INSTITUTO DE INVESTIGACIÓN MARQUÉS DE VALDECILLA (IDIVAL)

Grupo de Genómica del Cáncer.

UNIVERSIDAD DE CANTABRIA

INSTITUTO DE BIOMEDICINA Y BIOTECNOLOGÍA DE CANTABRIA (IBBTEC)

Departamento de Señalización Celular y Molecular.

Precision medicine in Merkel cell carcinoma and advanced cutaneous melanoma. Implications of molecular characterization in diagnosis, prognosis and targeted therapy.

Soraya Curiel del Olmo

Octubre de 2016

El **Dr. Miguel Ángel Piris Pinilla**, director científico de IDIVAL, jefe del servicio de Anatomía Patológica del Hospital Universitario Marqués de Valdecilla, jefe del grupo de Genómica del Cáncer de IDIVAL,

El **Dr. José Pedro Vaqué Díez**, investigador Ramón y Cajal de IBBTEC, jefe del grupo de Mecanismos Moleculares que Promueven el Cáncer Humano de IBBTEC,

CERTIFICAN:

Que la Lda. Soraya Curiel del Olmo ha realizado bajo su dirección el presente trabajo titulado "Precision medicine in Merkel cell carcinoma and advanced cutaneous melanoma. Implications of molecular characterization in diagnosis, prognosis and targeted therapy" ("Medicina de precision en el carcinoma de células de Merkel y el melanoma cutáneo avanzado. Implicaciones de la caracterización molecular en el diagnóstico, el pronóstico y la terapia dirigida").

Que consideran que este trabajo reúne los requisitos de originalidad y calidad científica necesarios para su presentación como Memoria de Doctorado por la interesada, con objeto de poder optar al grado de Doctor por la Universidad de Cantabria.

Y para que conste y surta los efectos oportunos, firman el presente certificado.

En Santander, a 5 de octubre de 2016.

Fdo.: Dr. Miguel Ángel Piris Pinilla

Fdo.: Dr. José Pedro Vaqué Díez

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Resumen

En este trabajo se han estudiado dos tipos de cáncer de piel agresivos; el carcinoma de células de Merkel (MCC) y el melanoma cutáneo avanzado. Su estudio se ha abordado partiendo de la caracterización molecular de los tumores mediante el uso de técnicas de secuenciación de ADN, cuyos resultados se han utilizado posteriormente para desarrollar posibles aplicaciones traslacionales para el diagnóstico y el tratamiento.

Tanto el melanoma cutáneo como el carcinoma de células de Merkel se originan a partir de la transformación maligna de células de la piel derivadas de la cresta neural embrionaria; los melanocitos [Sommer, L., 2011] y las células de Merkel [Merkel, F. S., 1875; Goessling, W. et al., 2002], respectivamente. Sin embargo, el origen del MCC está todavía en discusión y algunos autores defienden un origen epitelial [Halata, Z. et al., 2003; Morrison, K. M. et al., 2009]. De hecho, una de las principales diferencias entre estos dos tipos de cáncer es la gran cantidad de datos disponibles sobre el melanoma, tanto a nivel biológico como clínico, frente a la escasez de información y conocimiento existentes acerca del MCC.

Ambos tipos de cáncer suelen aparecer en zonas de la piel expuestas al sol y la radiación ultravioleta (UV) parece estar implicada en la carcinogénesis. En realidad, la exposición de la piel a este tipo de radiación se considera el mayor factor de riesgo para la aparición del melanoma cutáneo en personas de piel clara. La firma mutacional atribuida a la radiación UV (descrita como un porcentaje de cambios C→T en sitios dipirimidínicos superior al 60 %, o bien, un porcentaje de cambios en tándem CC→TT superior al 5 % respecto del total de mutaciones [Brash, D. E., 2015]) suele encontrarse en la gran mayoría de las lesiones tumorales de melanoma [The_Cancer_Genome_Atlas, 2015]. No está tan claro el papel que juega la radiación UV en la transformación que conduce a la aparición del MCC, pero se ha descrito recientemente la presencia de la firma ultravioleta en el ADN de una proporción considerable de tumores [Harms, P. W. et al., 2015; Goh, G. et al., 2016]. Por otro lado, muchas lesiones tumorales de MCC tienen integrado el ADN de un poliomavirus (poliomavirus de las células de Merkel, MCPyV), que parece estar también involucrado en la carcinogénesis [Feng, H. et al., 2008; Kassem, A. et al., 2008].

Cuando el melanoma y el MCC se detectan en estadios tempranos, la resección quirúrgica de la lesión tumoral suele ser el tratamiento estándar, con buenos resultados de supervivencia [Balch, C. M. et al., 2009; American_Cancer_Society, 2016]. Si la enfermedad se encuentra más avanzada, ambos tipos de cáncer carecen de terapias sistémicas efectivas, capaces de producir respuestas sostenidas en la mayoría de los pacientes. Para tratar el melanoma cutáneo avanzado se están utilizando terapias dirigidas destinadas a inhibir la señalización MAPK-ERK1/2 en aquellos casos que presentan mutaciones activantes en el gen *BRAF*, lo que ocurre en aproximadamente la mitad de los casos [Curtin, J. A. et al., 2005; Lee, J. H. et al., 2011]. Con este tipo de tratamiento se ha registrado una supervivencia libre

de progresión de 11,4 meses [Robert, C. et al., 2015] en ese 50 - 60 % de los pacientes. A pesar de que la inmunoterapia ha conseguido aumentar el tiempo de respuesta en muchos casos de melanoma, produciendo datos de supervivencia de hasta 4 años, incluso de 10 años, en algún caso reciente (revisado por [Sharma, P. et al., 2015]), la realidad es que más de la mitad de los pacientes tratados con este tipo de terapia no muestran respuesta alguna [Hodi, F. S. et al., 2010; Topalian, S. L. et al., 2012; Hamid, O. et al., 2013; Delyon, J. et al., 2015; Sharma, P. et al., 2015]. En cuanto al carcinoma de células de Merkel, no dispone de una terapia dirigida específica y pocos datos hay todavía sobre el uso de la inmunoterapia. Un reciente ensayo clínico con agentes anti-PD-1 en pacientes con MCC avanzado [Nghiem, P. T. et al., 2016] ha conseguido una tasa de respuesta objetiva del 56 % durante casi 10 meses. Por lo tanto, parece que el MCC y el melanoma pueden ser buenos candidatos para el desarrollo de estrategias destinadas a estimular la respuesta inmune, pero ambos tipos de cáncer carecen todavía de marcadores específicos predictivos de la respuesta de cada paciente a estos tratamientos. Por otro lado, las terapias dirigidas están prácticamente por descubrir en el caso del MCC, al contrario que en el caso del melanoma, donde, sin embargo, no están beneficiando a todos los pacientes y no están produciendo, de momento, respuestas sostenidas en el tiempo.

Los dos proyectos que componen esta tesis se han desarrollado partiendo de la hipótesis de que la caracterización molecular de casos de cáncer individuales puede conducir a la detección de mecanismos de enfermedad específicos. Esto podría desembocar en la identificación de dianas terapéuticas, así como de biomarcadores que puedan apoyar el diagnóstico y el pronóstico, o predecir la respuesta y la evolución ante determinados tratamientos. Las firmas mutacionales presentes en las células tumorales podrían originar redes de señalización aberrantes que, a su vez, podrían determinar la evolución clínica de un cáncer y su respuesta a diferentes terapias. Por lo tanto, la caracterización molecular del cáncer podría ser clave para conseguir una selección racional de los tratamientos.

En este trabajo, la información obtenida mediante técnicas de secuenciación se ha utilizado posteriormente para inferir rutas de señalización, procesos biológicos y/o biomarcadores con un posible papel en el desarrollo del melanoma o del MCC. Algunos de estos mecanismos y marcadores podrán, tal vez, ser utilizados en un futuro cercano para establecer pronósticos y diagnósticos más precisos, e incluso, para dirigir el uso de determinadas terapias.

PROCEDIMIENTOS, RESULTADOS Y DISCUSIÓN.

Teniendo en cuenta que el carcinoma de células de Merkel es relativamente poco conocido, y ante los escasos datos existentes acerca de los mecanismos moleculares implicados en esta enfermedad, su estudio se abordó mediante la secuenciación del exoma completo de una cohorte de 15 casos clínicamente caracterizados. Este abordaje exploratorio aportó una visión global de las mutaciones somáticas presentes en los tumores, cuyo análisis e interpretación posteriores desvelaron una serie de procesos biológicos y de mecanismos moleculares significativamente alterados en nuestra cohorte. La mayor parte de estos procesos no habían sido hasta ahora relacionados con este cáncer.

Aproximadamente la mitad de los casos de nuestra cohorte tenían el DNA del Poliomavirus de las células de Merkel (MCPyV) integrado en el genoma de las células tumorales. Las características genómicas esta serie de casos indicaban la presencia de dos poblaciones claramente diferentes: mientras que los casos que expresaban las proteínas virales tenían bajas tasas de mutación, los casos en los que no detectamos antígenos del poliomavirus tenían índices mutacionales muy altos, acompañados de la firma mutacional atribuida a la radiación ultravioleta. Esto sugería la existencia de dos etiologías distintas; el virus y la radiación ultravioleta.

El análisis bioinformático de los datos mutacionales reveló una serie de genes mutados con una frecuencia mayor de la esperable por azar, así como varios procesos biológicos y rutas de señalización celular significativamente alteradas en nuestra cohorte. La interpretación mecanística de estos datos nos llevó a seleccionar una serie de marcadores que sirvieran como indicadores de la actividad de estas rutas y mecanismos que habíamos encontrado alterados. Estos marcadores fueron analizados mediante inmunohistoquímica en secciones de tumores, lo cual nos permitió extender nuestro estudio a 48 casos con seguimiento clínico. Mediante este análisis encontramos mecanismos de enfermedad compartidos entre las dos etiologías, a pesar de las notables diferencias genéticas existentes entre ellas. Por otro lado, encontramos algunos mecanismos de enfermedad prácticamente exclusivos de los casos MCPyV-negativos.

Por último, descubrimos que dos de los biomarcadores seleccionados, P-CREB y P-STAT, que formaban parte de mecanismos de enfermedad comunes, estaban fuertemente asociados a una menor superviviencia de los pacientes. Sin embargo, un posterior análisis multivariante en el que se incluyeron otros factores que podían estar afectando a la supervivencia, reveló que sólo P-CREB era un factor pronóstico independiente del estadio de la enfermedad, el sexo, la edad, la integración del poliomavirus en el genoma tumoral y la expresión de P-STAT.

Para estudiar el melanoma cutáneo, se eligió una técnica de secuenciación dirigida para analizar las mutaciones presentes en las zonas codificantes de un grupo de 217 genes previamente seleccionado. Dado que esta enfermedad es mucho más conocida y descrita que la anterior, tanto a nivel clínico como molecular, nos basamos en la literatura para decidir los genes que nos interesaba estudiar. Mediante un primer análisis *in silico* de las mutaciones presentes en nuestra selección de genes en cohortes de paciente independientes, encontramos una media de 3,74 genes mutados que podían ser asociados con un inhibidor, directo o indirecto, en uso en la clínica. Además, identificamos un total de 8 grupos de genes y rutas de señalización frecuentemente alterados en estos pacientes.

Posteriores estudios *ex vivo* nos permitieron analizar los efectos sobre la proliferación celular de inhibidores escogidos en base a las mutaciones encontradas en cada caso. De esta forma comprobamos, en primer lugar, que la combinación de drogas era más eficaz que la monoterapia. En segundo lugar, que algunos inhibidores independientes de la ruta MAPK, que no afectaban a la activación de ERK1/2, tenían importantes efectos antiproliferativos. En tercer lugar, que utilizando estos inhibidores junto con los habituales inhibidores de MEK y $BRAF^{V600E}$, utilizados habitualmente en la clínica, podíamos potenciar sus efectos. Por último, que el efecto de las drogas sobre una línea celular estaba condicionado por el conjunto de mutaciones presentes en ella.

Estos estudios fueron complementados con experimentos *in vivo*, en los que utilizamos ratones con tumores en *xenograft*, por un lado, derivados de líneas celulares y, por otro lado, derivados de lesiones tumorales. Estos experimentos nos sirvieron para constatar que éramos capaces de reducir el crecimiento tumoral utilizando fármacos que no inhibían la activación de ERK1/2, igual que ocurría con la proliferación celular. Además, comprobamos que la combinación de drogas mejoraba los resultados de la monoterapia, confirmando de nuevo los resultados obtenidos *ex vivo*.

CONCLUSIONES.

Conclusiones del proyecto 1:

1. Se ha diseñado un abordaje novedoso y original para caracterizar molecularmente tumores de MCC, consistente en la combinación de análisis mutacionales e inmunohistoquímicos.
2. Las características genómicas de los tumores indican la existencia de 2 subtipos principales de MCC, lo que, a su vez, sugiere la existencia de 2 etiologías:
   a. Un subtipo se caracteriza por tener el ADN del MCPyV integrado en el genoma y presentar bajas tasas mutacionales.
   b. El otro subtipo se caracteriza por carecer de antígenos virales y presentar altas tasas mutacionales, acompañadas de firma ultravioleta.
3. En nuestra cohorte, se han encontrado significativamente alterados varios procesos biológicos y rutas de señalización que no habían sido hasta ahora relacionados con MCC.
4. A pesar de las diferencias genómicas, los casos positivos y negativos para el poliomavirus parecen desarrollar mecanismos de enfermedad comunes: RB, p53, p63, NFAT, P-STAT y P-CREB.
5. La expresión de P-CREB y P-STAT correlaciona significativamente con un peor pronóstico en un análisis univariante.
6. En un análisis multivariante, la expresión de P-CREB ha sido identificada como un marcador pronóstico independiente (del sexo, la edad, el estadio de la enfermedad, P-STAT y la presencia o ausencia del MCPyV).

Conclusiones del proyecto 2:

1. Se ha desarrollado una plataforma de análisis mutacional dirigido para caracterizar casos de melanoma cutáneo en un tiempo compatible con la práctica clínica (aproximadamente 15 días).
2. Cada lesión tumoral ha mostrado un perfil mutacional único y una media de entre 3 y 4 genes mutados por caso que pueden ser, directa o indirectamente, asociados con un inhibidor específico.
3. Las terapias que combinan inhibidores de mecanismos dependientes e independientes de MAPK han sido más efectivas que las monoterapias, tanto *ex vivo* como *in vivo*.
4. Utilizando este abordaje, se podrían sugerir terapias dirigidas para casos de melanoma avanzado independientemente de la presencia o ausencia de mutaciones en *BRAF*.

5. Las terapias dirigidas han sido más efectivas *ex vivo* cuando se han utilizado sobre una línea celular con un perfil mutacional adecuado.

PERSPECTIVAS.

Los resultados obtenidos en los dos proyectos que componen esta tesis nos han permitido ampliar los conocimientos existentes sobre las bases moleculares que intervienen en la aparición y el avance de estos dos tipos de cáncer. Además, estos resultados podrían acabar dando lugar a nuevas herramientas aplicables, en un futuro próximo, tanto al diagnóstico como a la selección de tratamientos.

En el caso concreto del carcinoma de células de Merkel, hemos encontrado una serie de procesos mecanísticos y biológicos alterados de manera significativa en nuestra cohorte. Cabe destacar que la mayoría de ellos no habían sido hasta ahora relacionados con este tipo de cáncer. Algunos de estos mecanismos desregulados podrían servir para dirigir terapia, por lo que, en este sentido, podrían ser muy útiles futuros experimentos con líneas celulares de MCC sobre las que probar el efecto de varios inhibidores, como podrían ser, por ejemplo:

- Tacrolimus (FK-506) para bloquear la señalización por calcio (uno de los mecanismos significativamente alterados en nuestra cohorte).
- Dacomitinib (PF299804, PF299), Vargatef (BIBF1120) y Regorafenib para inhibir varios receptores Tirosina-Kinasa, entre los cuales estarían ERBB4, FLT4 y KDR, que han aparecido mutados recurrentemente en nuestros casos.
- BKM120 −un inhibidor de PI3K y P-AKT− o Everolimus −un inhibidor de mTOR−, que podrían ser útiles en aquellos casos que presenten alteraciones en la vía PI3K-AKT-mTOR, significativamente alterada en nuestra cohorte.

Además, el abordaje utilizado en este proyecto podría trasladarse, en parte, a la caracterización molecular rutinaria de las lesiones tumorales en el momento del diagnóstico. El hecho de haber identificado una serie de biomarcadores que pueden ser analizados por inmunohistoquímica sobre cortes de los tumores, brinda la posibilidad de detectar mecanismos de enfermedad en cada caso concreto de MCC de una manera relativamente rápida, barata y eficaz.

Por otro lado, existe actualmente una necesidad real de encontrar biomarcadores que puedan servir como predictores de la respuesta a la inmunoterapia. Según los últimos datos publicados a este respecto, la respuesta de los pacientes con MCC al tratamiento con anticuerpos anti-PD-1 es similar entre los casos positivos y negativos para los antígenos virales [Nghiem, P. T. et al., 2016]. Por lo tanto, el estudio de los marcadores subrogados a la actividad de aquellos mecanismos de enfermedad que han sido identificados como comunes a las dos etiologías podría ofrecer una buena oportunidad para caracterizar casos de MCC y predecir cuáles podrían verse más beneficiados por este tipo de tratamientos.

Por último, un importante hallazgo de nuestro estudio ha sido la identificación de la expresión de P-CREB como un marcador pronóstico independiente. Sería interesante validar este dato en una cohorte independiente de casos, lo que podría ser el comienzo de un futuro trabajo con MCC. De confirmarse su capacidad predictiva, podría ser recomendable la inclusión de P-CREB entre los marcadores a analizar habitualmente en los tumores de MCC.

En el caso del melanoma cutáneo, este trabajo ha desvelado un amplio rango de procesos mecanísticos alterados en los tumores, cuya inhibición ha tenido efectos antiproliferativos, tanto *ex vivo* como *in vivo*. Esto podría aportar nuevas opciones terapéuticas, que podrían ser utilizadas en combinación con los actuales tratamientos, pero que, además, serían especialmente útiles para aquellos pacientes que no responden a la inmunoterapia (aproximadamente el 60 %) [Hamid, O. et al., 2013; Delyon, J. et al., 2015; Sharma, P. et al., 2015] y que además carecen de mutaciones activantes en *BRAF*. Por lo tanto, podría ser conveniente plantearse la posibilidad de utilizar este abordaje en ensayos clínicos que nos permitan conocer los resultados de la utilización de esta estrategia en pacientes.

