test. Quantitative data were compared between both groups using either a Student's t-test (for variables with a normal distribution) or a Mann-Whitney U test (for variables without a normal distribution). Pearson correlations were performed on quantitative data. To determine the specificity and sensitivity of FISH, histopathology was considered the gold standard and the 95% confidence intervals (CIs) of sensitivity and specificity were calculated with the VassarStats Clinical Calculator 1 (http://www.vassarstats.net/clin1.html; date of last accession: 23/10/2013), which uses the Wilson score method. The results were considered statistically significant at a P value of 0.05 or less (2-sided).

Results

Clinicopathological features of patients

The main characteristics of patients with ALMs are presented in Table 2. Seventeen patients were men and 17 were women (male-to-female ratio 1:1), with a median age of 67 years (range 39-91). In 30 patients (88.2%) the tumor occurred on the feet while in 4 (11.8%) it arose on the hands. The median Breslow thickness was 3.8 mm (range 0.40-12 mm). The Clark level was II in 2 lesions (5.9%), III in 4 (11.8%), IV in 19 (55.9%) and V in 9 (26.5%). Ulceration was present in 18 cases (52.9%) and the median mitotic count was 4 mitoses per mm2 (range 0-18).

In AN, 10 patients were men and 14 were women (male-to-female ratio 1:1.4), with a median age of 37 years (range 16-64). Twenty-two of the lesions (91%) occurred on the feet and histologically they were mostly compound (21 of 24, 87.5%).

FISH results

FISH data for ALMs are summarized in Table 3. Overall, 29 out of 34 ALMs had a positive result when evaluated using the commercial FISH multiprobe set, resulting in a sensitivity of 85.3% (95% CI, 68.2% to 94.4%). The RREB1 gain criteria was the most sensitive, observed in 25 out of 34 cases (73.5%). Twenty out of 34 (58.8%) met the RREB1/CEP6 gain criteria, 14 out of 34 (41.2%) met the MYB/CEP6 loss criteria (Figure 1) and 8 out of 34 (23.5%) met the CCND1 gain criteria. CCND1 gains were confirmed in all cases with the commercial probe LSI Cyclin D1/CEP 11, which showed that all of them were gene amplifications [CCND1/CEP11 ratio: median 4.16 (range 2.18-5.10)]. When using the non-commercial probes, 10 of 34 tumors (29.4%) gave positive results. TERT copy number gains were present in 8 cases (23.5%), and in 6 of these cases there were gene amplifications, with an average ratio above 2 (Figure 1). AURKA copy number gains were seen in 2 cases (5.9%). CCND1, TERT and AURKA gains were mutually exclusive. No MYC copy number gains were detected in any case. When combining the results of all probes, FISH had a positive result in 33 out of 34 ALMs, resulting in an overall sensitivity of 97% (95% CI, 82.9% to 99.8%).

We also evaluated the presence of gene copy number alterations in the in situ component of ALMs, which could be assessed confidently in 19 of the 34 cases. In all of them we could find RREB1, TERT and CCND1 gene copy number gains in the in situ portion, at similar levels to those in the invasive one (Figure 2). In addition, in 5 cases, CCND1 and TERT gene copy number gains were also visualized in scattered cells on the basal layer of histologically normal-appearing skin beyond the in situ component.

None of the 24 AN showed any significant copy number changes in any of the targeted genes (Figure 3), resulting in an overall specificity of 100% (95% CI, 82.8% to 100%).

Correlation of FISH results with clinicopathological features

The clinicopathological characteristics of patients with melanomas harbouring CCND1, TERT and AURKA copy number gains were compared and no differences between these groups were found. In addition, no histological differences were found when comparing tumor thickness, ulceration, mitotic rate, tumor cell type, amount of pigmentation or

presence of tumor-infiltrating lymphocytes of melanomas belonging to the different groups.

Immunohistochemistry results and correlation with FISH

Immunohistochemical data are summarized in Table 4. ALMs showed a significantly higher percentage of nuclear CCND1, TERT and AURKA positive cells, compared to AN. Moreover, AN presented a decreasing CCND1 immunopositivity in deep portions of the tumor, while ALMs showed a more homogeneous distribution of positive cells. TERT and AURKA nuclear immunoreactivity did not show any particular distribution pattern. All lesions (both ALM and AN) showed TERT and AURKA cytoplasmic positivity in nearly 100% of cells.

ALMs harbouring CCND1 gains showed a significantly higher percentage of CCND1 positive cells by immunohistochemistry, when compared to those ALMs without CCND1 gain, with a median of 75% (range 50-95) and 45% (range 30-80), respectively (P=0.002) (Figure 4). Moreover, in ALMs with CCND1 gains, a positive significant correlation was found between the percentage of nuclear positive cells and the average number of CCND1 signals (ρ=0.90, P=0.015). No correlation was found concerning TERT or AURKA gene copy number gains with their respective protein expression.

Discussion

ALMs are characterized as presenting frequent gene copy number gains, and we have detected 2 genes related to oncogenesis that can show an increased copy number in ALMs: TERT and AURKA. In this study we have confirmed that these genes do not show copy number gains in any benign AN, thus both are promising genes that can help in differentiating malignant from benign acral melanocytic tumors. Moreover we have tested for the first time the commercially developed FISH assay in a series of 58 acral melanocytic lesions, including both benign and malignant counterparts, as only few acral melanocytic lesions had been included in previously published studies.[183, 186, 191, 192] This FISH assay was able to identify 29 out of 34 ALMs, while all AN were negative. These results correlate to a sensitivity of 85.3% (CI, 68.2% to 94.4%) and a specificity of 100% (CI, 82.8% to 100%), similar rates to those reached in previous studies. However, variable sensitivity rates have been found using this FISH probe, ranging from 40 to 100%, mainly depending on the melanoma subtypes.[181-185] When we added the TERT and AURKA gene exploration in our series, four ALMs with negative results using the commercial multi-color probe set showed amplifications. This result increased the

sensitivity in differentiating ALM from AN to 97% (CI, 82.9% to 99.8%), and maintained the specificity of 100% (CI, 82.8% to 100%) as no nevi showed any alterations. Interestingly, in our series, CCND1, TERT and AURKA gene copy number gains were mutually exclusive. However, in a previous study,[189] 11q13 and 5p15 amplifications were concurrently detected in the invasive portion of few ALMs. The 6p25 (RREB1) gain criterion was the most frequent gene alteration in our series of ALM, and it was detected in 73.5% of cases, in agreement with previously published studies on non-acral melanomas. The second most frequent gene alteration was 6q23 (MYB) loss, which we detected in 41.2% of cases. This result reinforces the concept that the presence of a chromosome 6 imbalance is a very common event in the vast majority of melanomas,[183] including ALMs. CCND1 and TERT copy number gains were both detected in an equal percentage of cases, 23.5%, and AURKA copy number gains were found in 5.8% of cases. The 11q13 region amplification was first detected by CGH, and considered a common and distinct event in ALM.[188] This fact was subsequently supported by other studies although the frequency of CCND1 gene amplification in ALMs has been found to be variable, ranging from 23 to 44%,[101, 198, 199] in accordance with our results. This amplification rates variability could be accounted for by the different techniques and cut-offs used in its assessment. In the previous study by Puig-Butille et al, using MLPA methodology on frozen samples, a higher proportion of ALM cases with increased CCND1, TERT and AURKA gene copy number was found. This discrepancy with our results, based on FISH methology on formalin-fixed paraffin-embedded tissue, could be accounted for by the fact that different techniques have been used, with different cut-offs and types of samples. Moreover, there exists a major limitation concerning the low number of cases studied. Wang et al have recently shown that array-based comparative genomic hybridization has a higher sensitivity than FISH to detect malignant melanomas, but a good correlation between both techniques has been found. Moreover, they suggest that the exploration of additional genes along with the commercial four-colour FISH probe enhances the detection of melanoma,[200] in accordance with our findings. MYC, a gene located at 8q24, has been found to show copy number gains in a subset of melanomas with distinctive clinicopathological features.[196] Its detection has been recently added to improve the differential diagnosis between malignant melanoma and nevi.[186] However we did not detect an increase in MYC copy number in any ALM, in agreement with a previous study in which a large number of melanomas from different anatomic sites, including 23 ALMs, were studied by FISH.[198]