Por otro lado, estos datos invitan a iniciar un estudio similar con casos de melanoma en progresión, que han dejado de responder a las terapias actuales, los cuales podrían verse muy beneficiados por una posible alternativa al tratamiento después de la recaída. Poco se conoce sobre los cambios genéticos y mecanísticos que pueden estar implicados en la aparición de resistencias y pocas opciones terapéuticas existen, a día de hoy, para los pacientes a partir de ese momento.

En definitiva, este trabajo plantea la posibilidad mejorar nuestra capacidad de diagnóstico y tratamiento de los dos cánceres de la piel más agresivos. En el caso del MCC, a través del uso de una serie de biomarcadores con posibles implicaciones en pronóstico y en terapia. En el caso del melanoma avanzado, mediante un abordaje compatible con la práctica clínica que permite el diseño de terapias de combinación más efectivas, o bien, complementarias a las terapias actuales. Además, este trabajo deja abiertas varias posibilidades para continuar con el estudio de estas enfermedades, tanto desde un enfoque molecular y mecanístico, como desde un punto de vista más biológico y clínico.

1. Introduction

1.1. GENERAL INTRODUCTION.

According to World Health Organization estimations, cancer is currently being diagnosed in approximately 15 million people worldwide. The continuing global demographic and epidemiologic transitions signal a continuously rising incidence (as represented in figure 1), which is estimated to reach 20 million of cases within the next 15 years [World_Health_Organization., 2012; Stewart, B. W., Wild, C. P., 2014; Ferlay, J. et al., 2015; International_Agency_for_Research_on_Cancer, 2015].

During the last two decades our ability to diagnose and treat cancer has experienced great advances but, despite these efforts, the current rate of mortality is still about 50% of the total number of cancers diagnosed worldwide [World_Health_Organization., 2012; Ferlay, J. et al., 2015; International_Agency_for_Research_on_Cancer, 2015] (figure 1).

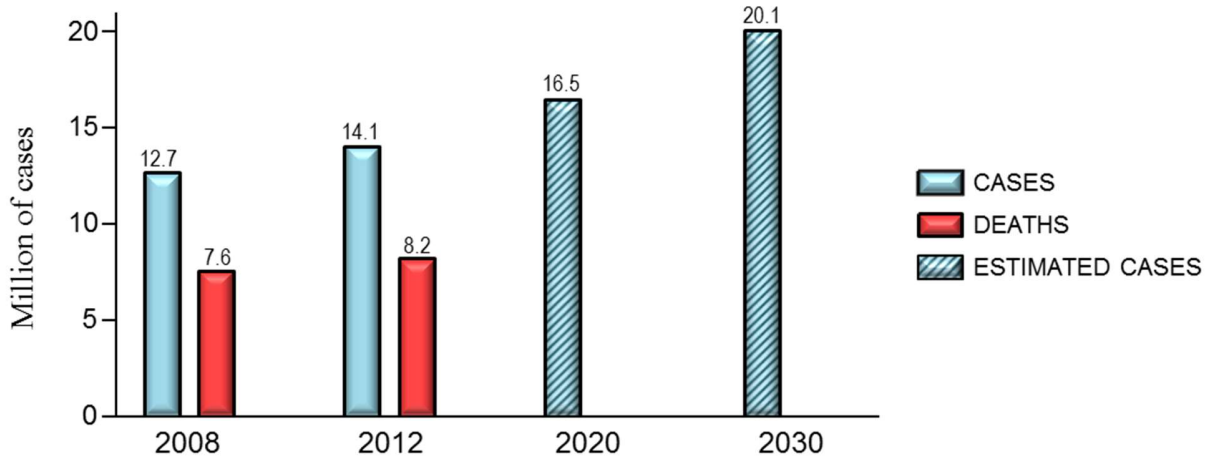

Figure 1. Incidence and mortality of cancer (based on data from World_Health_Organization, 2012 and from the International_Agency_for_Research_on_Cancer, 2015). Bars show the number of cancer cases diagnosed (in blue color) and the number of deaths (in red color) for the indicated year. Stripped bars indicate the number of estimated cases for the indicated years.

Classic and generalist approaches for cancer treatment, such as chemotherapy or radiotherapy, have been usually conceived empirically and, moreover, based on the conception of cancer as a monoclonal disease (as represented in figure 2), where each case would consist on a set of cells with similar genomic profiles, mechanisms and biological behaviors [Schea, R. A. et al., 1995; Wong, R. et al., 2006; Choueiri, T. K. et al., 2008; Crawford, S., 2013]. The development of many cytotoxic drugs (like, for example, paclitaxel, fludarabine, BCNU, carboplatin, cytosine arabinoside pentastatin, hydroxyurea, topotecan, and mitoxantrone, but to name a few) was the result of trial and error approaches [Marshall, E. K., Jr., 1964]. The success rate of these traditional cytotoxic chemotherapies in producing long-term responses, especially in terms of disease-free survival, still remains unsatisfactory in many cancers, fact which, however, does not dismiss their undeniable efficacy in the

treatment of some particular cancer types, such as testicular cancer or acute lymphoblastic leukemia [Crawford, S., 2013].

Recent advances in molecular characterization of cancer have increased our ability to diagnose and treat cancer but, even so, too many cases are still lacking an effective therapy [Stewart, B. W., Wild, C. P., 2014; Ferlay, J. et al., 2015]. We are now starting to learn the extent to which the understanding of molecular alterations leading to cellular transformation in specific cases can increase our chances to treat patients from a more rational perspective. This change of view is currently guiding the improvement in the efficacy of therapies in terms of overall survival and progression-free survival of patients [Reifenberger, G. et al., 2014; Robert, C. et al., 2015; Toss, A. et al., 2015; Fernandez-Cuesta, L. et al., 2016].

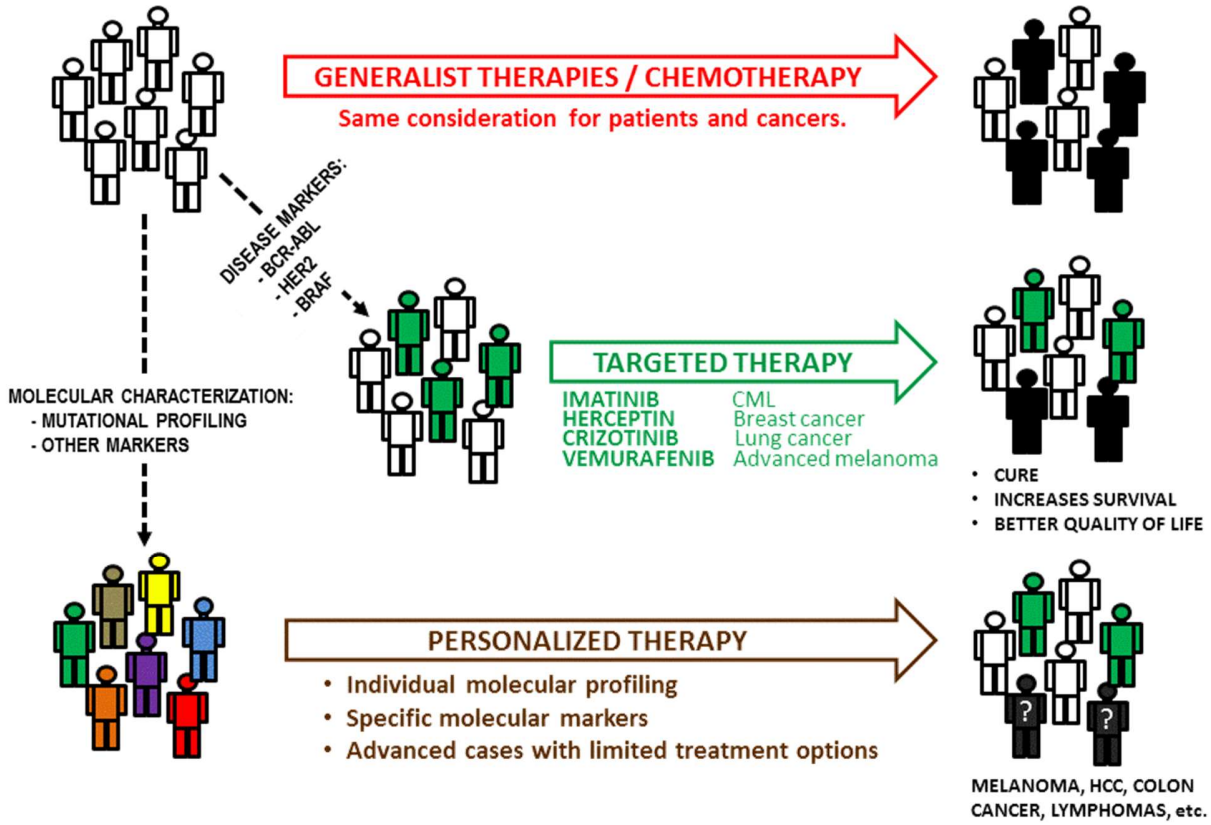

Figure 2. Impact of genomic analysis in the evolution of cancer therapies. Classic approaches for cancer treatment are based on the assumption of cancer patients to be a homogeneous population. Targeted therapies are currently using in some particular cases that harbor mutations in specific genes, considered markers (or biomarkers) of the disease, like, for example, BRAF, ER2 or BCR. These therapies have improved the effectiveness of generalist treatments and have increased the possibilities of treatment for some patients. Now, the idea of a very heterogeneous population of patients is been progressively consolidating, bringing hence the necessity of an individual molecular characterization of each cancer case and, hopefully, offering new opportunities of treatment.

As our knowledge about genetic bases of cancer evolves, it is becoming established that we are dealing with a very heterogeneous population of cancer patients [Lawrence, M. S. et al., 2013] (figure 2), with tumors presenting unique genomic alterations and mutational profiles and, therefore, private mechanisms of disease. In this line of evidence, the recent improvement in our ability to analyze cancer genomes through the use of Next Generation Sequencing techniques, has revealed extremely variable tumor mutational landscapes amongst patients, amongst different tumors within the same patient and, furthermore, between different regions of a single tumor lesion [Gerlinger, M. et al., 2012; Alexandrov, L. B. et al., 2013]. Moreover, we know now that most tumors are a mosaic of clones with varying population sizes, different genetic makeups and distinct phenotypic characteristics. In fact, the process of natural selection between clones is, precisely, what is currently believed to drive carcinogenesis and acquired therapeutic resistance in neoplasms [Nowell, P. C., 1976; Marusyk, A. et al., 2010; Andor, N. et al., 2016].

Thus, the complexity and heterogeneity shown by tumors, even within the same patient, imply a critical challenge for precision medicine. The wide variability of genomic signatures detected across cancers suggests that their resulting aberrant activities may be due to case-specific malignant networks of deregulated mechanisms, which would have the potential to control key steps in the pathogenesis and progression of human malignancies [Schwaederle, M. et al., 2015]. Moreover, current evidences indicate the necessity of resorting to combinatorial regimens, in order to address so complex molecular and biological signatures, which represent the hallmark of many cancers [Schwaederle, M. et al., 2015].

In the light of this, it could be conceivable to consider that the molecular characterization of specific tumor lesions could represent a useful approach to:

1. Support diagnosis, in the same way as the gene expression profiling was used to identify distinct subtypes of B-cell lymphomas, or the mutation L265P in *MYD88* gene is used for the differential diagnosis of this cancer type [Alizadeh, A. A. et al., 2000; Xu, L. et al., 2013; Martinez-Lopez, A. et al., 2015].
2. Determine prognosis, as done, per example, with the markers AMF and ST3GAL-A, whose overexpression has been associated with poor prognosis in renal cell carcinoma [Bai, Q. et al., 2015; Lucarelli, G. et al., 2015].
3. Guide therapy, in the same way as using EGFR inhibitors in cases of lung cancer, or Imatinib (a multi-target inhibitor of v-Abl, c-Kit and PDGFR) to treat chronic myeloid leukemia [Druker, B. J. et al., 2001; Maemondo, M. et al., 2010; Mitsudomi, T. et al., 2010].

Therefore, we have designed a precision medicine approach based on the individual molecular characterization of samples from patients at diagnosis, and the later use of this

information to potentially support diagnosis and/or guide the choice of therapies, often consisting of personalized combinatorial treatments. In this thesis, we have implemented such approach in two deadly types of skin cancer; advanced melanoma and Merkel cell carcinoma, both of them with a rising incidence and suboptimal therapies. We have used a combination of genomic analysis, deep sequencing and immunohistochemistry assays to study tumor samples originating from clinically-characterized patients. This allowed us to detect specific mechanisms of disease that may individually participate in the clinical course of each cancer case. In summary, our efforts have been aimed at developing novel tools to diagnose and treat specific cases of cancer from an individualized perspective by means of precision medicine.

1.2. DNA SEQUENCING TECHNIQUES USED TO CHARACTERIZE CANCER LESIONS.

The idea of using dideoxy nucleotides for stopping DNA synthesis by DNA polymerase [Sanger, F. et al., 1977] provided the basis for the development of automated Sanger sequencing, also known as first-generation sequencing [Smith, D. A., 1986; Ansorge, W. et al., 1987]. In this method, the mixture of deoxyribonucleotide-triphosphates (dNTPs) and dideoxyribonucleotide-triphosphates (ddNTPs) causes a random termination of the extension reaction, creating DNA strands extended until different lengths. Resulting molecules are then sorted by their molecular weight (depending of the point of termination) and the label attached to the terminating ddNTPs is, finally, read out by order, using (originally) gel electrophoresis. This process was very wearisome, owing to its limited automation, and allowed determining only a few hundred nucleotides per experiment.

Since the late 1980s, semi-automated sequencers provided a higher throughput and a lower tediousness, starting to use the capillary electrophoresis to order DNA fragments [Smith, L. M. et al., 1986; Swerdlow, H. et al., 1990]. Gently, this technology was enhanced to reach the sequencing of longer DNA fragments and to win a higher level of parallelism, making possible a simultaneous sequencing of 1000 base pairs (bp) per DNA fragment in 96 capillaries [Zagursky, R. J. et al., 1990; Kim, S. et al., 1996]. However, the throughput of automated Sanger sequencing is limited by the capacity of thermal cyclers and capillary electrophoresis analyzers, with the most advanced capillary analyzer capable of an output of approximately 500 kilobases (kb) in 24 h [Applied Biosystems]. Due to that, Sanger sequencing is not useful to analyze DNA in a high-throughput manner.

1.2.1. NEXT GENERATION SEQUENCING.

Since the completion of the first human genome sequence in 2003 [2003], the demand for cheaper and faster sequencing methods has increased quickly. The necessity for an extensive sequencing leaded to the development of new sequencing technologies, often called Next Generation Sequencing (NGS) or Massively Parallel Sequencing (MPS). Usually, the term Next Generation Sequencing is used to generically indicate 2nd or 3rd generation instruments. The term 2nd generation often refers to platforms that require amplification of the template molecules prior to sequencing while 3rd generation normally indicates platforms that directly sequence individual DNA molecules [Glenn, T. C., 2011; Rodríguez-Santiago, B. et al., 2012].

Some of such technologies (like Illumina platforms) use a DNA polymerase for catalyzing the incorporation of fluorescently labeled deoxyribonucleotide-triphosphates

(dNTPs) into a DNA pattern strand during sequential cycles. In each cycle, at the time of incorporation, nucleotides are identified by exciting fluorophores. The main difference between NGS and capillary electrophoresis-based sequencing is that, instead of sequencing a single DNA fragment, NGS extends this process across millions of fragments from a sample, which are sequenced in unison in a massively parallel way [Grada, A. et al., 2013]. Thus, NGS technologies gave rise to an unprecedented speed, together with a lower cost per base, and allowed an entire genome to be sequenced in less than one day [Koboldt, D. C. et al., 2010; Grada, A. et al., 2013]. For this reason, the number of sequencing related data publically available has drastically enlarged over last years and, nowadays, the mayor challenge lies in the storage, backup and analysis of such a volume of data [Koboldt, D. C. et al., 2010].

During the past decade, several NGS platforms have been developed, providing a low-cost, high-throughput sequencing, and they are continuously evolving. Currently, the major commercially available platforms are:

- 454 (Life Sciences/Roche), which was the first commercial NGS platform and is based on pyrosequencing.
- SOLiD 5500/5500xl (Applied Biosystems), which sequence by ligation.
- Ion Torrent Ion Proton/Ion PGM (Life Technologies), which sequence by monitoring pH, so does not need lasers, cameras or fluorescent dyes.
- MiSeq/NextSeq/HiSeq (Illumina), which sequence by synthesis.
- PacBioRS II (Pacific Biosciences), which sequences individual DNA molecules in real time.
- Starlight (Life Technologies), which uses quantum dots to achieve single-molecule sequencing.

Two of the most commonly used platforms in research and clinical laboratories today are the LifeTechnologies Ion Torrent™ Personal Genome Machine® (PGM) and the Illumina MiSeq®. However, for larger scale genomics it becomes necessary to choose an ultra-high-throughput sequencer, like the Illumina HiSeq System, which supports the broadest range of applications and study sizes. Both MiSeq and HiSeq sequencers are similar, differing from the other, mainly, in the run output. HiSeq is the main technology, as well as the most expensive one. It has the highest output and it will be the best choice in case of having a large number of samples or needing many reads per sample. The HiSeq 2500 System can process 8 human genomes at a depth of 30×, or 150 human exomes, assuming 4 Gb per exome at 2 × 75 bp. per run. MiSeq is a desktop instrument that performs runs in a faster and cheaper way. It should be the first choice when facing a small number of samples or when brief turnaround times are

needed. Genome Analyzer is the original machine, precursory of HiSeq and MiSeq, but it is no longer being updated, albeit is still being sold.

All these methods take advantage of sequencing by synthesis and have a similar base methodology, including template preparation, sequencing and imaging [Metzker, M. L., 2010]. The underlying workflow employed by different NGS technologies involves [Grada, A. et al., 2013; Nguyen, L. et al., 2014]:

1. Fragmentation of DNA or complementary DNA (cDNA) by enzymatic digestion, nebulization or sonication. Optimal fragment length is ranging from 50 to 20000 bp, depending on the platform.
2. Ligation to platform-specific adaptor sequences, which join to the ends of DNA fragments, creating the sequencing library.
3. Immobilization to a solid surface through the adaptor sequence.
4. Amplification (in order to increase the signal and enable the detection) by emulsion bead PCR or surface cluster PCR.
5. Sequencing by signal detection while base incorporation. The read length change according to the platform, but it is usually shorter than that for Sanger, and can be about 800-1000 bp. Sequencing may be paired-end or mate-paired. In paired-end sequencing, fragments with a length inferior to 1000 bp are sequenced from both ends. In mate-paired sequencing, fragments with a length larger than 1000 bp are circularized by ligating the ends to a single adaptor, then, fragmented into linear pieces with a central adaptor, and ligated to two additional adaptors by edges. Fragments are, finally, sequenced from both ends [Mardis, E. R., 2013].
6. Data analysis reassembling through overlapping sequences.

1.2.1.1. WHOLE GENOME SEQUENCING.

NGS can be used for the novo assembly of whole genomes, as well as for identifying genetic variations in a particular genome comparatively with a given reference genome. The sequencing of full genomes has the potential to answer specific diagnostic questions and, furthermore, to uncover clinically significant genetic findings, sometimes unrelated to the primary indication for sequencing.

Whole Genome Sequencing (WGS) is expected to provide a high coverage of almost all (95 %) genomic regions [Feero, W. G., 2014], including intronic and other noncoding zones [Dewey, F. E. et al., 2014]. WGS is also expected to bring a major shift in clinical practice in terms of diagnosis and understanding of diseases, ultimately enabling personalized medicine

[Majewski, J. et al., 2011]. WGS allows examination of single-nucleotide variants (SNVs), insertions and deletions (indels), structural variants (SVs) and copy number variants (CNVs) in both coding and non-coding regions of the genome, which include regulatory regions, such as promoters and enhancers.

Although NGS is much less costly in time and money than firs generation sequencing, performing WGS is still too expensive for many laboratories. Furthermore, data analysis can require a lot of time and, maybe, special skills in bioinformatics to harvest accurate information from such a volume of data

To quote an example that reflects the utility of WGS, Berger et al. [Berger, M. F. et al., 2012] discovered a new recurrently mutated gene in melanoma –PREX2– by sequencing the whole genome of 25 tumors (and their matched normal sample). Furthermore, in this same work, a genomic evidence of ultraviolet (UV) pathogenesis was revealed.

A much bigger project based on WGS is the 1000 Genomes Project [genomes]; the first project that have sequenced the genomes of a large number of people for providing a deep catalog of human genetic variation. 1000 genomes are just a start of this project, which aims to sequence 2500 genomes from 27 populations worldwide. Its goal is to find most genetic variants that have frequencies of at least 1% in the studied populations. This goal can be attained by sequencing many individuals lightly. Sequencing is still too expensive to deeply sequence the many samples being studied here. However, any particular region of the genome generally contains a limited number of haplotypes. Data can be combined across many samples to allow the efficient detection of most variants in a region. Combining the data from 2500 samples should allow highly accurate estimation (imputation) of the variants and genotypes for each sample that were not seen directly by the light sequencing [genomes; Patterson, K., 2011].

1.2.1.2. WHOLE EXOME AND TARGETED SEQUENCING.

Targeted sequencing lies in selecting a series of specific genomic regions to be sequenced, restricting analyses to specific genes, exons or punctual genomic regions. Thus, the data volume is reduced in comparison with WGS, whereupon the later bioinformatics analysis becomes easier and the time and expenses decrease, as well. Furthermore, the smaller target region size produces a higher depth of sequencing coverage per run, even if multiplexing many samples together. Targeted sequencing requires regions of interest to be captured and amplified by PCR during the library preparation [Nguyen, L. et al., 2014].

Sometimes, regions of interest are all exons of the genome. In this case sequencing is known as Whole Exome Sequencing (WES). As the human exome comprises about 1 % of

the whole genome, sequencing just protein-coding regions is much cheaper and faster than sequencing the entire genome. Reduced costs make it feasible to increase the number of samples to be sequenced, enabling large population based comparisons. Furthermore, analyzing data for the exome is easier in comparison to the whole genome, due to the smaller number of required sequence reads (and therefore smaller file sizes).

To perform WES, it is previously required a selective capture and enrichment of the exonic regions of genomic DNA. Several target-enrichment strategies have been developed with this purpose, based on different strategies that include PCR, hybrid capture, molecular inversion probes or, the most common and efficient method, in-solution capture [Kahvejian, A. et al., 2008; Turner, E. H. et al., 2009; Mamanova, L. et al., 2010; Mertes, F. et al., 2011]. This last strategy utilizes pools of probes bound to magnetic beads, whose sequence has been designed to hybridize to exonic regions. After binding to genomic DNA, these probes are pulled down and washed, leaving selected regions ready to be sequenced.

There are several commercial kits for exome capture, being TruSeq® Exome Library Prep Kit (Illumina) one of the most commonly used. TruSeq workflow begins with the fragmentation of DNA. A combination of fill-in reactions and exonuclease activity are used to generate blunt-end DNA fragments, followed by size selection with solid phase reversible immobilization beads. Then, an adenine nucleotide is added to the blunt ends of each strand, in preparation for ligation to the indexed adapters, which contain a thymine nucleotide overhang, allowing the ligation to the A-tailed fragmented DNA. These adapters contain the full complement of sequencing primer hybridization sites for single, paired-end, and indexed reads. Adapters are ligated to the fragments and ligated products are amplified using PCR. Next, libraries are pooled and denatured. Biotinylated probes are hybridized to the targeted regions and captured using streptavidin beads. After another PCR reaction, fragments are eluted from the beads. This workflow generates a target insert of approximately 150 bp ready for sequencing.

This kind of enrichment protocols allows the preparation of exome-containing libraries in less than three days, making the time which WES takes much lower than that required for WGS. Together with its lower cost and its easier data analysis, this fact is contributing to the increasingly use of WES in studies with large cohorts of patient and control samples.

Other important fact in favor of WES is that our current genomic perspective estimates that about 85 % of genomic alterations causing diseases are located in exons [Majewski, J. et al., 2011]. Moreover, nowadays we can understand better the functional consequences of mutations that directly affect the protein structure and activity. Therefore, focusing on only the protein-coding portion of the genome can place many advantages of the emerging technologies into the hands of researchers.

However, in comparison with WGS, sequencing just the exome has, on the other hand, a number of weaknesses. Some of them are:

1. WES omits regulatory regions, like promoters or enhancers.
2. Differences in the hybridization efficiency of WES capture probes can result in regions of the genome with little or no coverage, so WES has less reliable sequence coverage than WGS.
3. Coverage uniformity is also inferior with WES. Regions of the genome with low sequence complexity restrict the ability to design useful WES capture baits, resulting in off target capture effects.
4. Due to the bulk of DNA required, WES frequently requires PCR amplification.
5. Most target probes are designed to have a length lower than 120 nucleotides, making it meaningless to sequence using a greater read length.
6. WES capture probes tend to preferentially enrich reference alleles at heterozygous sites, producing false negative SNV calls.

As an example of WES implementation, Stark et al. [Stark, M. S. et al., 2012] discovered likely inactivating mutations in the protein-coding regions of either MAP3K5 or MAP3K9 affecting 24 % of studied melanoma cell lines by sequencing their whole exome.

Apart from WES, it is possible to sequence smaller amount of genomic regions (either all exons or specific fragments of a number of genes), as aforementioned. It can be very useful in the rapid diagnosis of genetic diseases and its results can assist the therapeutic decision in different cases, including those cancers for which the treatments can vary depending on the cancer type [Rehm, H. L., 2013].

Diagnostics of mutations in cancer susceptibility genes have benefited from the broad availability of next-generation sequencing analyses using targeted gene panels. Pre-designed panels that target common regions of interest are commercially available for clinical or research use, but also, on another side, there is the possibility of designing customized sets of primers and probes for desired targets [Sikkema-Raddatz, B. et al., 2013; Soukupova, J. et al., 2015]. Some commercial panels are prepared to detect selected somatic mutations considered “hotspots” for certain diseases [Rehm, H. L., 2013]. Hotspot cancer panels provide an affordable and efficient method for discovering rare somatic mutations, many of which have been identified as important cancer drivers.

Alternatively, different companies offer the possibility of designing customized panels for sequencing a number of genome regions chosen by the consumer. As an example of the use of this technology, Nikiforova et al., 2013 [Nikiforova, M. N. et al., 2013] developed an approach for genotyping thyroid samples by using a Life Technologies design tool to generate

a pool of 34 primers and creating a customized panel (ThyroSeq) to target 12 cancer genes with 284 mutational hot spots. Using this approach, they generated molecular profiles of all main types of thyroid cancer and identified point mutations in 30%–83% of specific cancer types.

In some of our studies, among them is one of the two projects that compound this thesis, we have used HaloPlex™ Custom Panels (by Agilent Technologies), which provide the possibility of focusing on any genomic regions of interest. Custom designs for this approach are created with the specific tool SureDesign (also by Agilent Technologies). Figure 3 shows a schematic representation of this methodology.

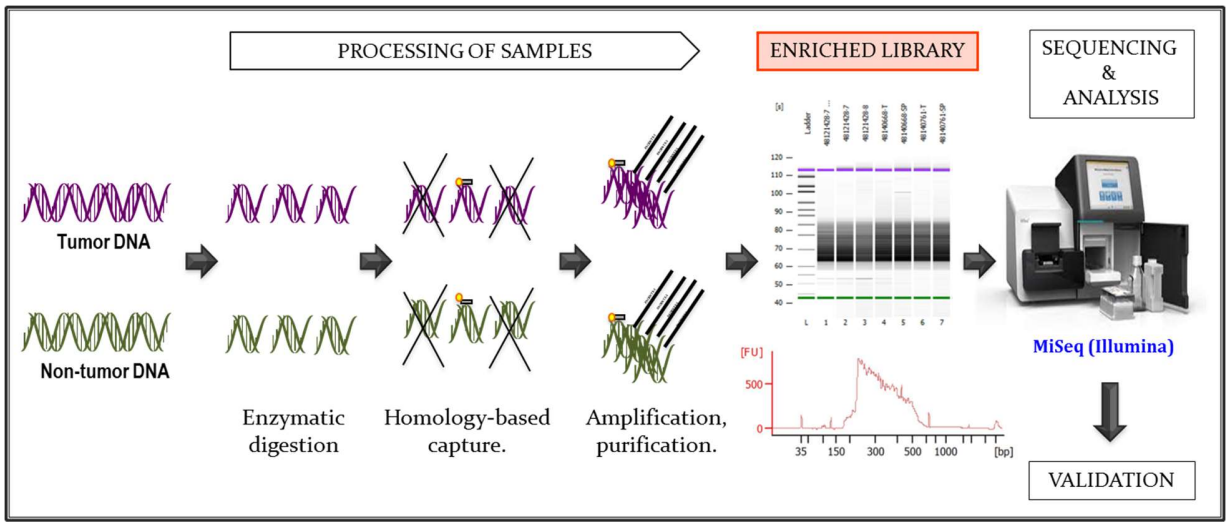

Figure 3. Workflow of preparation and analysis of HaloPlex libraries. The sample preparation for sequencing starts with the enzymatic digestion of DNA samples (paired tumor and normal DNA of each case). This digested DNA is then hybridized to biotinylated probes, which binds specifically to genomic areas of interest, and indexed. Target DNA is captured on streptavidin beads and amplified by PCR. After a purification step, samples with different indexes are pooled for multiplexed sequencing. The enriched library is assessed by means of a bioanalyzer and sequenced in a MiSeq platform. After the variant calling (using the non-tumor DNA sample as a reference), found mutations are validated by an independent sequencing.

1.2.1.3. ANALYSIS OF NEXT GENERATION SEQUENCING DATA.

Given the huge amount of sequencing data produced by NGS platforms, the development of accurate and efficient data handling and analysis pipelines is essential. Once sequencing is complete, raw sequence data must undergo several analysis steps, which requires extensive bioinformatics support and hardware infrastructure. Analysis of the sequences can include a wide variety of bioinformatics assessments, including the identification of both somatic and germline mutation events, the detection of SNPs or indels,

the discovery of novel genes or regulatory elements, and the analysis of transcript expression levels.

There are many free online tools and software packages to perform the data analysis [Gogol-Doring, A. et al., 2012]. A generalized pipeline for analyzing NGS data includes five primary operations [Grada, A. et al., 2013; Rehm, H. L. et al., 2013]:

1. Preprocessing data to remove adapter sequences and low-quality reads.
2. Base calling: identification of the specific nucleotide present at each position in a single sequencing read. This is typically integrated into the instrument software, given the technology-specific nature of the process. Files with this information are recommended to be in FASTQ and FASTA formats.
3. Read alignment: correctly positioning short DNA sequence reads along the genome in relation to a reference sequence or, otherwise, de novo alignment of the sequence reads.

   A reference genome (also known as a reference assembly) is a digital nucleic acid sequence used as a representative example of the specie. It is often assembled from the sequencing of DNA from a number of donors. The Genome Reference Consortium (GRC) regularly updates assembly references. The first major release of the human reference assembly made by the GRC was GRCh37 (from March, 2009) and the last one is GRCh38.p6 (from December, 2015).

   Aligned data are usually filed in two formats: SAM (Sequence Alignment/Map) and BAM (the binary version of a SAM file). These two formats are designed to contain the same information, but the SAM format is more human readable and easier to process by conventional text based processing programs and it can be used to store sequence data, both aligned as well as unaligned. The BAM format provides binary versions of most of the same data, and is designed to compress reasonably well.
4. Variant calling: detection of the DNA variants in the sequence analyzed as compared with a reference sequence. The accuracy of identifying variants greatly depends on the depth of sequence coverage; increased depth improves variant calling. Because some regions may have low sequence coverage, it is important to track positions where there is absent data or an ambiguous call, enabling test limitations to be defined. The VCF (Variant Call Format) format is a tab delimited format for storing variant calls and individual genotypes. It is able to store all variant calls from single nucleotide variants to large scale insertions and deletions.
5. Variant annotation: addition of information about each detected variant. For example, annotation pipelines will determine whether a variant is within or near a gene, where the variant is located within that gene (e.g., untranslated region, exon, intron), and whether the variant causes a change in an amino-acid within the encoded protein. Ideally, the annotation will also include additional information that facilitates interpretation of its

clinical significance. This information may include the presence of the variant in certain databases, the degree of evolutionary conservation of the encoded amino-acid, and a prediction of whether the variant is pathogenic due its potential impact on protein function using in silico algorithms. Genome annotations can be in GTF, GFF and BED format.

Alignment of sequence data to reference genomes/transcriptomes is an important step to analyze, interpret and take advantage of NGS results. Some tools exist which allow performing the alignment without requiring special computer knowledge, like the web-based platform Galaxy, which is a framework for integrating computational tools. To explore and visualize the resulting read pileups along with genome annotation features, IGV genome browser (from the Broad Institute) can be a useful simple tool.

1.2.1.4. GENOMIC CONCEPTS DERIVED FROM N.G.S.

A series of basic concepts and terms are frequently used to expose and explain sequencing results. Its understanding is necessary for the correct interpretation of the sequencing data and for the envisioning of their possible applications to clinic and research. Some of the most frequently used concepts, generally speaking and also specifically in the two projects which compose this thesis, are briefly described below.

1.2.1.4.1. COVERAGE AND DEPTH.

The term **depth** simply reflects the average number of times that a given region has been sequenced by independent reads. It describes the average number of reads that align to known reference bases. The NGS depth often determines whether variant discovery can be made with a certain degree of confidence at particular base positions. It is expressed as a number of times (for example, a study can have a 30× average depth).

The term **coverage** refers to the breadth of the genome that has been sequenced. It is defined as the percentage of target bases that has been sequenced a given number of times, *id est*, that has been "covered" by the sequencing. It is expressed as a percentage (per example, a study can achieve a 95 % breadth of coverage)

Sometimes, the term "depth of coverage" is used as a synonym of depth.

1.2.1.4.2. MUTATION RATE.

The mutation rate stands as the number of mutations identified by DNA sequencing in relation to the number of sequenced bases. It is usually expressed as the number of mutations per megabase of DNA (mutations/Mb).

Tumors have a notably variable mutation rate. Some cancer genomes carry more than 100000 somatic point mutations (or single nucleotide variants, SNVs), whereas others have fewer than 1000 [Cancer_Genome_Atlas_Network, 2012; Cancer_Genome_Atlas_Research_Network, 2012; The_Cancer_Genome_Atlas_Network, 2012; The_Cancer_Genome_Atlas, 2015]. Some of this variation can be accounted for by previous heavy mutagenic exposures or by the existence of known DNA repair defects. For example, melanoma and lung cancer are known to find their mutation burden increased by environmental mutagens; ultraviolet (UV) radiation and tobacco, respectively ([Cancer_Genome_Atlas_Research_Network, 2012; The_Cancer_Genome_Atlas, 2015; Fernandez-Cuesta, L. et al., 2016]). In addition, mutation rates can vary extraordinarily depending on which genes are mutated (e.g. tumors possessing mutations in mismatch repair genes).