In most cancers, including melanoma, gene amplifications have been linked to tumor progression since they occur frequently at the later stages.[201] In contrast, and in agreement with previous studies,[188, 202] we have detected gene copy number gains, mainly amplifications, in ALMs in both the invasive and the in situ components at similar levels. This suggests that gene amplifications might arise very early on during tumorigenesis in this melanoma subset. Furthermore, we have also identified CCND1 and TERT gene copy number gains in isolated cells lying on histologically normal-appearing skin, far from the invasive and in situ portions. This particular finding has already been described for some genes including CCND1 and TERT in ALMs, and these cells have been referred to as “field cells”. It has been suggested that these “field cells” arise even before the phase of melanoma in situ, representing clonally related melanocytes in the seemingly non-lesional epidermis and that they could explain local recurrences that take place even after an apparently complete melanoma excision.[188, 189] This finding confers a distinctive biological behaviour on ALMs. CCND1 is a proto-oncogene encoding cyclin D1, which plays an important role in the progression from the G1 to S phase and cell proliferation. Its amplification might alter cell cycle progression thus contributing to tumorigenesis.[203] TERT encodes for the catalytic subunit of telomerase reverse transcriptase, which stabilizes telomeric length that is essential for cellular proliferation. Increased telomerase activity has been reported in a majority of human tumors suggesting that telomeric stability maintenance plays a critical role in oncogenesis.[194] The existence of amplifications of these two genes in the earlier stages of ALMs would represent severe genomic instability thus clearly contributing to ALM oncogenesis.

In this study, a significant correlation between gene copy number gains and protein overexpression was only found for CCND1. However, half of the ALMs without CCND1 gene copy number gains also showed protein overexpression. Actually, protein overexpression of cyclins is not always caused by gene amplification, as other mechanisms could be involved in its post-transcriptional regulation, such as translocations [204] or mutations in upstream genes of the mitogen-activated protein kinase pathway.[97] Additionally, CCND1 expression was significantly lower in nevi than in melanomas. CCND1 expression in melanocytic tumors has given controversial results in previously published studies. Some studies have shown no differences in its expression between benign and malignant lesions, whereas others have demonstrated clear CCND1 overexpression in melanomas.[182, 205, 206] Interestingly, we have also found a different CCND1 expression pattern between nevi and melanomas. Nevi showed a clear zonal

pattern, with a higher proportion of immunopositive cells near the dermal-epidermal junction, in accordance with previous studies[206, 207] whereas melanomas showed a homogeneous pattern of immunostaining. We have also observed a significantly higher expression of nuclear TERT and AURKA in melanomas compared to nevi. However their expression was very low in both groups and some nevi showed higher expression than some melanomas, which limits their usefulness for differential diagnosis purposes.

In conclusion, we have shown that the addition of FISH probes including TERT and AURKA genes to the commercial four-color probe set could improve sensitivity for the diagnosis of ALM, while maintaining 100% specificity. Future efforts should be focused on validating these results by the analysis of larger series, including histopathologically ambiguous lesions.

REFERENCES

1. Buettner PG and Raasch BA. Incidence rates of skin cancer in Townsville, Australia. Int J Cancer. 1998; 78(5):587-593.

2. Lo JS, Snow SN, Reizner GT, Mohs FE, Larson PO and Hruza GJ. Metastatic basal cell carcinoma: report of twelve cases with a review of the literature. J Am Acad Dermatol. 1991; 24(5 Pt 1):715-719.

3. Robinson JK and Dahiya M. Basal cell carcinoma with pulmonary and lymph node metastasis causing death. Arch Dermatol. 2003; 139(5):643-648.

4. von Domarus H and Stevens PJ. Metastatic basal cell carcinoma. Report of five cases and review of 170 cases in the literature. J Am Acad Dermatol. 1984; 10(6):1043-1060.

5. Boyd AS, Shyr Y and King LE, Jr. Basal cell carcinoma in young women: an evaluation of the association of tanning bed use and smoking. J Am Acad Dermatol. 2002; 46(5):706-709.

6. Siegel R, Naishadham D and Jemal A. Cancer statistics, 2012. CA Cancer J Clin. 2012; 62(1):10-29.

7. Thompson JF, Scolyer RA and Kefford RF. Cutaneous melanoma in the era of molecular profiling. Lancet. 2009; 374(9687):362-365.

8. Goldstein AM, Chan M, Harland M, Hayward NK, Demenais F, Bishop DT, Azizi E, Bergman W, Bianchi-Scarra G, Bruno W, Calista D, Albright LA, Chaudru V, Chompret A, Cuellar F, Elder DE, et al. Features associated with germline CDKN2A mutations: a GenoMEL study of melanoma-prone families from three continents. J Med Genet. 2007; 44(2):99-106.

9. Puig S, Malvehy J, Badenas C, Ruiz A, Jimenez D, Cuellar F, Azon A, Gonzalez U, Castel T, Campoy A, Herrero J, Marti R, Brunet-Vidal J and Mila M. Role of the CDKN2A locus in patients with multiple primary melanomas. J Clin Oncol. 2005; 23(13):3043-3051.

10. Liontos M, Pateras IS, Evangelou K and Gorgoulis VG. The Tumor Suppressor Gene ARF as a Sensor of Oxidative Stress. Curr Mol Med. 2012.

11. Jenkins NC, Liu T, Cassidy P, Leachman SA, Boucher KM, Goodson AG, Samadashwily G and Grossman D. The p16(INK4A) tumor suppressor regulates cellular oxidative stress. Oncogene. 2011; 30(3):265-274.

12. Lang J, Boxer M and MacKie RM. CDKN2A mutations in Scottish families with cutaneous melanoma: results from 32 newly identified families. Br J Dermatol. 2005; 153(6):1121-1125.

13. Larre Borges A, Cuellar F, Puig-Butille JA, Scarone M, Delgado L, Badenas C, Mila M, Malvehy J, Barquet V, Nunez J, Laporte M, Fernandez G, Levrero P, Martinez-Asuaga M and Puig S. CDKN2A mutations in melanoma families from Uruguay. Br J Dermatol. 2009; 161(3):536-541.