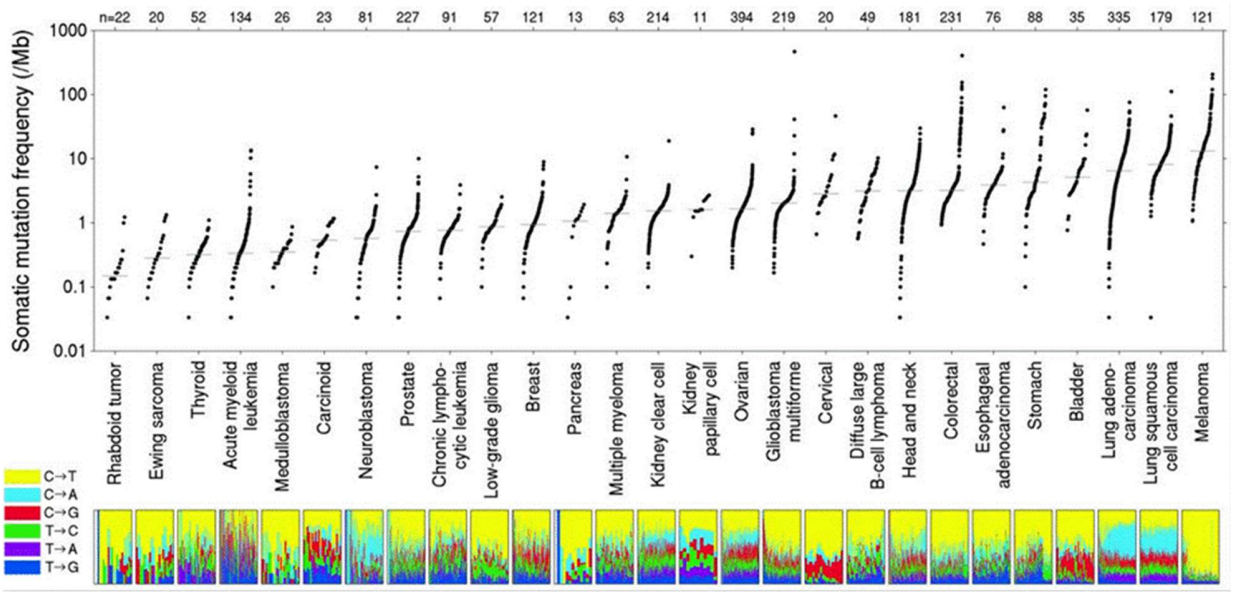

Figure 4: Somatic mutation frequencies observed in exomes from 3,083 tumor-normal pairs (from [Lawrence, M. S. et al., 2013]). Each dot corresponds to a tumor-normal pair, with vertical position indicating the total frequency of somatic mutations in the exome. Tumor types are ordered by their median somatic mutation frequency, with the lowest frequencies (left) found in hematological and pediatric tumors, and the highest (right) in tumors induced by carcinogens such as tobacco smoke and UV light. Mutation frequencies vary more than 1000-fold between lowest and highest mutation rates across cancer and also within several tumor types. The lower panel shows the relative proportions of the six different possible base-pair substitutions, as indicated in the legend on the left.

A large study performed at the Broad Institute consisting in a meta-analysis of 2957 whole exomes and 126 whole genomes from 27 cancer types [Lawrence, M. S. et al., 2013] illustrated the great variation existing in the mutational burden between different cancer types. As shown in figure 4, the median frequency of non-synonymous mutations varied by more than 1000 fold across cancer types: pediatric cancers showed frequencies as low as 0.1 somatic mutations/Mb (approximately one change across the entire exome) whereas, at the opposite extreme, some melanoma and lung cancer cases exceed 100. No less important is the fact that mutation frequencies varied dramatically across patients within a cancer type. For example, the mutational frequency of melanoma ranged from 1 to 100 somatic mutations per Mb and, despite the low median frequency of AML (0.37/Mb), the patient-specific frequencies similarly spanned three orders of magnitude, from 0.01 to 10 mutations/Mb. In some cases, variation may be due to key biological factors, such as melanomas not attributed to UV exposure (on unexposed skin) [Armstrong, B. K. et al., 1993], colon cancers with or without mismatch repair defects [The_Cancer_Genome_Atlas_Network, 2012], or head and neck tumors with viral or non-viral origin [Stransky, N. et al., 2011].

Similarly, Alexandrov et al. published in *Nature* [Alexandrov, L. B. et al., 2013] the mutation burden of 30 different classes of cancer (figure 5). They took into account somatic substitutions and small insertions/deletions (indels) coming from mutational catalogues of 7042 primary cancers (6535 of them analyzed by WES and the remaining 507, by WGS). Again, the prevalence of somatic mutations was highly variable between and within cancer classes, ranging from about 0.001 per Mb to more than 400 per Mb (figure 5). Also in agreement with Lawrence et al., certain childhood cancers carried the smallest number of mutations whereas cancers related to chronic mutagenic exposures, such as lung and malignant melanoma, exhibited the highest prevalence.

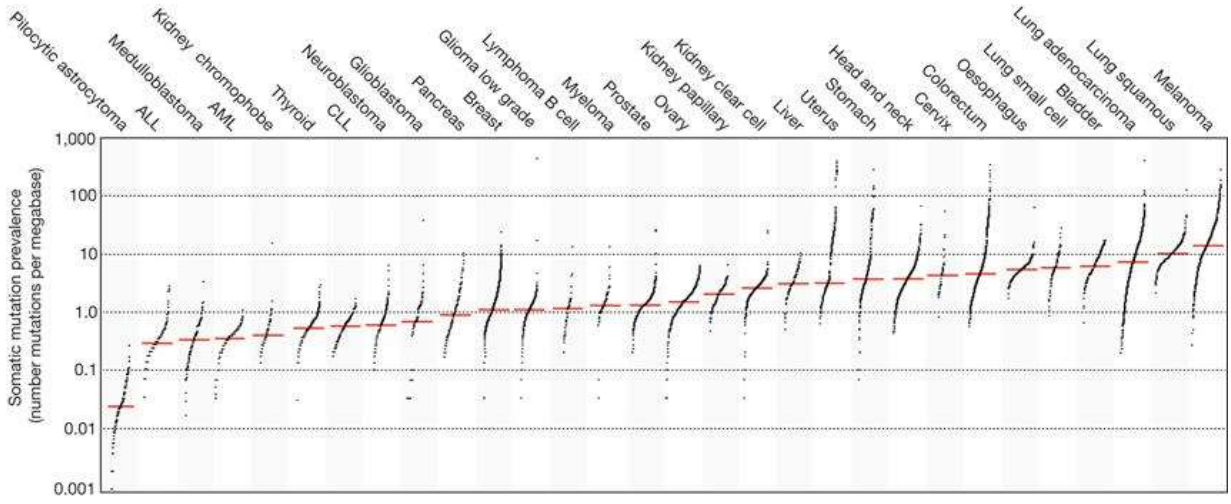

Figure 5. The prevalence of somatic mutations across 30 human cancer types (from [Alexandrov, L. B. et al., 2013]). Every dot represents a tumor sample (normalized with matching non-tumor sample) whereas the red horizontal lines are the median numbers of mutations in the respective cancer types. The vertical

axis (log scaled) shows the number of mutations per megabase. All different cancer types are ordered on the horizontal axis by their median numbers of somatic mutations. ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia.

Association of mutation rate with survival and response to treatments.

The total mutation burden of tumors has recurrently been proposed to correlate with better or poor prognosis and/or with response to treatments in different cancer types. For example, it has been observed in bladder cancers that invasive tumors normally have larger burdens of mutations of all types, including SNVs and copy number changes. Likewise, invasive tumors also show greater clonal diversity [Cazier, J. B. et al., 2014]. By contrast, a trend toward higher mutational load being associated with a better survival has been reported in melanoma by the consortium "The Cancer Genome Atlas" [The_Cancer_Genome_Atlas, 2015], although this association did not reach statistical significance. A later work with metastatic melanoma [Hugo, W. et al., 2016] reported a correlation between high mutational load and improved patient survival which, in this case, did achieve statistical significance.

Theoretically, one could expect that the higher number of total mutations in a tumor cell the higher number of new epitopes (**neoepitopes**) would presented such cell, becoming hence more recognizable by the immune system. In this case, intra-tumor infiltrating T cells −either stimulated or not by a treatment− would be more effective in controlling tumor growth.

In this line, Snyder et al. [Snyder, A. et al., 2014] hypothesized that an increased mutation burden in metastatic melanoma samples would correlate with a benefit from immunotherapy. They found a significant difference in mutational load between patients with a long-term clinical benefit and those with a minimal benefit or no benefit. Data indicate that a high mutational load correlates with a sustained clinical benefit from CTLA-4 blockade, albeit a high load alone is not sufficient to impart a clinical benefit [Snyder, A. et al., 2014]. In the same vein, a recently published report [Rizvi, N. A. et al., 2015] addresses the question of whether tumor mutation burden correlates with response to immunotherapy in non-small cell lung cancer (NSCLC). A clear and strong correlation was seen between the number of nonsynonymous mutations found in NSCLC tumors and the extent of response to therapy with the human monoclonal antibody Pembrolizumab, an anti–PD-1 agent.

But not only with response to immunotherapy is related the mutational load. Regarding to chemotherapy, observations in ovarian cancer suggest that the total mutation burden, coupled with BRCA1 or BRCA2 mutations, is a predictor of treatment response and outcome [Birkbak, N. J. et al., 2013].

Anyway, it is important to underscore that, although the overall mutation load has been correlated with clinical responses to therapies in different cancer types [Le, D. T. et al., 2015;

Rizvi, N. A. et al., 2015; Van Allen, E. M. et al., 2015], actually, the range of somatic mutation and neoepitope loads of the responding pretreatment tumors overlapped considerably with that of the non-responding tumors. In this line, a recent work reported differences between the mutational rate of responders and non-responders to anti-PD-1 treatment which not met the statistical significance cutoff [Hugo, W. et al., 2016]. As such, the presence of a low mutational load does not necessarily preclude clinical responses, and conversely, the presence of a high mutational load does not always correlate with responses.

1.2.1.4.3. MUTATIONAL SIGNATURES.

Besides described differences in the mutational burdens, the mutational spectrums also vary across cancer types. Different mutational processes often generate different combinations of mutation types, termed "signatures". Clustering analysis on all possible mutations have found natural groupings between mutational spectrum and cancer types which are consistent with signatures [Watson, I. R. et al., 2013]. Certain mutational signatures are associated with the age of patient at the time of diagnosis, with known mutagenic exposures or with defects in DNA maintenance, but many are of cryptic origin [Alexandrov, L. B. et al., 2013]. Thus, lung tumors possess a high fraction of G→T transversions, attached to exposure to polycyclic aromatic hydrocarbons from tobacco [Hainaut, P. et al., 2001]; melanomas possess a high fraction of C→T transitions in dipyrimidines, associated with UV-induced DNA damage and misrepair [The_Cancer_Genome_Atlas, 2015]; gastrointestinal tumors (esophageal, colorectal and gastric) possess a high frequency of transition mutations at CpG dinucleotides that may be a reflection of elevated methylation levels in these tumors [The_Cancer_Genome_Atlas_Network, 2012]; bladder, some head-and-neck, ovarian and breast cancers possess frequent mutations at Cs in the context of TpC, characteristically caused by the APOBEC family of cytidine deaminases [Nik-Zainal, S. et al., 2012; Burns, M. B. et al., 2013; Taylor, B. J. et al., 2013]; leukemic samples (acute myeloid leukaemia (AML) and chronic lymphocytic leukemia (CLL)) possess A to T mutations in the TpA context [Lawrence, M. S. et al., 2013].

In total, 21 distinct mutational signatures have been revealed [Alexandrov, L. B. et al., 2013] (all of them shown in figure 6), manifesting hence a considerable diversity. In most cancer classes at least two mutational signatures were detected, with a maximum of six in cancers of the liver, uterus and stomach [Alexandrov, L. B. et al., 2013]. Data hint at some cancers as having a more complex repertoire of mutational processes than others. Cancers show many different combinations of signatures and the patterns of contribution to the mutational burden of each sample vary markedly between signatures.

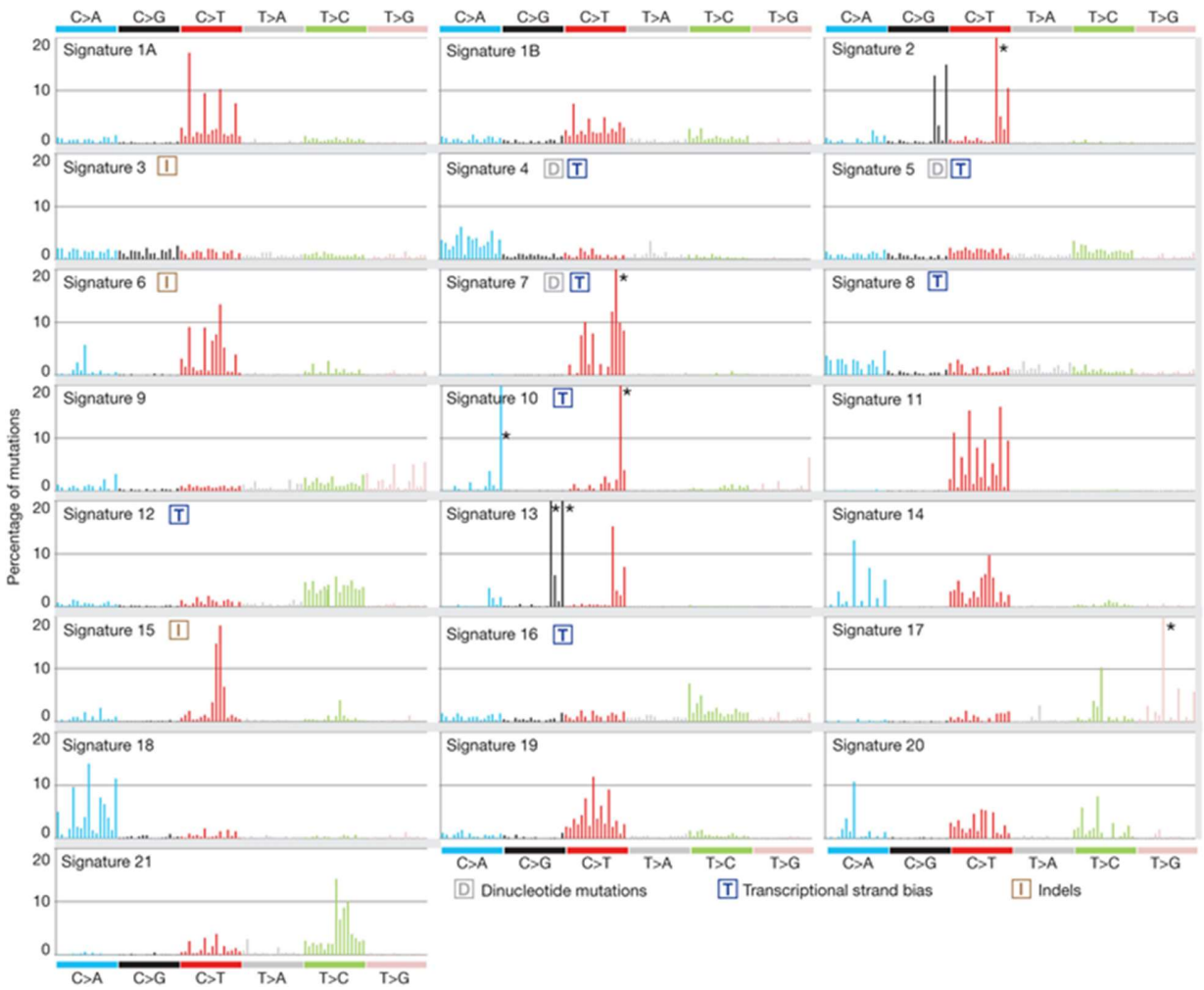

Figure 6. Validated mutational signatures found in human cancer (from [Alexandrov, L. B. et al., 2013]). Each signature is displayed according to the 96 substitution classification defined by the substitution class and sequence context immediately 3′ and 5′ to the mutated base. The probability bars for the six types of substitutions are displayed in different colors. The mutation types are on the horizontal axes, whereas vertical axes depict the percentage of mutations attributed to a specific mutation type. All mutational signatures are displayed on the basis of the trinucleotide frequency of the human genome. Asterisk indicates mutation type exceeding 20%.

1.3. CLINICAL, BIOLOGICAL AND MECHANISTIC CHARACTERISTICS OF CUTANEOUS MELANOMA AND MERKEL CELL CARCINOMA.

Cutaneous melanoma and Merkel cell carcinoma (MCC) are two types of aggressive cancer of the skin, whose mortality rate is rising each year. Both are thought to be originated from the malignant transformation of cells derived from the embryonic neural crest; melanocytes [Sommer, L., 2011] and Merkel cells [Merkel, F. S., 1875; Goessling, W. et al., 2002], respectively. However, the origin of Merkel cell carcinoma and Merkel cells is still controversial and some authors argue in favor of an epithelial origin [Halata, Z. et al., 2003; Morrison, K. M. et al., 2009].

In fact, one big difference between melanoma and MCC is the large amount of available data about the first disease, both biological and clinical, and the shortage of information and knowledge about the second one. Virtually everything about MCC is currently under discussion and supported by few data.

Another dissimilarity between these two types of cancer lies in the age of patients; whereas MCC usually affects elderly people, being the median age of patients around 70 cases [Llombart, B. et al., 2005; Kukko, H. et al., 2012], melanoma often appears in young people and the median age of patients is lower than that of most cancers [Tamir, G. et al., 1996; Evans, M. S. et al., 2013].

Both melanoma and MCC lack an effective systemic therapy, able to produce sustained responses. The standard approach to the initial management of primary tumors is the surgical resection [Lebbe, C. et al., 2015; Teng, J. et al., 2015], but its effectiveness and the possibility of using this technique, depends, amongst others circumstances, on the spread of the cancer and the age and physical conditions of patients. Survival rates are low in melanoma and MCC cases with regional or distant metastases [Sandel, H. D. t. et al., 2006; Robert, C. et al., 2015] and also in those cases that shows high depth (in melanoma and MCC) [Vollmer, R. T. et al., 2001; Santonocito, C. et al., 2007; Smith, F. O. et al., 2015] or diameter (in MCC) [American_Joint_Committee_on_Cancer, 2010; Smith, F. O. et al., 2015; Haymerle, G. et al., 2016] (more information in tables 1 and 2).

Both cancer types frequently appear in sun-exposed areas of skin and ultraviolet radiation likely plays a role in their carcinogenesis. Indeed, the exposure to UV radiation is considered the major risk factor for melanoma in fair-skinned populations [Mc, G. V., 1952; Balch, C. M. et al., 2001]. Less clear was the importance of UV radiation in the malignant transformation that leads to MCC. It has been recently published that a high proportion of MCC tumors shows a pattern of genomic alterations attributable to UV light effect [Goh, G. et al., 2015; Harms, P. W. et al., 2015]. Usually, the presence of UV signature is coupled with

the presence of a high mutational burden [Goh, G. et al., 2015; Harms, P. W. et al., 2015; The_Cancer_Genome_Atlas, 2015].

Ultraviolet signature.

DNA and RNA contain chromophores strongly absorbent for UVA and UVB, with the aromatic heterocyclic nitrogen bases absorbing with maxima wavelength at 260–265 nm. Photoproducts known as cyclobutane pyrimidine dimers (CPDs) and pyrimidine 6-4 pyrimidines are generated upon saturation of the 5,6 double bonds and formation of a four-membered cyclobutyl ring, creating C→T and CC→TT mutations [Brash, D. E. et al., 1982; Ravanat, J. L. et al., 2001; Runger, T. M., 2008].

Thus, it has been proposed that a high proportion of C→T transitions or CC→TT tandem substitutions in a DNA sample signals UV radiation mutagenic effects [Howard, B. D. et al., 1964; Miller, J. H., 1985; Brash, D. E., 2015]. Most melanoma samples have such kind of mutational profile [The_Cancer_Genome_Atlas, 2015], likewise a considerable proportion of Merkel cell carcionomas [Harms, P. W. et al., 2015; Goh, G. et al., 2016].

However, C→T transitions can be caused not only by the exposure to UV light, but also by other mechanisms, such as aging and impaired mismatch repair [Alexandrov, L. B. et al., 2013]. Interestingly, both aging and UV radiation can be involved together in Merkel cell carcinoma, so described patterns of mutations that can specifically be attributable to one particular process are especially useful in this disease. In this regard, C→T mutation events located specifically at dipyrimidine sites have been specifically ascribed to UVA and UVB radiation [Rochette, P. J. et al., 2003; Runger, T. M., 2008; Brash, D. E., 2015]. Therefore, if a high proportion of C→T mutations occur at such sites the sample are usually considered as displaying UV effects. Likewise, the presence of CC→TT tandem substitutions has been specifically associated with UV radiation [Brash, D. E., 2015].

Thus, a confirmed UV signature would be the presence of C→T transitions at dipyrimidine sites accounted for more than 60%, or the presence of CC→TT tandem substitutions accounted for more than 5% of the total mutational burden [Brash, D. E., 2015]. In addition, according to the work of Alexandrov et al [Alexandrov, L. B. et al., 2013], signature 7, mainly found in malignant melanoma, shows a higher prevalence of C→T mutations on the untranscribed compared to the transcribed strands, consistent with the formation, through ultraviolet exposure, of pyrimidine dimers and other lesions which are known to be repaired by transcription-coupled NER. This signature can be specifically attributable to the exposure to UV light [Alexandrov, L. B. et al., 2013].

1.3.1. DEVELOPMENT, STAGING AND INCIDENCE OF MERKEL CELL CARCINOMA.

Merkel cell carcinoma (MCC) has been proposed to be a cutaneous neuroendocrine carcinoma originated from the transformation of Merkel Cells [Wick, M. R. et al., 1983; Sibley, R. K. et al., 1985; Hitchcock, C. L. et al., 1988; Goessling, W. et al., 2002], which are located in the basal layer of the epidermis, associated with nerve endings [Gould, V. E. et al., 1985] (Figure 7). Merkel cells were first identified by the German anatomist Friedrich Sigmund Merkel in 1875 [Merkel, F. S., 1875] and were referred to as "Tastzellen" or "touch cells.". They are the only cells in the skin known to contain neurosecretory granules in their cytoplasm, the same kind of granules described in several tumors of neuroendocrine origin [Tang, C. K. et al., 1978; Walsh, N. M., 2001]. In fact, neuroendocrine tumors (NET) are characterized by their capacity to produce and secrete multiple biologically active substances, which can have critical effects in the tumor microenvironment, as well as in distant tissues [Rindi, G. et al., 2000; Papotti, M. et al., 2005; No_authors_listed, 2016]. Merkel cells are clear and oval, with lobulated nuclei that contain intermediate cytokeratin and neurofilaments. They have spike-like protrusions that enable them to interdigitate with the surrounding keratinocytes [Munger, B. L., 1965; Iggo, A. et al., 1969]. After a long-standing controversy about the role of Merkel cells within the mechanotransduction process, experimental evidence pointed at their direct involvement in the transformation of mechanical stimuli to action potentials in the afferent nerve fiber [Chan, E. et al., 1996; Senok, S. S. et al., 1997; Fagan, B. M. et al., 2001].

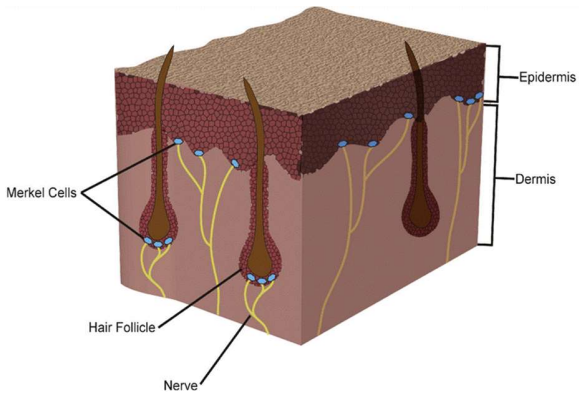

Figure 7. Schematic representation of Merkel cells location (adapted from [Spurgeon, M. E. et al., 2013]). Cutaneous Merkel cells are located in the basal layer of the epidermis, associated with the terminal ends of nerves, and also enriched in the bulge region of hair follicles.

However, whether Merkel cell carcinoma truly derives from Merkel cells is still to date controversially discussed. Another proposed hypothesis defends that this cancer arises from a pluripotent stem cell in the epidermis [McCardle, T. W. et al., 2010]. More studies directed to elucidate the origin of this malignancy are needed.

Merkel cell carcinoma is an infrequent disease which affects especially aged and immunodepressed patients who, at the time of diagnosis, often have metastasis [Sarnaik, A. A. et al., 2010]. Although rare, the reported incidence of MCC has tripled over the past 20 years, due to an improved detection and a rise in the number of elderly and immunosuppressed individuals who are at risk [Hodgson, N. C., 2005]. Median age of patients with MCC is around 70 and patients below 50 compose only 5 % of cases [Llombart, B. et al., 2005; Kukko, H. et al., 2012]. This cancer is characterized by a high incidence of local recurrence, regional nodal metastases, distant metastases, and by a high mortality rate [Coit, D. G., 2001; Goessling, W. et al., 2002; Agelli, M. et al., 2010; Sarnaik, A. A. et al., 2010] (Table 1).

Epidemiologic data suggest a strong link between MCC and the immune system. Individuals with T cell dysfunction, such as solid organ transplant recipients [Penn, I., 1999; Agelli, M. et al., 2003], HIV-infected patients [Engels, E. A. et al., 2002] or chronic lymphocytic leukemia patients [Heath, M. et al., 2008] are at 5- to 50-fold increased risk of developing MCC. Tumors sometimes regress following improvement in immune function [Burack, J. et al., 2003; Muirhead, R. et al., 2007] underscoring the importance of immune surveillance in the development of MCC. Additionally, there are several reported cases of complete spontaneous regression in the MCC literature (a far greater number than expected for its rarity), fact wich suggest a sudden recognition by the immune system leading to the clearance of MCC [Miller, R. W. et al., 1999; Ciudad, C. et al., 2010]. These epidemiologic data raised the possibility of an infectious etiology for MCC. Indeed, the discovery of the Merkel cell polyomavirus (MCPyV) provided the missing link between MCC and its association with immune suppression [Feng, H. et al., 2008] (broader information in section 1.3.2.).

A significant proportion of MCCs have been reported to occur in association with other malignant epithelial neoplasms, mainly squamous cell carcinomas [Brenner, B. et al., 2001; Walsh, N. M., 2001]. In addition, divergent differentiation within these tumors, particularly of squamous and eccrine types, is not infrequent. In a survey of 67 patients with MCC, 25 % of them had a second neoplasm before, concomitant with, or after the diagnosis of MCC [Brenner, B. et al., 2001].

Regarding to staging, different systems have been used for MCC over time. A staging system is a standard way to assess how far a cancer has spread and it is useful to predict its evolution and to select a therapy. Nowadays, the most frequently used staging system is the “TNM classification of malignant tumors “of the American Joint Committee on Cancer (AJCC) [American_Joint_Committee_on_Cancer, 2010]. This system is based on the primary tumor size and potential growth into close tissues, the spread to nearby lymph nodes and the spread to distant organs: “T” describes the size of the original (primary) tumor, “N” describes

nearby (regional) lymph nodes that are involved and "M" describes distant metastasis. As MCC is an uncommon type of cancer, it is hard to get accurate and updated survival statistics for this disease, especially by individual stages. Table 1 shows data recorded in the USA National Cancer Data Base, available in the web page www.cancer.org [American_Cancer_Society, 2016].

STAGE	SPREAD	5-YEAR SURVIVAL RATE
IA	≤ 2 cm	80 %
IB		60 %
IIA	> 2 cm, not sowing metastases	60 %
IIB		50 %
IIC		50 %
IIIA	Lymph node metastasis	45 %
IIIB		25 %
IV	Distant metastasis	20 %

Table 1. Merkel cell carcinoma staging and associated survival rates (based on data recorded in the USA National Cancer Data Base). Survival rates are calculated based upon outcomes of nearly 3,000 patients diagnosed with MCC from 1986 to 2000 [American_Cancer_Society, 2016]. Local disease is classified as stage I for tumors ≤ 2 cm and as stage II for tumors > 2 cm, with A, B or C sub-classification based on pathologic versus clinical evaluation of lymph nodes. Regional nodal disease is stage IIIA when nodes are examined by pathology only and stage IIIB when clinically apparent by examination or radiologic study. Stage IV denotes distant metastatic disease.

In addition to staging, other factors can affect survival. For example, several studies have shown that people with a weakened immune system, such as those who have had organ transplants or who are infected by HIV, tend to have a worse outlook [Fukumoto, H. et al., 2013; Nghiem, P., 2015]. Older age has also been linked with an unfavorable outcome [Agelli, M. et al., 2010; Tarantola, T. I. et al., 2013]. By contrast, intratumoral CD8+ lymphocyte invasion has been shown to be a significant biomarker for improved survival in MCC patients [Paulson, K. G. et al., 2011]. Surprisingly, tumor size seems to have no impact on survival [Tarantola, T. I. et al., 2013].

There is no "gold standard" for the diagnosis of MCC, which is sometimes difficult to distinguish of other cancer types with similar cellular and tissular characteristics, particularly, of small cell lung carcinoma (SCLC). Indeed, these two pathologies can only be distinguished by histopathologic analyses of the expression of specific biomarkers, like cytokeratin 20 [Becker, J. C., 2010], as further explained below. Therefore, a better awareness of each disease will probably be very helpful in the development of strategies for differential diagnosis.

Likewise, there is still uncertainty about the etiology of MCC. Several lines of evidence suggest a strong link between MCC and ultraviolet light exposure. For example, the incidence of MCC is higher at more equatorial latitudes [Agelli, M. et al., 2003], 81% of primary tumors occur on sun-exposed skin, and Caucasians have the greatest risk [Heath, M. et al., 2008]. Owing to these data, some authors argue in favor of UV radiation as a main cause [Lunder, E. J. et al., 1998; No_authors_listed, 2009], as it has been proposed for other malignant skin tumors, like melanoma [Davies, H. et al., 2002]. On another hand, Merkel cell polyomavirus (MCPyV) is present in 50 - 90 % MCC tumors, whereas it is in just 16 % of control tissue samples [Feng, H. et al., 2008; Kassem, A. et al., 2008]. Therefore, MCPyV has been also recurrently proposed to be a major etiological factor of MCC.

1.3.2. MERKEL CELL POLYOMAVIRUS.

Polyomaviruses are a genus of non-enveloped viruses with a circular double-stranded DNA genome of approximately 5000 base pairs. The ability of certain polyomaviruses to transform mammalian cells is well known. The best studied example is the SV40 polyomavirus that was originally discovered in the primary monkey kidney cells used to prepare polio vaccines. SV40 was found to induce multiple tumors in newborn hamsters [Eddy, B. E. et al., 1962].

In 2008, a novel polyomavirus was identified in Merkel cell carcinoma tissues [Feng, H. et al., 2008], which was called Merkel cell polyomavirus (MCPyV). A direct sequencing approach, called digital transcriptome subtraction (DTS) [Feng, H. et al., 2007], was used to identify foreign transcripts with homology to polyomavirus T antigens in the Merkel cell tumor genome. The viral genome was found to be clonally-integrated into the tumor genomes, indicating that it was present prior to the cancer cell clonal expansion. Two full-length viral genomes, MCC350 and MCC339, were deposited in Genbank (EU375803 and EU375804, respectively) and DNA clones are available through the AIDS Reasearch Reagent Repository.

Merkel cell polyomavirus (MCPyV) shares significant homology to viruses belonging to the Murine polyomavirus (MuPyV) subgroup. It is also very closely related to the African Green Monkey lymphotrophic polyomavirus (LPyV) and more distantly to SV40 and other known human polyomaviruses.

In humans, MCPyV is the first polyomavirus with demonstrated integration into genomic DNA. Several observations suggest that this virus contributes to the pathogenesis of MCC (more deeply expounded in next section). Some of these evidences are:

1) It is present in a substantial portion of MCC tumors [Garneski, K. M. et al., 2009; Foulongne, V. et al., 2010],

2) It is monoclonaly integrated in MCC tumor cells [Feng, H. et al., 2008],
3) The MCPyV LT antigen expressed in MCC tumors is often truncated due to mutations that preserve critical cell-cycle progression functions, but eliminate cell-lethal virus-replication activities [Shuda, M. et al., 2008].

Like all polyomaviridae, MCPyV is a small, circular, nonenveloped, double-stranded DNA virus that integrates into the tumor genome in a clonal manner (figure 8). It has a T antigen locus, which encodes for four differentially spliced mRNA transcripts (figures 8 and 9), corresponding to a large T antigen (LT), a small T antigen (ST), encoded by two transcripts, besides an additional isoform called 57kT, which may represent an analogue to the SV40 17kT transcript [Shuda, M. et al., 2008]. A Rb binging domain are conserved in large T and 57 kT antigens, suggesting a potential ability of this virus to modify RB tumor suppressor activity and promote tumorigenesis through still poorly defined mechanisms [Shuda, M. et al., 2009; Stakaityte, G. et al., 2014].

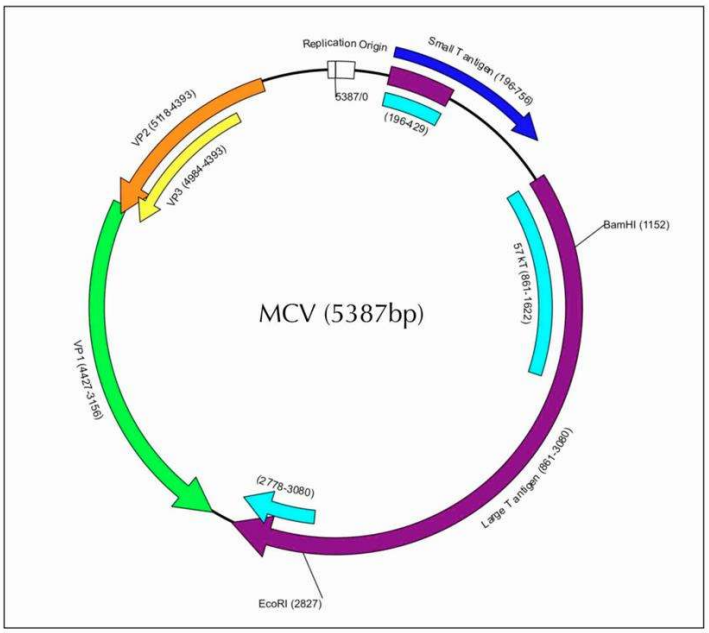

Figure 8. Schematic of MCPyV genome (adapted from [Feng, H. et al., 2008]). The genome encodes typical features of a polyomavirus, including large T (purple), small T (dark blue) and 57KT (light blue) open reading frames. Also shown are predicted VP1 (green) and overlapping VP2 (orange) and VP3 (yellow) genes.

The MCPyV LT antigen appears to retain the major conserved features of other polyomavirus LT antigens, including the DnaJ motif (which binds to heat-shock proteins), the LXCXE motif (which inactivates retinoblastoma family proteins), and the origin-binding and helicase/ATPase domains (which promote viral replication) [Shuda, M. et al., 2008]. These various domains allow the polyomaviruses to use host cell machinery for viral genome

replication, but can also target tumor suppressor proteins, perhaps contributing to cell transformation [Ali, S. H. et al., 2001].

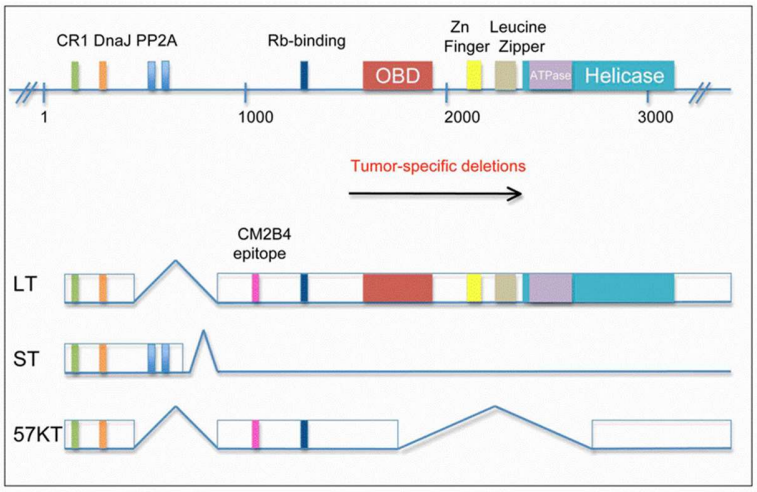

Figure 9. Transcript mapping of the multiply spliced MCPyV T antigen locus (adapted from [Shuda, M. et al., 2009]. Three T antigens have been identified as large T, small T and 57 KT. Four different transcripts are encoded, each one with CR1 (green) and DnaJ (orange) domains. ST protein contains two PP2A binding motifs (blue). Rb binding (dark blue) domain are conserved in large T and 57KT. Large T contains unique domains including origin binding (red), zing finger (yellow), leucine zipper (blue) and helicase (cyan)/ATPase (purple).