14. Yakobson E, Eisenberg S, Isacson R, Halle D, Levy-Lahad E, Catane R, Safro M, Sobolev V, Huot T, Peters G, Ruiz A, Malvehy J, Puig S, Chompret A, Avril MF, Shafir R, et al. A single Mediterranean, possibly Jewish, origin for the Val59Gly CDKN2A mutation in four melanoma-prone families. Eur J Hum Genet. 2003; 11(4):288-296.

15. Majore S, De Simone P, Crisi A, Eibenschutz L, Binni F, Antigoni I, De Bernardo C, Catricala C and Grammatico P. CDKN2A/CDK4 molecular study on 155 Italian subjects with familial and/or primary multiple melanoma. Pigment Cell Melanoma Res. 2008; 21(2):209-211.

16. Eliason MJ, Larson AA, Florell SR, Zone JJ, Cannon-Albright LA, Samlowski WE and Leachman SA. Population-based prevalence of CDKN2A mutations in Utah melanoma families. J Invest Dermatol. 2006; 126(3):660-666.

17. Ciotti P, Struewing JP, Mantelli M, Chompret A, Avril MF, Santi PL, Tucker MA, Bianchi-Scarra G, Bressac-de Paillerets B and Goldstein AM. A single genetic origin for the G101W CDKN2A mutation in 20 melanoma-prone families. Am J Hum Genet. 2000; 67(2):311-319.

18. Jefferies S, Edwards SM, Hamoudi RA, A'Hern R, Foulkes W, Goldgar D and Eeles R. No germline mutations in CDKN2A (p16) in patients with squamous cell cancer of the head and neck and second primary tumours. Br J Cancer. 2001; 85(9):1383-1386.

19. Cabanillas R, Astudillo A, Valle M, de la Rosa J, Alvarez R, Duran NS and Cadinanos J. Novel germline CDKN2A mutation associated with head and neck squamous cell carcinomas and melanomas. Head Neck. 2011.

20. Rees JL. The genetics of sun sensitivity in humans. Am J Hum Genet. 2004; 75(5):739-751.

21. Valverde P, Healy E, Jackson I, Rees JL and Thody AJ. Variants of the melanocyte-stimulating hormone receptor gene are associated with red hair and fair skin in humans. Nat Genet. 1995; 11(3):328-330.

22. Bastiaens MT, ter Huurne JA, Kielich C, Gruis NA, Westendorp RG, Vermeer BJ and Bavinck JN. Melanocortin-1 receptor gene variants determine the risk of nonmelanoma skin cancer independently of fair skin and red hair. Am J Hum Genet. 2001; 68(4):884-894.

23. Box NF, Duffy DL, Irving RE, Russell A, Chen W, Griffyths LR, Parsons PG, Green AC and Sturm RA. Melanocortin-1 receptor genotype is a risk factor for basal and squamous cell carcinoma. J Invest Dermatol. 2001; 116(2):224-229.

24. Box NF, Duffy DL, Chen W, Stark M, Martin NG, Sturm RA and Hayward NK. MC1R genotype modifies risk of melanoma in families segregating CDKN2A mutations. Am J Hum Genet. 2001; 69(4):765-773.

25. Goldstein AM, Chaudru V, Ghiorzo P, Badenas C, Malvehy J, Pastorino L, Laud K, Hulley B, Avril MF, Puig-Butille JA, Miniere A, Marti R, Chompret A, Cuellar F, Kolm I, Mila M, et al. Cutaneous phenotype and MC1R variants as modifying factors for the development of melanoma in CDKN2A G101W mutation carriers from 4 countries. Int J Cancer. 2007; 121(4):825-831.

26. Demenais F, Mohamdi H, Chaudru V, Goldstein AM, Newton Bishop JA, Bishop DT, Kanetsky PA, Hayward NK, Gillanders E, Elder DE, Avril MF, Azizi E, van Belle P, Bergman W, Bianchi-Scarra G, Bressac-de Paillerets B, et al. Association of MC1R variants and host phenotypes

with melanoma risk in CDKN2A mutation carriers: a GenoMEL study. J Natl Cancer Inst. 2010; 102(20):1568-1583.

27. Herraiz C, Jimenez-Cervantes C, Zanna P and Garcia-Borron JC. Melanocortin 1 receptor mutations impact differentially on signalling to the cAMP and the ERK mitogen-activated protein kinase pathways. FEBS Lett. 2009; 583(19):3269-3274.

28. Le Pape E, Passeron T, Giubellino A, Valencia JC, Wolber R and Hearing VJ. Microarray analysis sheds light on the dedifferentiating role of agouti signal protein in murine melanocytes via the Mc1r. Proc Natl Acad Sci U S A. 2009; 106(6):1802-1807.

29. Sviderskaya EV, Hill SP, Evans-Whipp TJ, Chin L, Orlow SJ, Easty DJ, Cheong SC, Beach D, DePinho RA and Bennett DC. p16(Ink4a) in melanocyte senescence and differentiation. J Natl Cancer Inst. 2002; 94(6):446-454.

30. Eisinger M and Marko O. Selective proliferation of normal human melanocytes in vitro in the presence of phorbol ester and cholera toxin. Proc Natl Acad Sci U S A. 1982; 79(6):2018-2022.

31. Herlyn M, Rodeck U, Mancianti M, Cardillo FM, Lang A, Ross AH, Jambrosic J and Koprowski H. Expression of melanoma-associated antigens in rapidly dividing human melanocytes in culture. Cancer Res. 1987; 47(12):3057-3061.

32. Elder DE, Rodeck U, Thurin J, Cardillo F, Clark WH, Stewart R and Herlyn M. Antigenic profile of tumor progression stages in human melanocytic nevi and melanomas. Cancer Res. 1989; 49(18):5091-5096.

33. Valyi-Nagy IT, Hirka G, Jensen PJ, Shih IM, Juhasz I and Herlyn M. Undifferentiated keratinocytes control growth, morphology, and antigen expression of normal melanocytes through cell-cell contact. Lab Invest. 1993; 69(2):152-159.

34. Hsu MY, Meier FE, Nesbit M, Hsu JY, Van Belle P, Elder DE and Herlyn M. E-cadherin expression in melanoma cells restores keratinocyte-mediated growth control and down-regulates expression of invasion-related adhesion receptors. Am J Pathol. 2000; 156(5):1515-1525.

35. Phillips J, Gawkrodger DJ, Caddy CM, Hedley S, Dawson RA, Smith-Thomas L, Freedlander E and Mac Neil S. Keratinocytes suppress TRP-1 expression and reduce cell number of co-cultured melanocytes - implications for grafting of patients with vitiligo. Pigment Cell Res. 2001; 14(2):116-125.

36. Haass NK, Smalley KS, Li L and Herlyn M. Adhesion, migration and communication in melanocytes and melanoma. Pigment Cell Res. 2005; 18(3):150-159.

37. Hirobe T. Role of keratinocyte-derived factors involved in regulating the proliferation and differentiation of mammalian epidermal melanocytes. Pigment Cell Res. 2005; 18(1):2-12.

38. Nindl I, Dang C, Forschner T, Kuban RJ, Meyer T, Sterry W and Stockfleth E. Identification of differentially expressed genes in cutaneous squamous cell carcinoma by microarray expression profiling. Mol Cancer. 2006; 5:30.

39. Scatolini M, Grand MM, Grosso E, Venesio T, Pisacane A, Balsamo A, Sirovich R, Risio M and Chiorino G. Altered molecular pathways in melanocytic lesions. Int J Cancer. 2010; 126(8):1869-1881.

40. de Snoo FA and Hayward NK. Cutaneous melanoma susceptibility and progression genes. Cancer Lett. 2005; 230(2):153-186.

41. Ogbah Z, Puig-Butille JA, Simonetta F, Badenas C, Cervera R, Mila J, Benitez D, Malvehy J, Vilella R and Puig S. Molecular characterization of human cutaneous melanoma-derived cell lines. Anticancer Res. 2012; 32(4):1245-1251.

42. Herbert BS, Chanoux RA, Liu Y, Baenziger PH, Goswami CP, McClintick JN, Edenberg HJ, Pennington RE, Lipkin SM and Kopelovich L. A molecular signature of normal breast epithelial and stromal cells from Li-Fraumeni syndrome mutation carriers. Oncotarget. 2012; 1(6):405-422.

43. Gu F, Qureshi AA, Niu T, Kraft P, Guo Q, Hunter DJ and Han J. Interleukin and interleukin receptor gene polymorphisms and susceptibility to melanoma. Melanoma Res. 2008; 18(5):330-335.

44. Howell WM, Turner SJ, Bateman AC and Theaker JM. IL-10 promoter polymorphisms influence tumour development in cutaneous malignant melanoma. Genes Immun. 2001; 2(1):25-31.

45. Yang XR, Pfeiffer RM, Wheeler W, Yeager M, Chanock S, Tucker MA and Goldstein AM. Identification of modifier genes for cutaneous malignant melanoma in melanoma-prone families with and without CDKN2A mutations. Int J Cancer. 2009; 125(12):2912-2917.

46. Guo J, Wen DR, Huang RR, Paul E, Wunsch P, Itakura E and Cochran AJ. Detection of multiple melanoma-associated markers in melanoma cell lines by RT in situ PCR. Exp Mol Pathol. 2003; 74(2):140-147.

47. Raposo G and Marks MS. The dark side of lysosome-related organelles: specialization of the endocytic pathway for melanosome biogenesis. Traffic. 2002; 3(4):237-248.

48. Ramanathan HN and Ye Y. The p97 ATPase associates with EEA1 to regulate the size of early endosomes. Cell Res. 2011; 22(2):346-359.

49. Sakai C, Kawakami Y, Law LW, Furumura M and Hearing VJ, Jr. Melanosomal proteins as melanoma-specific immune targets. Melanoma Res. 1997; 7(2):83-95.

50. Hearing VJ. Biochemical control of melanogenesis and melanosomal organization. J Investig Dermatol Symp Proc. 1999; 4(1):24-28.

51. Schouwey K, Delmas V, Larue L, Zimber-Strobl U, Strobl LJ, Radtke F and Beermann F. Notch1 and Notch2 receptors influence progressive hair graying in a dose-dependent manner. Dev Dyn. 2007; 236(1):282-289.

52. Okuyama R, Tagami H and Aiba S. Notch signaling: its role in epidermal homeostasis and in the pathogenesis of skin diseases. J Dermatol Sci. 2008; 49(3):187-194.

53. Xin Y, Lu Q and Li Q. IKK1 control of epidermal differentiation is modulated by notch signaling. Am J Pathol. 2011; 178(4):1568-1577.

54. Lin HY, Kao CH, Lin KM, Kaartinen V and Yang LT. Notch signaling regulates late-stage epidermal differentiation and maintains postnatal hair cycle homeostasis. PLoS One. 2011; 6(1):e15842.

55. Panelos J and Massi D. Emerging role of Notch signaling in epidermal differentiation and skin cancer. Cancer Biol Ther. 2009; 8(21):1986-1993.

56. Chang YM, Newton-Bishop JA, Bishop DT, Armstrong BK, Bataille V, Bergman W, Berwick M, Bracci PM, Elwood JM, Ernstoff MS, Green AC, Gruis NA, Holly EA, Ingvar C, Kanetsky PA, Karagas MR, et al. A pooled analysis of melanocytic nevus phenotype and the risk of cutaneous melanoma at different latitudes. Int J Cancer. 2009; 124(2):420-428.

57. Bishop JA, Wachsmuth RC, Harland M, Bataille V, Pinney E, Mac KP, Baglietto L, Cuzick J and Bishop DT. Genotype/phenotype and penetrance studies in melanoma families with germline CDKN2A mutations. J Invest Dermatol. 2000; 114(1):28-33.

58. Sanchez-Laorden BL, Herraiz C, Valencia JC, Hearing VJ, Jimenez-Cervantes C and Garcia-Borron JC. Aberrant trafficking of human melanocortin 1 receptor variants associated with red hair and skin cancer: Steady-state retention of mutant forms in the proximal golgi. J Cell Physiol. 2009; 220(3):640-654.

59. Mitra D, Luo X, Morgan A, Wang J, Hoang MP, Lo J, Guerrero CR, Lennerz JK, Mihm MC, Wargo JA, Robinson KC, Devi SP, Vanover JC, D'Orazio JA, McMahon M, Bosenberg MW, et al. An ultraviolet-radiation-independent pathway to melanoma carcinogenesis in the red hair/fair skin background. Nature. 2012; 491(7424):449-453.

60. Catania A. Neuroprotective actions of melanocortins: a therapeutic opportunity. Trends Neurosci. 2008; 31(7):353-360.

61. Liu R, Gao X, Lu Y and Chen H. Meta-analysis of the relationship between Parkinson disease and melanoma. Neurology. 2011; 76(23):2002-2009.

62. Olsen JH, Friis S and Frederiksen K. Malignant melanoma and other types of cancer preceding Parkinson disease. Epidemiology. 2006; 17(5):582-587.

63. Olsen JH, Friis S, Frederiksen K, McLaughlin JK, Mellemkjaer L and Moller H. Atypical cancer pattern in patients with Parkinson's disease. Br J Cancer. 2005; 92(1):201-205.

64. Gao X, Simon KC, Han J, Schwarzschild MA and Ascherio A. Genetic determinants of hair color and Parkinson's disease risk. Ann Neurol. 2009; 65(1):76-82.

65. Meng S, Song F, Chen H, Gao X, Amos CI, Lee JE, Wei Q, Qureshi AA and Han J. No association between Parkinson disease alleles and the risk of melanoma. Cancer Epidemiol Biomarkers Prev. 2011; 21(1):243-245.

66. Hassin-Baer S, Laitman Y, Azizi E, Molchadski I, Galore-Haskel G, Barak F, Cohen OS and Friedman E. The leucine rich repeat kinase 2 (LRRK2) G2019S substitution mutation. Association with Parkinson disease, malignant melanoma and prevalence in ethnic groups in Israel. J Neurol. 2009; 256(3):483-487.