In addition, the MCPyV genome expresses a 22-nucleotide viral miRNA (MCV-miR-M1-5p) from the late strand that most likely autoregulates early viral gene expression during the late phase of infection, as it was shown to reduce the level of reporter transcripts containing MCPyV early region sequences [Seo, G. J. et al., 2009; Lee, S. et al., 2011]. This miRNA has complete reverse complementarity to a sequence in LT adjacent to the LXCXE motif, implying that it targets this early protein for autoregulation, and it is also predicted to affect a variety of cellular targets with potentially relevant functions in transformation [Seo, G. J. et al., 2009; Johnson, E. M., 2010; Lee, S. et al., 2011]. One study [Lee, S. et al., 2011] reported that miRNA expression is preserved in approximately 50% of MCPyV-positive MCC tumors, and the level of miRNA expression correlates with viral genome copy number in the tumor. The presence of MCPyV miRNA in MCC tumors certainly warrants further investigation into its role, if any, in the pathogenesis of MCC.

1.3.2.1. ROLE OF MERKEL CELL POLYOMAVIRUS IN THE PATHOGENESIS OF MCC.

Since its discovery in 2008, epidemiological studies have established Merkel cell polyomavirus as a common virus infecting the human population. Enzyme immunoassays

specific for the immunogenic determinant of MCPyV, the major capsid protein VP1, have been used to determine that up to 80 % of the adult population contains serum antibodies to MCPyV [Stolt, A. et al., 2003; Carter, J. J. et al., 2009; Kean, J. M. et al., 2009; Tolstov, Y. L. et al., 2011; Gossai, A. et al., 2016]. By monitoring overt symptoms alongside seroconversion of MCPyV-seronegative individuals, it appears that this infection is mostly asymptomatic [Tolstov, Y. L. et al., 2011].

Viral DNA has been detected in a wide variety of anatomical locations, including respiratory tract [Babakir-Mina, M. et al., 2010], saliva [Foulongne, V. et al., 2010], lymphoid tissues [Sharp, C. P. et al., 2009], urine [Husseiny, M. I. et al., 2010] and gastrointestinal tract [Campello, C. et al., 2011]. Viral DNA detection in these locations is relatively low compared to the skin, where both viral DNA and encapsidated virions can be detected [Feng, H. et al., 2008; Foulongne, V. et al., 2010; Loyo, M. et al., 2010; Schowalter, R. M. et al., 2010].

As Merkel cell polyomavirus was originally isolated from Merkel cell carcinoma tissue samples, in the hallmark report announcing its discovery, Feng and colleagues established the initial framework for a causal relationship between this virus and this cancer [Feng, H. et al., 2008]. Since then, several lines of evidence have been suggesting that MCPyV is an etiological agent of MCC [Sastre-Garau, X. et al., 2009; Houben, R. et al., 2010; Chang, Y. et al., 2012]. Perhaps, one of the more persuasive pieces of evidence supporting this theory is the clonal integration of the viral genome into the cell genomes of MCC tumors [Feng, H. et al., 2008; Laude, H. C. et al., 2010; Martel-Jantin, C. et al., 2012], fact that suggests that the infection precedes the initial tumor development and the monoclonal expansion of cells. This conclusion was reached based on the circumstance that many tumor-derived polyomaviruses possess premature stop codon mutations that result in truncation of the large T protein. The truncative mutations eliminate the T antigen helicase activity but retain the LXCXE–retinoblastoma protein-binding motif, as well as other N-terminal motifs found in the T antigens [Shuda, M. et al., 2008]. Hence, these viruses retain the ability to regulate the host cell cycle and inhibit the Retinoblastoma protein gene family, but can no longer initiate replication, so the virus would be lost if it they were not integrated into the host tumor cell genome. For this reason, MCPyV cannot be a passenger virus that secondarily infects the tumor. These two independent mutational events (virus integration and T antigen truncation) seem to play a mechanistic role in the development of MCC and may also explain why this cancer is relatively rare. It could be also possible that skin exposure to UV or ionizing radiation increases risk for MCC by enhancing T antigen and virus integration mutations.

Cancer-associated viruses may contribute to carcinogenesis directly, via expression of viral oncogenes that promote cell transformation, or indirectly, via chronic infection and inflammation, which may predispose host cells to acquire carcinogenic mutations (reviewed by [Moore, P. S. et al., 2010]).

MCPyV integration is analogous to that seen in high-risk human papillomavirus integration in cervical cancer cells, and occurs at different sites in the genome in different individual cases [Feng, H. et al., 2008]. In one of the cases studied by Feng et al. a metastatic tumor had the same integration pattern as the primary tumor, showing that the metastasis arose from a single cancer cell derived from the primary tumor. Although MCPyV integrates at different sites, it is currently unknown whether or not virus integration interrupts common cellular pathways involved in tumor cell growth. Likewise, the precise mechanisms that direct the integration of the MCPyV genome and the contribution of exogenous influences in this process are not yet understood.

Different research works have shown that MCPyV small and large T antigens can inhibit apoptosis, via upregulation of survivin [Schrama, D. et al., 2013], inhibit proteasomal degradation, via inhibition of ubiquitin ligases [Kwun, H. J. et al., 2013], and augment cap-dependent translation of mRNA [Shuda, M. et al., 2011].

Given the preponderance of evidences associating MCPyV with MCC, as well as the transforming potential of MCPyV genes, the World Health Organization's International Agency for Research on Cancer has recently classified this polyomavirus as a group 2A carcinogen, a designation meaning it is “probably carcinogenic to humans” [Bouvard, V. et al., 2012]. However, it is important to note that not all MCC tumors are positive for MCPyV antigens, but infection has been described in a cohort-dependent proportion which range from 50 % to 90 %. Interestingly, North American MCC tumors are more frequently MCPyV-positive than Australian MCC tumors [Garneski, K. M. et al., 2009]. This difference may be due to the increased sun exposure in Australia, making, perhaps, a possible viral contribution less frequent. Thus, the infection with MCPyV appears not to be a mandatory and required condition for the emergence and development of MCC.

On the other hand, we should not dismiss the fact that, although most individuals are naturally exposed to MCPyV, very few have MCC. Therefore, other factors, such as an immunosuppressed state, likely contribute to viral integration, mutagenesis, and carcinogenesis. In this regard, it has been mentioned before in this thesis that the incidence of MCCs is dramatically elevated in immunosuppressed patients [Harms, P. W. et al., 2015].

1.3.3. SUBCLASSES AND ETIOLOGIES OF MERKEL CELL CARCINOMA.

It has been recently described that the number of somatic single nucleotide variants (SSNVs) in MCC varies widely, ranging from 3 to 4707 SSNVs per exome [Goh, G. et al., 2015; Harms, P. W. et al., 2015]. The distribution of SSNVs is bimodal and representes extreme cases in cancer biology, showing a huge difference between the average of the mutational rates of those MCCs with few SSNVs and the average of MCCs with a high

burden [Goh, G. et al., 2015; Harms, P. W. et al., 2015]. Interestingly, those MCCs with an elevated mutational burden corresponded with MCPyV-negative cases whereas MCCs with a low mutational burden represented MCPyV-positive tumors [Goh, G. et al., 2015; Harms, P. W. et al., 2015].

In addition, tumors with high mutational burden (and without detected viral antigens) were significantly enriched for C → T transitions [Goh, G. et al., 2015; Harms, P. W. et al., 2015], characteristic that can be a result of ultraviolet light, although they can be also owing to other causes (as aforesaid), such as aging and impaired mismatch repair [Alexandrov, L. B. et al., 2013]. Therefore, Goh et al. [Goh, G. et al. 2015] used a developed algorithm that extracts mutational signatures from somatic mutations [Alexandrov, L. B. et al., 2013] and found that a median of 66 % of SSNVs per each MCPyV-negative MCC could be attributed to signature 7, described by Alexandrov et al. as due to UV exposure. In a different way, Harms et al. [Harms, P. W. et al. 2015] examined the bases immediately 5' and 3' next to mutated nucleotides, finding an enrichment of C → T transitions at dipyrimidine sites −a pattern also reported to be induced by UV radiation [Alexandrov, L. B. et al., 2013; Brash, D. E., 2015]− only in MCPyV-negative cases. Furthermore, the majority of tandem substitutions found in non-infected cases were CC → TT transitions, likewise consistent with a UV signature. These data were comparable with those found in other UV-induced cancers, such as melanoma, and significantly higher than those observed in MCPyV-positive MCCs, where the UV signature was not present at all [Goh, G. et al., 2015; Harms, P. W. et al., 2015; Wong, S. Q. et al., 2015].

Thus, Merkel cell carcinoma seems to have a viral and a non-viral etiology, so it could be segregated into two distinct molecular classes:

1. MCPyV-negative MCCs, which show high mutational burdens with UV mutational signatures, supporting thus the UV-induced damage as etiology.
2. MCPyV-positive MCCs, which harbor relatively few mutations (much lower than MCPyV-negative tumors) and do not display a definitive UV-signature, supporting an oncogenic role for MCPyV T antigens as primary drivers for these tumors.

These findings are analogous to those achieved in head and neck squamous cell carcinomas, where tumors lacking human papillomavirus display higher mutational burdens [Stransky, N. et al., 2011].

The effect of the polyomavirus on the clinical course of the disease is unclear. Some studies have shown that individuals with MCPyV-positive tumors have a better prognosis than those with MCPyV-negative tumors [Inoue, T. et al., 2000; Paulson, K. G. et al., 2011; Higaki-Mori, H. et al., 2012; Sihto, H. et al., 2012]. Likewise, Touzé and colleagues found that high antibody titers of MCPyV were a significant predictor for a longer progression-free

survival [Touze, A. et al., 2011]. Accordingly with those results, virus-negative MCCs normally harbor alterations affecting the tumor suppressor gene TP53, which have been linked to worse outcomes in different cancer types [Molina-Vila, M. A. et al., 2014; Said, R. et al., 2014], although not, so far, in MCC [Sihto, H. et al., 2011; Waltari, M. et al., 2011]. In contrast, other studies have reported not to have found statistically significant differences in survival between patients with virus-positive and virus-negative tumors [Handschel, J. et al., 2010; Schrama, D. et al., 2011]. Perhaps, these conflicting findings could be explained by differences in the geographical origin or even the timing of patient cohort collection, amongst other reasons.

In any case, pooling data from many different studies, MCC development seems to have two distinct etiologic ways: MCPyV-mediated and non-MCPyV-mediated (Reviewed by [Ma, J. E. et al., 2014]).

1.3.4. MAIN DISEASE MECHANISMS OF MERKEL CELL CARCINOMA.

The genetic mechanisms underlying the development and tumor progression of MCC are still poorly understood. Comparative genomic hybridization (CGH) studies have shown that the pattern of chromosomal abnormalities in MCC is similar to that seen in small cell lung cancers (SCLC) [Van Gele, M. et al., 1998; Van Gele, M. et al., 2002]. The most frequent genetic alterations in MCC involve the short arm of chromosome 1, where deletions and unbalanced translocations occur in as many as 40 % of cases [Vortmeyer, A. O. et al., 1998; Van Gele, M. et al., 2002]. Deletion mapping studies pointed to a more than one possible tumor suppressor locus for MCC in chromosome 1p32–1p36, but the candidate genes are yet to be identified [Leonard, J. H. et al., 2000]. Deletions involving the same location have also been investigated for genetic changes in pheochromocytoma, neuroblastoma, and (arguably) melanoma. This suggests that Merkel cell carcinogenesis may share some pathogenetic mechanisms with other neoplasms of neural crest derivation [Vortmeyer, A. O. et al., 1998].

1.3.4.1. CELL CYCLE- AND APOPTOSIS-RELATED GENES

In multicellular organisms, cell proliferation and death must be finely regulated to maintain tissue homeostasis. Indeed, deregulation of cell proliferation can result in pathologic conditions, including neoplasias. Different observations suggest that this regulation may be achieved, in part, by connecting the control of cell cycle progression and programmed cell death. Several instances in which apoptosis is regulated by genes that are involved in cell cycle progression support the theory of a link between both mechanisms [Evan, G. I. et al.,

1995]. This linkage has been recognized for tumor suppressor genes such as *TP53* and *Retinoblastoma* (*RB)*, the dominant oncogene *c-Myc*, and several cyclin-dependent kinases (CDKs), which play a role in proliferative pathways and, in addition, may participate in leading cells to apoptosis [Jacks, T. et al., 1992; Amati, B. et al., 1993; Meikrantz, W. et al., 1994; Graeber, T. G. et al., 1996; Polyak, K. et al., 1996; Harvey, K. J. et al., 1998].

Multiple checkpoints in the eukaryotic cell cycle ensure that division occurs only after sufficient growth and faithful DNA replication, and only when favorable conditions exist. At each checkpoint, numerous proteins engage in a series of carefully coordinated biochemical reactions. This complexity allows for precise regulation of all steps in the cell cycle and is essential to preventing the devastating consequences of cell division gone awry. Cell external signals and cell intrinsic information together determine whether cells enter a division cycle. In general, external signals affect this decision only until cells commit to go through the entire cycle, at a time in G1 known as "restriction point" in mammals. From there on, progression through the cell cycle is controlled intrinsically by the cell-cycle machinery [van den Heuvel, S., 2005].

Of the many proteins involved in cell cycle control, Cyclin-dependent Kinases (CDKs) are amongst the most important. CDKs are small serine/threonine protein kinases able to modify various protein substrates involved in cell cycle progression. As well, the transcription factor p53 regulates the fate of the cell by inducing either death or cell cycle arrest through specific pathways chosen depending on a variety of factors (such as the extent of DNA damage and on the genetic background of the cell). In addition, the presence of functional p21 and the cross-talk with RB are critical determinants in the role of P53 [Polyak, K. et al., 1996]. Finally, Retinoblastoma (RB) family of proteins is not only a growth suppressor, but also has a protective function against apoptosis, by means of interactions with caspases and participation in E2F activation [Jacks, T. et al., 1992].

1.3.4.1.1. TP53.

Tumor Protein p53 (*TP53*) is a gene which codifies a transcription factor that was given its name due to its apparent molecular mass (53 kDa) at the moment of its discovery, in 1979 [Lane, D. P. et al., 1979]. The p53 protein level is usually low in healthy cells due to its short half-life and rapid turnover, so normal functioning p53 is typically undetectable at the protein level. However, it increases in response to DNA damage and other stress signals (like hypoxia or spindle damage).

Activation of p53 begins through a number of mechanisms, including phosphorylation by ATM, ATR, Chk1 and MAPKs. By contrast, MDM2 and MDM4 (the major negative

regulators of p53) are ubiquitin ligases that binds p53 and target it for proteasomal degradation.

p53 regulates a large number of genes (over 100 genes) that control a number of key tumor suppressing functions, such as cell cycle arrest, DNA repair, senescence and apoptosis [Science, W. I. o.]. Whereas the activation of p53 often leads to apoptosis, p53 inactivation facilitates tumor progression. Accordingly, inactivating *TP53* somatic mutations occur in over 50% of cancers of different types [Hollstein, M. et al., 1991].

Nevertheless, mutations in *TP53* gene have not often been found in MCC, ranging the percentage of cases with mutations in this gene from 0 % to 28 % [Carson, H. J. et al., 2000; Lassacher, A. et al., 2008; Lill, C. et al., 2011; Waltari, M. et al., 2011]. In addition, some of these mutations represent SNPs or silent mutations with unknown or no clinical significance. Of note, Waltari et al. [Waltari, M. et al., 2011] analyzed 87 MCC tumors and found no *TP53* mutations. p53 protein expression was detected in only 22.8% of samples. Tumor p53 expression was shown to be associated with absence of detectable MCPyV DNA and with unfavorable survival. Likewise, Lassacher et al. [Lassacher, A. et al., 2008] evaluated 21 MCC tumors for mutations in tumor suppressors and oncogenes commonly seen in skin cancers, founding only three mutations in *TP53* (14 % of samples). In contrast, the tumor suppressor protein p73, a structural homologue of p53, appeared mutated in 4 of 15 MCC samples (27 %), with unclear significance. These rates are relatively low in comparison with other cancer types. Although in certain cases missense mutations in *TP53* can prevent protein degradation and tumor suppressive function, the majority of MCC samples studied to date had wild-type *TP53*, and therefore, other mechanisms are likely at play.

Curiously, the same trend has been observed in melanoma, the other skin cancer that constitutes this thesis: mutations in *TP53* gene have been found in only 15 – 19 % of melanoma samples [Hodis, E. et al., 2012; The_Cancer_Genome_Atlas, 2015]. This fact suggests the possibility that MCCs and melanomas use some alternative way to overcome p53-mediated tumor suppression. Indeed, several alterations in genes affecting p53 activity have already been discovered in melanoma. For example, mutations in p14ARF [Hussussian, C. J. et al., 1994], overexpression of MDM2 [Polsky, D. et al., 2001], p63 [Matin, R. N. et al., 2013] and iASPP [Bergamaschi, D. et al., 2006] or amplifications affecting MDM4 [Gembarska, A. et al., 2012] evoke different mechanisms that could replace the lack of *TP53* mutations.

Some of these mechanisms could perhaps be acting in Merkel cell carcinomas. In this regard, the mouse double minute 2 homolog (MDM2) protein may be of therapeutic significance. This protein forms a complex with p53 in the cytoplasm, preventing the tumor suppressor from binding its responsive element and initiating anti-proliferative and DNA repair mechanisms. Due to this sequestration, p53 accumulates and remains nonfunctional

[Momand, J. et al., 1992]. This process has already been described in sarcoma, where it was shown that MDM2 was amplified in one third of 47 samples, and was specifically associated with detectable expression of p53 [Oliner, J. D. et al., 1992]. Houben et al. [Houben, R. et al., 2013], looking specifically at MCC, studied whether T antigens contributed to p53 stabilization, founding that viral knockdown did not lead to resumed p53 function. However, they found that inhibition of MDM2 by the compound Nutlin-3a did induce *TP53* transcriptional activation, resulting in tumor cell apoptosis in 5 out of 7 lines with wild-type *TP53*.