67. Shinozuka K, Uzawa K, Fushimi K, Yamano Y, Shiiba M, Bukawa H, Yokoe H and Tanzawa H. Downregulation of carcinoembryonic antigen-related cell adhesion molecule 1 in oral squamous cell carcinoma: correlation with tumor progression and poor prognosis. Oncology. 2009; 76(6):387-397.

68. Quintanilla-Dieck MJ, Codriansky K, Keady M, Bhawan J and Runger TM. Cathepsin K in melanoma invasion. J Invest Dermatol. 2008; 128(9):2281-2288.

69. Rheinwald JG and Green H. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. Cell. 1975; 6(3):331-343.

70. Llames SG, Del Rio M, Larcher F, Garcia E, Garcia M, Escamez MJ, Jorcano JL, Holguin P and Meana A. Human plasma as a dermal scaffold for the generation of a completely autologous bioengineered skin. Transplantation. 2004; 77(3):350-355.

71. Del Rio M, Larcher F, Serrano F, Meana A, Munoz M, Garcia M, Munoz E, Martin C, Bernad A and Jorcano JL. A preclinical model for the analysis of genetically modified human skin in vivo. Hum Gene Ther. 2002; 13(8):959-968.

72. Larcher F, Dellambra E, Rico L, Bondanza S, Murillas R, Cattoglio C, Mavilio F, Jorcano JL, Zambruno G and Del Rio M. Long-term engraftment of single genetically modified human epidermal holoclones enables safety pre-assessment of cutaneous gene therapy. Mol Ther. 2007; 15(9):1670-1676.

73. De Luca M, Franzi AT, D'Anna F, Zicca A, Albanese E, Bondanza S and Cancedda R. Coculture of human keratinocytes and melanocytes: differentiated melanocytes are physiologically organized in the basal layer of the cultured epithelium. Eur J Cell Biol. 1988; 46(1):176-180.

74. Guerra L, Capurro S, Melchi F, Primavera G, Bondanza S, Cancedda R, Luci A, De Luca M and Pellegrini G. Treatment of "stable" vitiligo by Timedsurgery and transplantation of cultured epidermal autografts. Arch Dermatol. 2000; 136(11):1380-1389.

75. Garcia M, Llames S, Garcia E, Meana A, Cuadrado N, Recasens M, Puig S, Nagore E, Illera N, Jorcano JL, Del Rio M and Larcher F. In vivo assessment of acute UVB responses in normal and Xeroderma Pigmentosum (XP-C) skin-humanized mouse models. Am J Pathol. 2010; 177(2):865-872.

76. Livak KJ and Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001; 25(4):402-408.

77. Bolstad BM, Irizarry RA, Astrand M and Speed TP. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics. 2003; 19(2):185-193.

78. Benjamini Y, Drai D, Elmer G, Kafkafi N and Golani I. Controlling the false discovery rate in behavior genetics research. Behav Brain Res. 2001; 125(1-2):279-284.

79. Al-Shahrour F, Arbiza L, Dopazo H, Huerta-Cepas J, Minguez P, Montaner D and Dopazo J. From genes to functional classes in the study of biological systems. BMC Bioinformatics. 2007; 8:114.

80. Medina I, Carbonell J, Pulido L, Madeira SC, Goetz S, Conesa A, Tarraga J, Pascual-Montano A, Nogales-Cadenas R, Santoyo J, Garcia F, Marba M, Montaner D and Dopazo J. Babelomics: an integrative platform for the analysis of transcriptomics, proteomics and genomic data with advanced functional profiling. Nucleic Acids Res. 2010; 38(Web Server issue):W210-213.

81. Chaudru V, Laud K, Avril MF, Miniere A, Chompret A, Bressac-de Paillerets B and Demenais F. Melanocortin-1 receptor (MC1R) gene variants and dysplastic nevi modify penetrance of CDKN2A mutations in French melanoma-prone pedigrees. Cancer Epidemiol Biomarkers Prev. 2005; 14(10):2384-2390.

82. Cerroni L, Barnhill R, Elder D, Gottlieb G, Heenan P, Kutzner H, LeBoit PE, Mihm M, Jr., Rosai J and Kerl H. Melanocytic tumors of uncertain malignant potential: results of a tutorial held at the XXIX Symposium of the International Society of Dermatopathology in Graz, October 2008. The American journal of surgical pathology. 2010; 34(3):314-326.

83. Bastian BC, Olshen AB, LeBoit PE and Pinkel D. Classifying melanocytic tumors based on DNA copy number changes. The American journal of pathology. 2003; 163(5):1765-1770.

84. Gerami P, Jewell SS, Morrison LE, Blondin B, Schulz J, Ruffalo T, Matushek Pt, Legator M, Jacobson K, Dalton SR, Charzan S, Kolaitis NA, Guitart J, Lertsbarapa T, Boone S, LeBoit PE, et al. Fluorescence in situ hybridization (FISH) as an ancillary diagnostic tool in the diagnosis of melanoma. The American journal of surgical pathology. 2009; 33(8):1146-1156.

85. Dalton SR, Gerami P, Kolaitis NA, Charzan S, Werling R, LeBoit PE and Bastian BC. Use of fluorescence in situ hybridization (FISH) to distinguish intranodal nevus from metastatic melanoma. The American journal of surgical pathology. 2010; 34(2):231-237.

86. Diaz A, Valera A, Carrera C, Hakim S, Aguilera P, Garcia A, Palou J, Puig S, Malvehy J and Alos L. Pigmented spindle cell nevus: clues for differentiating it from spindle cell malignant melanoma. A comprehensive survey including clinicopathologic, immunohistochemical, and FISH studies. The American journal of surgical pathology. 2011; 35(11):1733-1742.

87. Gerami P, Mafee M, Lurtsbarapa T, Guitart J, Haghighat Z and Newman M. Sensitivity of fluorescence in situ hybridization for melanoma diagnosis using RREB1, MYB, Cep6, and 11q13 probes in melanoma subtypes. Archives of dermatology. 2010; 146(3):273-278.

88. Kutzner H, Metzler G, Argenyi Z, Requena L, Palmedo G, Mentzel T, Rutten A, Hantschke M, Paredes BE, Scharer L, Hesse B, El-Shabrawi-Caelen L, Fried I, Kerl H, Lorenzo C, Murali R, et al. Histological and genetic evidence for a variant of superficial spreading melanoma composed predominantly of large nests. Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc. 2012; 25(6):838-845.

89. Newman MD, Mirzabeigi M and Gerami P. Chromosomal copy number changes supporting the classification of lentiginous junctional melanoma of the elderly as a subtype of melanoma. Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc. 2009; 22(9):1258-1262.

90. Curtin JA, Fridlyand J, Kageshita T, Patel HN, Busam KJ, Kutzner H, Cho KH, Aiba S, Brocker EB, LeBoit PE, Pinkel D and Bastian BC. Distinct sets of genetic alterations in melanoma. The New England journal of medicine. 2005; 353(20):2135-2147.

91. Gerami P, Li G, Pouryazdanparast P, Blondin B, Beilfuss B, Slenk C, Du J, Guitart J, Jewell S and Pestova K. A highly specific and discriminatory FISH assay for distinguishing between benign and malignant melanocytic neoplasms. The American journal of surgical pathology. 2012; 36(6):808-817.

92. LeBoit P.E. BG WD, Sarasain A, ed. World Health Organization Classification of Tumours. Pathology and Genetics of Skin Tumours (Lyon: IARC Press).

93. Bastian BC, Kashani-Sabet M, Hamm H, Godfrey T, Moore DH, 2nd, Brocker EB, LeBoit PE and Pinkel D. Gene amplifications characterize acral melanoma and permit the detection of occult tumor cells in the surrounding skin. Cancer research. 2000; 60(7):1968-1973.

94. Bastian BC, LeBoit PE, Hamm H, Brocker EB and Pinkel D. Chromosomal gains and losses in primary cutaneous melanomas detected by comparative genomic hybridization. Cancer research. 1998; 58(10):2170-2175.

95. North JP, Kageshita T, Pinkel D, LeBoit PE and Bastian BC. Distribution and significance of occult intraepidermal tumor cells surrounding primary melanoma. The Journal of investigative dermatology. 2008; 128(8):2024-2030.

96. Puig-Butille JA, Badenas C, Ogbah Z, Carrera C, Aguilera P, Malvehy J and Puig S. Genetic alterations in RAS-regulated pathway in acral lentiginous melanoma. Experimental dermatology. 2013; 22(2):148-150.

97. Gaiser T, Kutzner H, Palmedo G, Siegelin MD, Wiesner T, Bruckner T, Hartschuh W, Enk AH and Becker MR. Classifying ambiguous melanocytic lesions with FISH and correlation with clinical long-term follow up. Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc. 2010; 23(3):413-419.

98. Morey AL, Murali R, McCarthy SW, Mann GJ and Scolyer RA. Diagnosis of cutaneous melanocytic tumours by four-colour fluorescence in situ hybridisation. Pathology. 2009; 41(4):383-387.

99. Hosler GA, Moresi JM and Barrett TL. Nevi with site-related atypia: a review of melanocytic nevi with atypical histologic features based on anatomic site. Journal of cutaneous pathology. 2008; 35(10):889-898.

100. Shay JW and Wright WE. Telomerase therapeutics for cancer: challenges and new directions. Nature reviews Drug discovery. 2006; 5(7):577-584.

101. Fu J, Bian M, Jiang Q and Zhang C. Roles of Aurora kinases in mitosis and tumorigenesis. Molecular cancer research : MCR. 2007; 5(1):1-10.

102. Pouryazdanparast P, Cowen DP, Beilfuss BA, Haghighat Z, Guitart J, Rademaker A and Gerami P. Distinctive clinical and histologic features in cutaneous melanoma with copy number gains in 8q24. The American journal of surgical pathology. 2012; 36(2):253-264.

103. Zhu CQ, Cutz JC, Liu N, Lau D, Shepherd FA, Squire JA and Tsao MS. Amplification of telomerase (hTERT) gene is a poor prognostic marker in non-small-cell lung cancer. British journal of cancer. 2006; 94(10):1452-1459.

104. Glatz-Krieger K, Pache M, Tapia C, Fuchs A, Savic S, Glatz D, Mihatsch M and Meyer P. Anatomic site-specific patterns of gene copy number gains in skin, mucosal, and uveal melanomas detected by fluorescence in situ hybridization. Virchows Archiv : an international journal of pathology. 2006; 449(3):328-333.

105. Sauter ER, Yeo UC, von Stemm A, Zhu W, Litwin S, Tichansky DS, Pistritto G, Nesbit M, Pinkel D, Herlyn M and Bastian BC. Cyclin D1 is a candidate oncogene in cutaneous melanoma. Cancer research. 2002; 62(11):3200-3206.

106. Takata M, Goto Y, Ichii N, Yamaura M, Murata H, Koga H, Fujimoto A and Saida T. Constitutive activation of the mitogen-activated protein kinase signaling pathway in acral melanomas. The Journal of investigative dermatology. 2005; 125(2):318-322.

107. Wang L, Rao M, Fang Y, Hameed M, Viale A, Busam K and Jhanwar SC. A Genome-Wide High-Resolution Array-CGH Analysis of Cutaneous Melanoma and Comparison of Array-CGH to FISH in Diagnostic Evaluation. The Journal of molecular diagnostics : JMD. 2013; 15(5):581-591.

108. Lengauer C, Kinzler KW and Vogelstein B. Genetic instabilities in human cancers. Nature. 1998; 396(6712):643-649.

109. Yamaura M, Takata M, Miyazaki A and Saida T. Specific dermoscopy patterns and amplifications of the cyclin D1 gene to define histopathologically unrecognizable early lesions of acral melanoma in situ. Archives of dermatology. 2005; 141(11):1413-1418.

110. Musgrove EA, Caldon CE, Barraclough J, Stone A and Sutherland RL. Cyclin D as a therapeutic target in cancer. Nature reviews Cancer. 2011; 11(8):558-572.

111. Bondi J, Husdal A, Bukholm G, Nesland JM, Bakka A and Bukholm IR. Expression and gene amplification of primary (A, B1, D1, D3, and E) and secondary (C and H) cyclins in colon adenocarcinomas and correlation with patient outcome. Journal of clinical pathology. 2005; 58(5):509-514.

112. Georgieva J, Sinha P and Schadendorf D. Expression of cyclins and cyclin dependent kinases in human benign and malignant melanocytic lesions. Journal of clinical pathology. 2001; 54(3):229-235.

113. Ramirez JA, Guitart J, Rao MS and Diaz LK. Cyclin D1 expression in melanocytic lesions of the skin. Annals of diagnostic pathology. 2005; 9(4):185-188.

114. Nagasaka T, Lai R, Medeiros LJ, Brynes RK, McCourty A, Harada T and Saddik M. Cyclin D1 overexpression in Spitz nevi: an immunohistochemical study. The American Journal of dermatopathology. 1999; 21(2):115-120.

TABLES

Table 1. Elaborated FISH-probes from bacterial artificial chromosomes (BACs) and colour fluorochromes (spectrum) used to visualize the specific gene.

Gene	Locus	BAC	Spectrum
TERT	5p15.33	RP11-117B23	Green
*MCTP1**	5q15	RP11-73K22	Red
AURKA	20q13	RP11-158O17	Green

BAC, bacterial artificial chromosome

* The gene *MCTP1*, located on 5q15, was used as chromosome 5 number control instead of a centromeric probe.

Table 2 Main clinicopathological features of patients with acral-lentiginous melanomas.

Case #	Age	Sex	Site	Clark level	Breslow thickness (mm)
1	61	M	Sole	V	12
2	76	F	Sole	IV	5
3	49	F	Sole	V	6
4	89	F	Heel	IV	3.2
5	67	M	Ring finger	III	0.7
6	68	F	Sole	IV	0.9
7	82	F	Sole	IV	6
8	58	M	Sole	V	4.5
9	87	F	Heel	V	10
10	51	F	Sole	V	4.6
11	58	M	Palm	V	8
12	54	M	Sole	V	12
13	67	M	Heel	V	6.5
14	91	F	Heel	IV	2
15	71	F	Palm	IV	1.4
16	83	M	Sole	II	0.9
17	58	M	Index finger	IV	1
18	74	M	Great toe	II	0.4
19	63	M	Sole	III	1
20	78	F	Heel	IV	3

21	74	F	Heel	III	1
22	77	M	Sole	IV	4
23	78	F	Sole	IV	3
24	39	M	Sole	IV	2
25	40	M	Sole	V	2
26	61	M	Great toe	III	0.5
27	75	F	Heel	IV	5
28	60	M	Sole	IV	2
29	73	M	Sole	IV	3.3
30	90	F	Sole	IV	5
31	66	F	Sole	IV	1.2
32	45	M	Heel	IV	5
33	58	F	Heel	IV	3.5
34	83	F	Sole	IV	2.8

M, male; F, female.