One putative mechanism by which Merkel cell polyomavirus could contribute to transformation of Merkel cells is, precisely, the interference with the p53 tumor suppressor pathway. The usual functions of p53 are not conducive to viral replication, as p53 transactivates genes that lead to cell cycle arrest, which could deprive the virus of essential replication factors. Additionally, active p53 could lead to cellular apoptosis in response to the presence of viral or cellular oncoproteins. The polyomaviruses have developed the ability to block p53 function through several mechanisms, ability which allows them to complete their normal infectious cycles. In this regard, it is known that the bipartite domain of the SV40 (a polyomavirus close to MCPyV (more information in section 1.3.2.)) LT antigen can bind directly to the specific DNA binding domain of p53, hence interfering with p53-dependent gene transcription [Jiang, D. et al., 1993; Segawa, K. et al., 1993].

As the MCPyV LT antigen has been sometimes found prematurely truncated in the MCC tumor cells, lacking the helicase domain and the supposed p53-binding sites [Shuda, M. et al., 2008], the significance of the p53 pathway in pathogenesis of MCPyV-associated MCC is unclear. However, even if the truncated T-antigen does not bind to p53, MCPyV may play a role in suppressing p53 function in MCC tumors via other mechanisms. For example, there is evidence that the binding of T-antigen to p53 in SV40 may not be sufficient to block p53 function and that other indirect mechanisms (involving small T-antigen and/or the J- and Rb-binding domains of the LT antigen) are also important in functional suppression of p53 (reviewed by [Pipas, J. M. et al., 2001] and by [Ahuja, D. et al., 2005]).

Consistent with the possibility that MCPyV could somehow disable p53 function in MCC tumors is the previously mentioned fact that inactivating mutations in *TP53* gene and/or changes in p53 protein levels have been detected in a small subset of MCC tumors. Moreover, several studies have indicated an inverse relationship between p53 expression and MCPyV viral abundance in MCC tumors, as well as between p53 overexpression and poor outcome [Bhatia, K. et al., 2010; Waltari, M. et al., 2011].

1.3.4.1.2. RB.

The Retinoblastoma susceptibility family of genes (*RB*) is composed of three members; RB1/p105, p107 and RB2/p130, collectively referred to as "pocket proteins". It encodes nuclear phosphoproteins which act as negative regulators of cellular proliferation. *RB* genes are tumor suppressors that were firstly identified in a malignant tumor of the retina, known as retinoblastoma. RB (or Rb) proteins are responsible for a major G1 checkpoint (restriction point) blocking S-phase entry and cell growth, and promoting terminal differentiation by inducing both cell cycle exit and tissue-specific gene expression [Weinberg, R. A., 1995].

The interaction of Merkel cell polyomavirus with retinoblastoma tumor suppressor proteins appears to be critical to the observed growth-promoting effects of LT antigen [Houben, R. et al., 2012]. Immunohistochemical (IHC) data from human MCC tumors show a strong positive association between tumor RB expression and MCPyV LT antigen expression, with most LT antigen-positive MCC tumors also expressing RB and most LT antigen-negative tumors being RB-negative as well [Bhatia, K. et al., 2010; Sihto, H. et al., 2011].

Similar to the well characterized interactions between SV40 LT antigen and the RB family of proteins (RB1, p107 and p130), the MCPyV LT antigen is likely to sequester hypophosphorylated RB that usually binds to E2F transcription factors. This sequestration of RB allows E2F-mediated transcription that leads to the entry of the cell into S-phase. The integrity of the DnaJ- and the LXCXE-motifs is required for this mechanism in SV40, and the retention of these domains (with intact RB-binding ability) in the truncated MCPyV LT-antigen is consistent with this mechanism being relevant to MCC pathogenesis. Merkel cell polyomavirus has a large T antigen LXCXE domain that, when expressed, binds directly retinoblastoma protein [Shuda, M. et al., 2008]. Several lines of evidence have suggested that in polyomavirus-positive cases, retinoblastoma dysregulation occurs secondarily to maintenance and expression of the large T antigen, and, specifically, the retinoblastoma protein-binding region of the large T antigen [Sastre-Garau, X. et al., 2009; Sihto, H. et al., 2011; Chang, Y. et al., 2012; Angermeyer, S. et al., 2013]. The large T antigen of integrated Merkel cell polyomavirus has been shown to have varying mutations among Merkel cell carcinoma cases, however, such mutations invariably spare the retinoblastoma protein-binding domain [Houben, R. et al., 2010].

Similarly to described data about p53 protein, several groups have reported a correlation between RB protein expression and Merkel cell polyomavirus copy number [Bhatia, K. et al., 2010; Harms, P. W. et al., 2013; Cimino, P. J. et al., 2014]. At the human genome level, a number of studies have provided evidences of loss of RB1 in subsets of Merkel cell carcinoma. In 1997, Leonard and Hayward [Leonard, J. H. et al., 1997] demonstrated loss of heterozygosity of 13q14, the chromosomal region of *RB1* locus, in 18/24 (75%) of Merkel cell carcinoma cases. Additionally, they used western blot analyses to show that cell lines

derived from 9 out of 18 patients had an absence of detectable retinoblastoma protein. Later, array comparative genomic hybridization studies by Van Gele et al [Van Gele, M. et al., 1998] and Paulson et al [Paulson, K. G. et al., 2009] involving the *RB1* locus showed the deletion of 13q in 8/24 (33%) MCC cases and the deletions 13q14 or 13q21 in 6/23 (26%) cases, respectively. More recently, it has been found that cases with no detectable polyomavirus by sensitive real-time polymerase chain reaction had truncating, nonsense *RB1* mutations [Cimino, P. J. et al., 2014]. Even though two of the five MCPyV-positive cases included in such work showed *RB1* genetic alterations (a deletion and a copy number variation, respectively), there were no single-nucleotide variation truncating nonsense mutations within polyomavirus-positive cases. This suggests a unique genetic mechanism to *RB1* inactivation occurring within polyomavirus-negative cases.

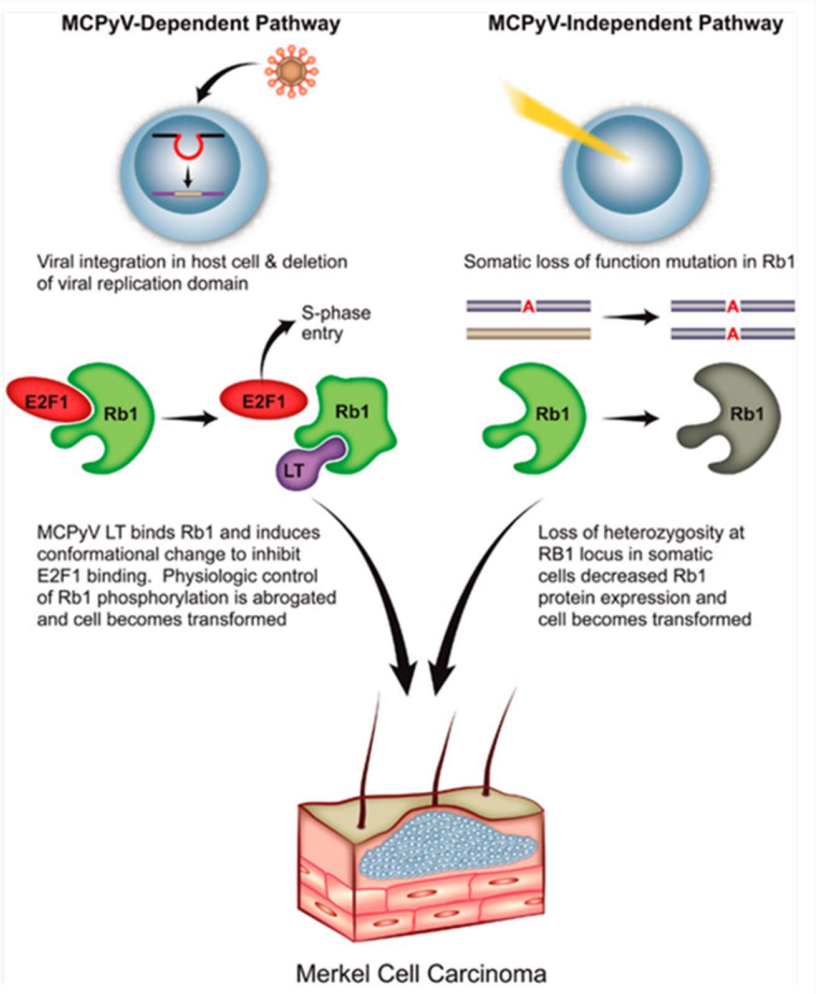

Figure 10. Proposed mechanism of Merkel cell carcinoma oncogenesis involving the retinoblastoma pathway (from [Cimino, P. J. et al., 2014]). In this model, the RB pathway is dysregulated in both polyomavirus-positive and polyomavirus-negative cases, leading to an indistinguishable morphological and clinical phenotype.

On the other hand, the presence of decreased retinoblastoma protein expression has been linked to specific genetic mutations that tend to occur in MCPyV-negative MCC cases, arguing thus that RB abrogation is required for Merkel cell carcinoma pathogenesis in the absence of polyomavirus [Cimino, P. J. et al., 2014]. These findings reinforce the proposed model of MCC oncogenesis consisting in two separate ways leading to the same pathology; a polyomavirus-dependent route, in which retinoblastoma protein is functionally inactivated, and a polyomavirus-independent path, in which *RB1* sustains somatic mutations. In both cases dysregulation of RB pathway would be required to produce an overlapping Merkel cell carcinoma phenotype (see figure 10). In any case, the retinoblastoma signaling appears to have an important role in Merkel cell carcinoma.

1.3.4.2. c-KIT.

Receptor Tyrosine Kinases (RTKs) are a family of cell-surface receptors which will be further explained in the section 1.3.8.5.1. of this thesis. One of these receptors is the v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog, commonly known as KIT, or c-KIT. It is activated by binding the cytokine stem cell factor (SCF). Signaling through KIT activate unless two signal-transduction pathways; MAPK and PI3K, that ultimately lead to cell proliferation.

KIT has been described as over-expressed in MCC [Su, L. D. et al., 2002; Sattler, M. et al., 2004; Strong, S. et al., 2004], as well as it occurs in other cancer types, where this receptor is a pharmacological target of Imatinib. c-KIT overexpression has sometimes been linked to poor prognosis in MCC [Andea, A. A. et al., 2010]. In addition, the Huntingtin Interacting Protein 1 (HIP1), which has the ability to physically interact with c-KIT receptor and stabilize it, has been reported to constitute a MCC Marker, which increases KIT levels in the cell surface, likely promoting tumorigenesis. The interaction between HIP1 and KIT would be modulated by the c-KIT ligand, SCF [Ames, H. M. et al., 2011].

Similarly to p53 expression, KIT expression is inversely correlated with the presence of the DNA of Merkel cell polyomavirus in the tumor and with unfavorable survival when its expression is stratified by presence of MCPyV. MCCs that do not express KIT contain higher number of copies of MCPyV DNA [Waltari, M. et al., 2011].

However, mutations in this gene are barely found in MCC [Brunner, M. et al., 2008; Kartha, R. V. et al., 2008], meaning that they are rare events in MCC. Furthermore, despite the described overexpression of this RTK in some MCC cases [Su, L. D. et al., 2002; Sattler, M. et al., 2004; Strong, S. et al., 2004], phosphorylated KIT and SCF are infrequently expressed in this cancer, signaling that KIT receptor is not usually activated in MCC tumors

[Waltari, M. et al., 2011]. These findings are supported by a recent multicenter trial, in which 23 patients with advanced KIT-positive MCC were treated with Imatinib mesylate (an inhibitor of KIT, PDGFRs and a few other kinases). Only one (4 %) of the patients (who had an unknown tumor mutational status) responded to Imatinib, and another patient (4 %) had a prolonged disease stabilization [Samlowski, W. E. et al., 2010].

1.3.4.3. PI3K – AKT – mTOR PATHWAY.

Alterations in PI3K pathway (which will be described in section 1.3.8.4. of this thesis) have been involved in multiple cancers types, including melanoma (section 1.3.8.4.), liver, colon, urothelial, gastric, and brain, among others [Karakas, B. et al., 2006; Lopez-Knowles, E. et al., 2006; Kantrow, S. M. et al., 2007; Dankort, D. et al., 2009]. PI3K serves as an intracellular tyrosine kinase that activates AKT downstream to stimulate cell cycle progression via mTOR, cellular proliferation via NF-κb, and inhibition of apoptosis via deactivation of tumor suppressors, including p53, p21, p27 and GSK3b (pathway represented in figure 14).

Nardi and colleges [Nardi, V. et al., 2012] sequenced selected mutational hotspots of 60 MCC tumors, founding 6 activating SSNVs (10 %) in *PIK3CA* (the alpha subunit of the kinase). 5 of these 6 observed *PIK3CA* mutations were within the helical domain of the p110a subunit, which is mutated in a wide variety of skin cancers [Hafner, C. et al., 2007]. These mutations were found in tumors from both primary and recurrent disease cases. All these tumors were MCPyV-negatives, except for one case. The authors were unable to correlate these findings to prognosis, likely due to limited power [Nardi, V. et al., 2012]. However, they tested multiple PIK3 and mTOR inhibitors and were able to inhibit phosphorylation and activation of AKT *ex vivo* and induce apoptosis. Although no mutations have been detected in the *AKT* gene, a subset of MCC samples displayed high AKT activity in the setting of wild-type PI3K, suggesting upstream activation either through an unknown oncogene or through a disinhibition of mutated tumor suppressor, such as PTEN [Nardi, V. et al., 2012].

Hafner et al. [Hafner, C. et al., 2012] also evaluated the PI3K pathway in MCC and found *PIK3CA* mutations in 2 of 46 (4 %) tumors, whereas none had mutations in *AKT*. Surprisingly, activating phosphorylation of AKT was found in a much higher proportion (88 % of tumors) and, furthermore, cells were sensitive to the PI3K inhibitor inhibitor LY-294002. AKT activation was uncorrelated with MCPyV status [Hafner, C. et al., 2012]. Based on these data, upstream or epigenetic aberrations could be, perhaps, driving the pathway given the lack of intrinsic mutations.

PTEN (phosphatase and tensin homologue) is a tumor suppressor implicated also in many cancer types (see section 1.3.8.4. of this thesis), which acts by inactivating AKT via dephosphorylation. Chromosomal analysis of 21 MCC samples showed hemizygous mutations in 9 (43 %) samples of the10q23 region of Ch10, where *PTEN* gene is located. However, homozygous deletions or point mutations of the remaining allele were quite rare, suggesting alternate mechanisms of PTEN inactivation or the involvement of other tumor suppressors in MCC [Van Gele, M. et al., 2001].

1.3.5. BIOMARKERS AND DIFFERENTIAL DIAGNOSIS OF MCC.

An interesting fact concerning the protein expression profile of Merkel cell carcinomas is that epithelial proteins, like cytokeratins, but also neuroendocrine markers, like neuron-specific enolase, can be detected in them. Furthermore, synaptophysin and chromogranin A can be immunohistochemically found in Merkel cells, as well [Halata, Z. et al., 2003].

In particular, cytokeratin 20 (CK20) has a significant value as a highly specific marker for Merkel cell carcinoma [Moll, R. et al., 1992]. The assessment of the expression of this protein allows distinguishing between MCC and other cancer types, like for example small cell lung carcinoma (SCLC), which shares many cellular and tissular characteristics with MCC. However, some studies showed that approximately 5 % of all Merkel cell carcinoma specimens lack CK20 expression [Paik, J. Y. et al., 2011]. Because of that, some "negative markers" must be used to assist the distinction between MCC and other cancer types. For example, the thyroid transcription factor-1 (TTF-1) and cytokeratin-7 (CK7) are diagnostic markers for small-cell lung carcinoma which are not expressed by Merkel cell carcinomas [Dancey, A. L. et al., 2006]. Similarly, the differentiation between MCC and lymphoma can be helped by the assessment of the leucocyte common antigen (LCA), which is usually positive in lymphoma [Becker, J. et al., 2008; Becker, J. C. et al., 2008] but not in MCC [Bobos, M. et al., 2006]. In the same way, segregating malignant melanoma and Merkel cell carcinoma can be done based on MCCs positivity for CK20, but negativity for HBM45, NKI/C3 and S-100, which are usually positive in melanomas [Kontochristopoulos, G. J. et al., 2000].

There are few more markers which may be useful diagnostically and/or prognostically in MCC. One of them is DEK, a chromatin architectural factor involved in pathogenesis of several cancers, which consistently presents diffuse expression in MCC [Patel, R. M. et al., 2012]. Likewise, the K homology domain-containing protein overexpressed in cancer (KOC), also known as IMP3, is expressed in the vast majority of MCCs (90%), and in most cases its expression is high and diffuse. IMP3 expression correlates with metastases [Pryor, J. G. et al., 2009].

In addition, several studies have been done with the aim of finding markers that could identify patients with poor prognosis at the time of diagnosis; these patients could benefit from increased or modified postsurgical therapy. In order to discover these biomarkers, Masterson and collages [Masterson, L. et al., 2014] examined differences in the global gene expression between patients that demonstrated good prognosis or poor prognosis at 24 months after resection. They found some genes upregulated in the poor prognosis tumors, among them KRT20, TPD52, MUC1 and KIT. By contrast, HOXB1 was downregulated in these samples. However, although studies like this have identified several biomarkers, their prognostic value needs to be completely determined yet.

1.3.6. CURRENT TREATMENT OPTIONS IN MERKEL CELL CARCINOMA.

Although several modalities of treatment have been proposed for MCC [Nathu, R. M. et al., 1998; Wong, K. C. et al., 1998; Allen, P. J. et al., 1999; Akhtar, S. et al., 2000; Coit, D. G., 2001; Goessling, W. et al., 2002], none have been shown to significantly improve survival of patients. The rarity of this neoplasm provokes a shortage of clinical trials, contributing to hindering any progress on this issue [Eng, T. Y. et al., 2007]. Searches of the Food and Drug Administration (FDA) website (http://www.fda.gov/) and of the Center Watch (http://www.centerwatch.com/) yield no approved agents for this cancer.

Treatment recommendations are based upon observational studies that consist primarily of single institution series gathered over many years. In most cases with a primary lesion, a wide local excision with negative margins is the initial approach. Many patients receive radiation following the wide surgical excision, although patients with positive lymph nodes or metastatic disease at time of diagnosis are candidates for chemotherapy. However, the optimal management of the regional lymph nodes at the initial presentation is less clear [Masterson, L. et al., 2014].