Table 3 FISH results of acral-lentiginous melanomas.

Case #	% cells with *RREB1* gain	% cells with *RREB1*/CEP6 gain	% cells with *CCND1* gain	% cells with *MYB*/CEP6 loss	*TERT*/*MCTP1* ratio	*AURKA*/CEP20 ratio
1	**76**	**70**	20	26	1.02	**1.66**
2	**93**	**83**	**93**	10	0.96	0.93
3	**46**	53	16	20	**1.61**	1.02
4	10	10	13	**70**	0.98	1.07
5	20	30	16	10	**2.58**	1.18
6	**66**	**60**	10	3	1.03	0.96
7	**80**	**60**	**53**	**43**	1.16	1.23
8	**60**	40	3	**43**	1.02	0.96
9	**36**	33	**80**	**70**	0.96	1.09
10	**86**	**66**	10	**56**	1.01	1.00
11	16	10	10	**50**	**4.50**	1.07
12	**46**	**56**	13	26	**4.81**	1.06
13	**93**	**83**	**100**	**83**	1.24	1.13
14	**80**	**53**	3	**50**	1.02	0.95
15	**90**	**56**	6	**56**	1.18	1.06
16	10	16	10	16	**3.10**	0.98
17	**70**	50	3	20	0.96	1.10
18	16	16	13	20	1.00	0.91
19	**70**	**76**	**93**	**66**	0.94	1.02
20	**80**	**80**	26	16	1.03	1.00
21	**80**	**76**	3	6	1.02	1.03
22	**70**	**60**	20	30	**1.88**	1.00

23	10	6	20	**50**	1.00	1.33
24	**66**	**60**	13	16	0.91	0.98
25	16	10	0	16	**4.16**	1.00
26	**80**	**76**	**90**	**60**	1.23	1.02
27	**96**	**90**	**96**	10	0.98	1.18
28	**90**	**90**	13	10	1.02	1.33
29	**70**	50	16	13	0.91	0.98
30	16	16	6	10	0.98	**2.24**
31	**53**	**73**	3	10	1.13	0.98
32	**66**	**66**	20	26	**2.49**	1.05
33	10	10	13	**60**	1.08	0.93
34	**93**	**90**	**100**	**46**	0.93	0.93

Bold lettering if met criteria

Table 4 Immunohistochemical results of acral-lentiginous melanomas (ALM) and acral nevi (AN)

	% of nuclear positive cells mean (range)		*P*
	ALM	AN	
CCND1	57.35 (30-95)	19.58 (5-40)	<0.001*
TERT	4.24 (0-20)	0.92 (0-10)	<0.001†
AURKA	2.65 (0-15)	0.50 (0-5)	0.001*

ALM, acral-lentiginous melanoma; AN, acral nevi.

* U Mann-Whitney

† Student T-test

FIGURE LEGENDS

Figure 1 Examples of two acral lentiginous melanomas (ALMs) showing different gene alterations by fluorescence in situ hybridization (FISH) (cases 4 and 25, respectively). (**A**) Low-power magnification showing a large non-pigmented melanocytic tumor arising on acral skin, with marked associated acanthosis and elongated rete ridges [hematoxylin and eosin (HE), X40]. (**B**) The same lesion at a higher magnification. Melanocytes in the dermis are predominantly epithelioid and show moderate atypia. A lentiginous proliferation of malignant melanocytes in the dermal-epidermal junction is also evident (HE, X200). (**C**) FISH picture of the same case showing *MYB* loss (gold signal) in relation to CEP6 (aqua signal) with the commercial multicolour probe set (X1000). (**D**) Ulcerated ALM arising in the sole of a 40-year-old man, with moderate pigmentation (HE, X40). (**E**) Higher power magnification shows nests of malignant melanocytes with marked pleomorphism. (HE, X2000). (**F**) FISH image of the same case showing *TERT* amplification (green signal) in relation to the control gene *MCTP1* (red signal) (X1000).

Figure 2 Two examples of acral lentiginous melanomas (ALMs) in which chromosomal aberrations were also detected in the in situ portion by fluorescence in situ hybridization (FISH). (**A**) ALM arising in the palm of a 58 year-old man, which shows a marked lentiginous proliferation pattern at the dermal-epidermal junction [hematoxylin and eosin (HE), X40]. (**B**) High-power magnification highlighting the typical lentiginous proliferation of atypical melanocytes at the border of the tumor (HE, X200). (**C**) FISH picture of the same case in which large nuclei at the dermal-epidermal junction showing *TERT* amplification (green signal) in relation to the control gene *MCTP1* (red signal) can be identified (X1000). (**D**) Histological section of another ALM showing marked epidermal acantosis (HE, X40) and (**E**) a continuous proliferation of atypical melanocytes adopting a lentiginous pattern (HE, X200). (**F**) This case showed amplification of *CCND1* (green signal) in the invasive portion, which was also detected in scattered large nuclei at the dermo-epidermal junction (X1000).

Figure 3 Example of an acral nevus. (**A**) Low power magnification showing a non-atypical melanocytic proliferation, with evident maturation (hematoxylin and eosin, X40). (**B**) Fluorescense in situ hybridization image of the same case hybridized with the commercial multicolour probe set illustrating a normal chromosomal profile (X1000).

Figure 4 A representative case of an (**A**) acral lentiginous melanoma [hematoxylin and eosin (HE), X200]. showing (**B**) CCND1 protein overexpression by immunohistochemistry (Cyclin D1, X2000) and (**C**) *CCND1* gene (green signals) amplification by fluorescence in situ hybridization (X1000)

ACKNOWLEDGEMENTS

The authors wish to thank Elena Gonzalvo for performing the technical procedures and Marta Sánchez for secretarial assistance.

ANEXO V. FIGURAS COMPLEMENTARIAS TRABAJO IV

1. Análisis de Componentes Principales (CPA)

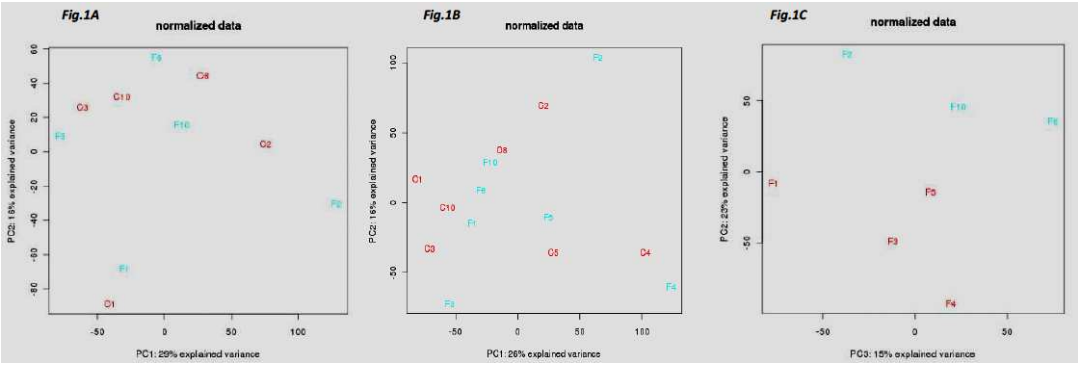


Figura 1. Gráficas del CPA de los datos normalizados: Cada individuo está codificado con un valor numérico, las muestras están dentificadas como lesión no tratada (tiempo inicial) con la letra "C" y como lesión después del tratamiento (tiempo final) con la letra "F. Figura 1A: Imagen del CPA incluyendo todos los individuos; Figura 1B: Imagen del CPA excluyendo los individuos afectos con el Sindrome Xeroderma Pigmentosum. En la figura 1A y 1B se indican en rojo las muestras a tiempo inicial y en azul las muestras a tiempo final. Figura 1C: Imagen del CPA comparando las muestras a tiempo final de todos los individuos , en base a la respuesta al tratamiento (muestras con una buena respuesta marcadas en rojo y muestras con una respuesta parcial marcadas en azul).

Se realizó el análisis de componentes principales para evaluar las causas de la variabilidad del conjunto de valores de expresión normalizados. El CPA no muestra una agrupación definida de las lesiones en base al tratamiento (muestras pre-tratadas vs muestras después del tratamiento, figura 1A y 1B), por lo que la muestras tienden a agruparse en mayor medida por el hecho de proceder del mismo paciente (factor "individuo"), que por ser muestras tratadas o no con el compuesto con fotoliasa (factor "tratamiento").

Por el contrario, sí que se identifica una agrupación de las muestras a tiempo final en base a la respuesta al tratamiento (el factor "respuesta al tratamiento" es mayor que el factor "individuo")

2. Expresión del gen CPI-17

En base a las diferencias de respuesta al tratamiento con fotoliasa entre los pacientes incluidos en el estudio, se evaluó la expresión del gen CPI-17 entre las lesiones antes del tratamiento y las mismas después del tratamiento.

De forma interesante, los pacientes con una respuesta completa al tratamiento, presentan unos niveles superiores de la expresión del gen CPI-17 en el campo de cancerización antes del tratamiento comparado con la expresión detectada en las lesiones de los pacientes con una respuesta parcial.

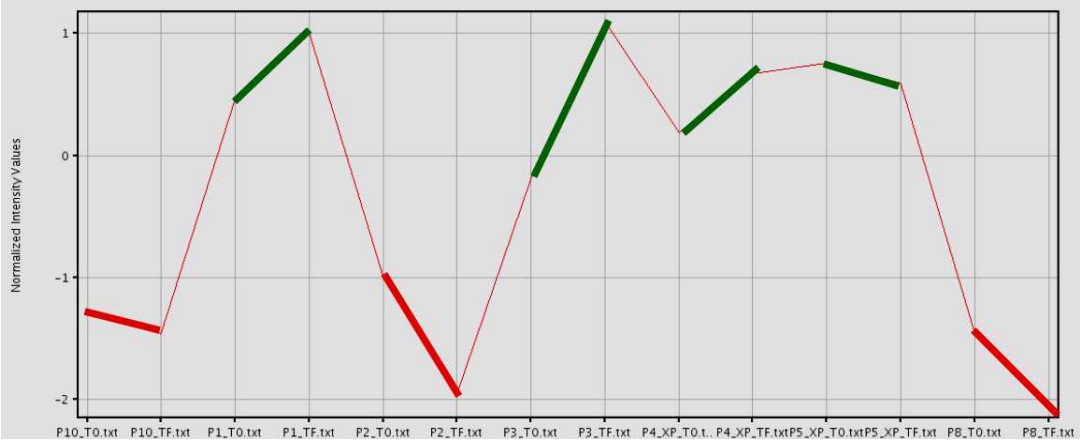


Figura 2: Valores de expresión del gen *CP1-17* en el campo de cancerización antes del tratamiento y después del tratamiento en cada paciente: La variación de la expresión entre el tiempo inicial (T0) y tiempo final (TF) de los pacientes con una respuesta parcial al tratamiento con fotoliasa se indican en rojo; y la variación en los pacientes con una respuesta completa en verde.

AGRADECIMIENTOS

En primer lloc, vull agrair a tots els membres de la Unitat de Melanoma de l'Hospital Clínic que han format part activa en el treballs inclosos en aquesta tesis. Gràcies per la vostra feina i per aportar coneixement i esperit crític sobre les qüestions científiques així com la vostra ajuda en qüestions més humanes. Sobretot als qui han estat "en primera línea" de foc desde l'inici (les dermatòlogues Cristina Carrera, Paula Aguilera, la biòloga Zighe Ogbah, els infermers Pablo Iglesias i Dani Gabriel i les tècnics Estefania Martinez i Remedios Cervantes) i a les incorporacions més recents, les becaries pre-doctorals Miriam Potrony i Gemma Tell, que han estat claus per a poder finalitzar part del estudis.

Vull agrair de forma molt especial a la Dra.Susana Puig, el Dr.Josep Malvehy i la Dra.Celia Badenas; primer, per encoratjar-me a fer el camí fins a aconseguir aquesta tesi i segon, per la seva dedicació, pel seu esforç i per acollir-me en el grup de recerca de la forma que ho han fet. Gràcies per ajudar-me a crèixer professionalment i també com a persona.

En segon lloc, agrair als membres del Servei de Genètica i Bioquímica Molecular que m'han acompanyat durant aquests anys de formació en el el camp de la genètica molecular per l'ambient creat en el laboratori i sobretot, per demostrar en fets que a més de tenir companys de feina també he tingut amics. Laia, Irene, Bernat, Alba i Maribel sou una part fonamental per fer aquests afirmacions, gràcies de tot cor. En especial agrair a la Dra.Montserrat Milà el seu tracte, la seva actitud positiva en el dia a dia dins el laboratori, i per donar-me l'oportunitat de formar part de la familia "Ciber".

Per últim vull agrair a la meva gent; la meva familia i amics; cada mostra d'interés sobre el meu treball i aquesta tesi així com les mostres d'ànims. Agrair a ma germana Marta la manera en que m'ha acompanyat durant aquests anys; i als meus pares el seu suport incondicional i generós i per ensenyar-me que es pot aconseguir allò que un es proposa amb esforç, constància i humilitat.

Agrair també a tots aquells amb els qui, gràcies a aquesta feina, ens hem conegut i compartit experiencies i feina (Dr.Eduardo Nagore, Dra.Àngels, Fabra, Dra. Maria Gonzalez, Marta Diví, Gabriel Salerni, Dei Elurbe, Francisca Torres, Eva Pastor, Anna Planagumà, Jordi Bruguera, i un llarg etc.)