For metastatic or unresectable disease, cytotoxic chemotherapy is the dominant mode of treatment. MCC is generally considered a chemotherapy-sensitive tumor [Wynne, C. J. et al., 1988] because temporary regression is observed in the majority of cases treated with first-line chemotherapy. However, it is virtually never curative and it causes a significant toxicity; therapy-related death occurs in up to 16 % of older patients [Voog, E. et al., 1999]. No standard chemotherapy protocol has been yet established for the treatment of MCC. Because of its morphological and immunohistochemical similarity to small cell lung carcinoma (SCLC), chemotherapy regimens for MCC are mostly extrapolated from protocols used for SCLC [Miller, N. J. et al., 2013]. A wide variety of chemotherapeutic agents have been discussed, including cytostatic drugs such as cyclophosphamide, doxorubicin, epirubicin, vincristine, etoposide, cisplatin, carboplatin, 5-fluorouracil, dacarbazine, mitoxantrone,

bleomycin, and iphosphamide. However, reports to date consist of only small studies and anecdotal evidence [Clark, J. R. et al., 2007; Jabbour, J. et al., 2007; Desch, L. et al., 2013]. The role of chemotherapy as part of a combined modality approach remains uncertain.

From a different approach, a clinical trial identified as NCT00068783 (https://clinicaltrials.gov) used Imatinib for treating patients with metastatic or unresectable MCC, yielding unsuccessful results. Currently, a new clinical research study that uses Cabozantinib for the treatment of Merkel cell carcinoma, amongst other skin cancers, is ongoing (NCT02036476). This is an open-label, non-randomized, phase II study directed to assess the feasibility of using this drug in recurrent/metastatic Merkel cell carcinoma patients that progressed after a platinum-based therapy.

The main treatment options currently used in MCC are briefly detailed following:

1.3.6.1. SURGERY.

Excision is the standard approach to the initial management of primary tumors [Lebbe, C. et al., 2015]. In many cases, a skin biopsy is done to remove a suspicious spot even before the doctor suspects it might be MCC. This can be thought of as a type of surgery, but it is not an adequate treatment for MCC [American_Cancer_Society, 2016]. If MCC is diagnosed from the biopsy, a wide excision should be done to remove more skin and other tissues in the area. A margin of at least 1 cm of normal appearing skin is generally recommended. Because MCC often shows extensive vertical growth, and sometimes extends into muscle, deep margins are a potential site of failure. Mohs micrographic surgery has been advocated to improve local tumor control compared with standard wide excision. With this approach, 100 percent of all major borders, including the deep margins, are evaluated histologically. However, adjuvant radiation therapy still has a role in preventing loco-regional recurrence, even when Mohs surgery is used [O'Connor, W. J. et al., 1997; Boyer, J. D. et al., 2002].

Even in people who have MCC with no obvious spread of the cancer to nearby lymph nodes (or distant organs), about 1 out of 3 will have cancer cells in their lymph nodes when the nodes are looked at with a microscope [American_Cancer_Society, 2016]. Because of this, a sentinel lymph node biopsy is typically a very important part of determining the stage of the cancer. If the sentinel lymph node biopsy result is negative, no more lymph node surgery is needed because it is very unlikely the cancer would have spread beyond this point, although radiation therapy might still be given to the nearby lymph nodes just preventively. If cancer cells are found in the sentinel node, the remaining lymph nodes in this area are often also removed and looked at. Radiation therapy might be given to the area after the lymph node dissection.

The role of surgery as a definitive modality is challenged by the high propensity of this disease for loco-regional and systemic recurrence and by the high anesthetic risk in an elderly patient population, who frequently have multiple comorbidities. In addition, the location of the tumor can pose difficulties with obtaining wide surgical margins, as is the case with head and neck sites, where functional and cosmetic sequelae are important considerations, and in limb locations, where definitive surgery with adequate margins carries a significant risk of postoperative functional impairment and lymphedema. All these circumstances become the radiation therapy a habitual choice, either itself or as adjuvant therapy after surgery [Warner, R. E. et al., 2008].

1.3.6.2. RADIATION THERAPY.

Radiation therapy, also referred to as radiotherapy or x radiotherapy (XRT), consists on the use of penetrating beams of energy waves or streams of particles delivered to the cancer cells and a small margin of surrounding normal tissue, sometimes called the radiation field.

Radiotherapy may be given:

- Before surgery to try to shrink or slow the growth of the cancer and give doctors a greater chance of removing it all with surgery. This is called “neo-adjuvant radiotherapy”.
- After surgery to try to reduce the chance of eventual cancer cells staying in adjacent tissues to proliferate and start a new tumor. This is called “adjuvant radiotherapy”.
- To shrink or slow the growth of the cancer for patients with localised, inoperable cancer. In these cases, XRT can be the first line treatment.
- To help to relieve symptoms such as pain. This is called “palliative radiotherapy”.

Numerous studies have shown a marked improvement in control of Merkel cell carcinoma at the primary site and draining lymph node basin when radiation therapy is added after surgery [Lewis, K. G. et al., 2006; Lee, J. et al., 2012]. In these cases, XRT is used as adjuvant therapy, (intended to destroy any cancer cell that may remain after a previous treatment). Local adjuvant treatment to the primary tumor site and any positive lymph node regions has been associated with lower rates of locoregional recurrence (reviewed by [Lee, J. et al., 2012].

Thus, XRT is commonly used as adjuvant therapy in MCC, but, in addition, it provides an alternative for those patients in whom surgical resection is not technically feasible, for example, in cases with margins close or involved with tumor or who are medically unfit for surgery [Veness, M. et al., 2010; Harrington, C. et al., 2014]. With this approach, Harrington

et al. [Harrington, C. et al., 2014] reported a tumor control of 88 % at 12 months and of 82 % at 2 years. The 5-year local relapse-free survival (RFS) was 90 % and the overall survival, 39 %. However, systemic relapse occurs in most cases after radiation therapy [Lien, M. H. et al., 2010].

1.3.6.3. CHEMOTHERAPY.

Basically, as aforesaid, MCC is assumed to be a chemosensitive tumor [Steinstraesser, L. et al., 2011; Schrama, D. et al., 2012], but to date no broadly accepted treatment algorithm exists. Supportively to primary excision, chemotherapy is used on MCC stages III (lymph node metastasis) and IV (distant metastases) after the AJCC staging system. It may be applied either alone or in combination with radiotherapy (see next section). Furthermore, chemotherapeutics are also used in locally advanced disease as palliative measure or in case of recurrences [Becker, J. et al., 2008].

Owing to difficulties to manage studies large enough to show which chemotherapeutic drugs work best against MCC, doctors often use drugs that are useful against other types of tumors, especially those with neuroendocrine origin [American_Cancer_Society, 2016]. The most commonly used drugs for MCCs that have spread include Cisplatin, Carboplatin, Etoposide and Topotecan. Most often, either cisplatin or carboplatin is used, sometimes along with etoposide. Topotecan tends to have fewer serious side effects, so it might be a better option for some people who are older or have serious health problems [American_Cancer_Society, 2016]. These drugs can often shrink MCC tumors for a time (or at least, slow their growth and spread) and help relieving some symptoms. However, tumors usually start growing again. Indeed, for advanced Merkel-cell carcinoma, cytotoxic chemotherapy offers a median progression-free survival of only 3 months [Tai, P. T. et al., 2000].

Intralesional chemotherapy consists on injecting small amounts of a drug, such as Bleomycin, directly into the site of the tumor. For some early skin tumors, it has been tried a few times after surgery. One advantage of this approach is the low probability of having side effects, often seen with systemic chemotherapy. Intralesional chemotherapy has been reported to be effective in some patients, albeit it has not been studied enough to be sure. Per example, Ely H. and colleges [Ely, H. et al., 2008] treated four cases of stage-I Merkel cell carcinoma with surgery followed by intralesional Bleomycin and followed these cases for up to five years with no evidence of recurrence or metastasis. Intralesional Bleomycin caused complete regression of one tumor with minimal scarring and long term cure. It so happens that Bleomycin, besides being a potent chemotherapy agent, has direct antiviral effects [Takeshita,

M. et al., 1977; Georgiou, N. A. et al., 2006], fact which may be related with the effectiveness of this drug in treating Merkel cell carcinoma.

1.3.6.4. CHEMORADIOTHERAPY.

Chemoradiotherapy (CRT or CRTx) consist of using some drugs, like Fluorouracil (5FU) or Capecitabine, which have the property of making cancer cells more sensitive to the radiation. Thus, this therapy has the theoretical advantage of radiosensitization and, therefore, might be more effective to enhance loco-regional control and eradicate micrometastases. On the other hand, lower doses of radiation could lead to the same outcome, diminishing thereby side effects.

However, chemoradiotherapy remains experimental in Merkel cell carcinoma [Hruby, G. et al., 2013] and more definitive information is needed to define the optimal approach.

1.3.6.5. IMMUNOTHERAPY.

The bases of the use of the immune system to control cancer will be broader explained in the section 1.3.9.2., which correspond to melanoma, as this cancer was a pioneering in the development of immunotherapeutic strategies. Likewise, the association of mutational burden with the response to immunotherapy will be also explained in such section, and it was already addressed in the section 1.2.1.4.2.

Theoretically, a higher number of total mutations in a tumor cell is expected to produce a higher number of neoepitopes presented by such cell, becoming it more recognizable by the immune system (section 1.2.1.4.2.). Therefore, under these conditions, intra-tumor infiltrating T cells −either stimulated or not by a treatment− would be more effective in controlling tumor growing.

Epidemiologic data suggest a strong link between Merkel cell carcinoma and the immune system [Calzavara-Pinton, P. et al., 2010; Sihto, H. et al., 2012; Ma, J. E. et al., 2014; Nghiem, P., 2015]. The increasing recognition of the importance of the immune system in MCC pathogenesis is leading to think that rational immunotherapeutic approaches can possibly improve outcomes for this aggressive disease.

Until recently, the use of different kinds of immunotherapies, like the immune checkpoint blockade, was being proposed for MCC mainly based on the concept that virus-associated cancers are intrinsically immunogenic because they express foreign (viral) antigens, which should be recognized by the host lymphocytes [Paulson, K. G. et al., 2010;

Lyngaa, R. et al., 2014]. This circumstance makes MCPyV-positive MCCs good candidates for immunotherapy. However, recent publications (including our work) have demonstrated a much higher incidence of somatic mutations in MCPyV-negative cases [Harms, P. W. et al., 2015; Goh, G. et al., 2016], fact which, as aforesaid, is usually associated with a higher number of neoantigens. Therefore, the use of immunotherapies would be supported not only in MCPyV-positive cases, but also in those MCPyV-negatives.

A possible therapeutic option for MCPyV-positive MCCs could lie in the fact that T-antigen specific antibody response is confined to a 78 amino acid N-terminus domain shared by the small- and large- T-antigens [Paulson, K. G. et al., 2010], which could provide a suitable vaccine or adoptive T-cell therapy target. Similarly, other non-viral tumor associated-antigens, such as Surviving [Kim, J. et al., 2008] or the oncoprotein HIP1, which interacts with c-KIT [Ames, H. M. et al., 2011], may also be suitable immunotherapeutic targets. Immunostimulatory cytokines, such as interferons or interleukins (IL-2, IL-12, IL-15 and IL-21), could be delivered, systemically or intratumorally, to counteract immune evasion mechanisms employed by MCC tumors.

Other therapeutic agents that look appealing to investigate for MCC treatment include CTLA-4 receptor blocking agents such as Ipilimumab (recently approved by FDA for metastatic melanoma), drugs targeting the PD-1/PDL-1 pathway to reverse immune exhaustion of infiltrating lymphocytes or drugs targeting the costimulatory 4-1BB pathways that could promote T cell infiltration, proliferation and cytokine production [Curran, M. A. et al., 2011; Palazon, A. et al., 2011]. Several studies have shown that approximately 50 % of Merkel cell carcinomas express PD-1 on tumor-infiltrating lymphocytes and express PD-L1 on tumor cells or infiltrating macrophages in an “adaptive resistance” pattern (with expression concentrated at the leading edges of the tumor) [Afanasiev, O. K. et al., 2013; Dowlatshahi, M. et al., 2013; Lipson, E. J. et al., 2013], fact which suggests an endogenous tumor-reactive immune response that might be unleashed by anti–PD-1 or anti–PD-L1 drugs [Afanasiev, O. K. et al., 2013]. In this regard, a phase II clinical trial has recently been undertaken with the main objective of assessing the efficacy of Pembrolizumab, an anti–PD-1 agent, in patients with advanced Merkel-cell carcinoma who had not previously received systemic therapy [Nghiem, P. T. et al., 2016]. In this study, 14 of 25 patients with at least one tumor assessment during treatment had a confirmed response (4 with a complete response and 10 with a partial response), representing an objective response rate of 56 %. Tumor regressions occurred in multiple organ sites and in patients with bulky disease. Regressions appeared to be durable within an observation period of up to 9.7 months after initial documentation of a response. Furthermore, responses were observed in both MCPyV-positive and MCPyV-negative Merkel-cell carcinomas. Surprisingly, neither PD-L1 expression on tumor cells nor its expression on infiltrating immune cells correlated in this work with clinical response to anti-PD-1. Furthermore, the observation that both MCPyV-positive and -negative cases

experienced similar clinical benefits to treatment with Pembrolizumab, argues against the role that MCPyV viral antigens or neo-epitopes caused by high mutational burdens could be playing in patient response to immunotherapy.

Thus, Merkel cell carcinoma appears to be a good candidate for immunotherapy but, like other tumors, including advanced melanoma or lung cancer, it still lacks specific biomarkers to predict patient response.

1.3.7. DEVELOPMENT, STAGING AND INCIDENCE OF CUTANEOUS MELANOMA.

Melanoma is a malignant neoplasm which arises from the transformation of a type of pigment-producing cells, called melanocytes [Patterson, M. S. R. C., 2006]. This cell type develops from the pluripotent neural crest and their immature form, called melanoblast, migrates along characteristic pathways to various destinations, such as dermis and epidermis, inner ear, choroids of the eye, hair, meninges, and ectodermal mucosa [Hall, B. K., 1999; Kalcheim, N. L. D. a. C., 2009].

Cutaneous melanoma occurs when the division of melanocytes located in the basal layer of the epidermis (see figure 11) escapes control mechanisms, so they proliferate unchecked, with the possibility of invading the underlying connective tissue compartment and, finally, metastasize to numerous distant locations throughout the body [Patterson, M. S. R. C., 2006].

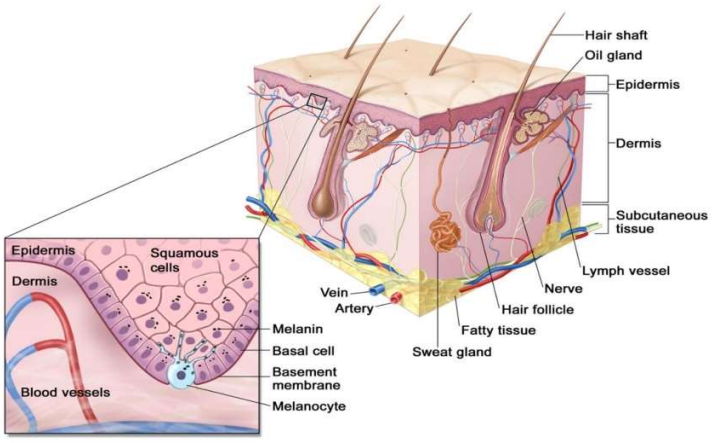

Figure 11. Schematic representation of healthy skin (from USA National Cancer Institute, NIH). Melanocytes are the source cell for melanoma. Cutaneous melanocytes are located near the area of separation between epidermis and dermis, which occurs at the basement membrane zone, located just inferior to the basal cell keratinocytes.

Only a small subset (about 5 %) of skin cancers is melanoma, but this kind of neoplasm is the deadliest amongst those affecting the skin. It is an increasingly common malignancy which appears in a younger population than most cancers [Evans, M. S. et al., 2013]. For this reason, it is the second cancer type in the ranking of causes for loss of potential years of life [Tran, T. T. et al., 2008].

As aforementioned, exposure to solar ultraviolet radiation is the major risk factor for melanoma in fair-skinned populations [Mc, G. V., 1952; Balch, C. M. et al., 2001]. Several studies suggest an increased melanoma risk related to short periods of intensive sun exposure in early adult life. In contrast, regular outdoor occupation seems to confer a decreased risk and a longer survival [Berwick, M. et al., 2005]. These apparent beneficial effects of habitual sun exposure on survival might be mediated through improvement in vitamin D status [Mocellin, S. et al., 2008].

Related with the effects of UV radiation on the skin is probably the wide range of point-mutation rates observed in melanoma. Indeed, as shown in figures 4 and 5, melanoma is the type of cancer with the highest number of mutations and, therefore, the most heterogeneous one, genetically speaking. Melanomas derived from primary lesions arising on non-UV-exposed hairless skin of the extremities have the lowest mutational burden (ranging from 3 to 14 mutations / Mb). Intermediate rates are found in those melanomas originating from hair-bearing skin of the trunk (from 5 to 55 mutations / Mb). The highest burdens are found in patients with a documented history of chronic sun exposure (up to 111 mutations / Mb) [Berger, M. F. et al., 2012]. Interestingly, melanomas that are wild-type for both *BRAF* and *NRAS* jointly have almost five times higher mutational loads than tumors with just one of these oncogenes mutated [Mar, V. J. et al., 2013; The_Cancer_Genome_Atlas, 2015].

Staging and survival.

As explained above, the stage of a cancer is a description of how widespread it is, having a great importance for planning a treatment and estimating a prognosis. The American Joint Committee on Cancer (AJCC) stablished a staging system for cutaneous melanoma on the basis of three main independent prognostic factors; thickness of the lesion, ulceration status and mitotic rate [Balch, C. M. et al., 2009]. Survival rates depend on the melanoma stage, with a 10-year survival varying from 95 % (Stage 1A) to 10 % (Stage IV) [Balch, C. M. et al., 2009]. Melanoma stages, as well as their associated survival rates are represented in Table 2.

The **Breslow** index (or Breslow thickness) indicates the microscopic measurement of the distance (in millimeters) between the upper layer of the epidermis and the deepest point of

tumor spread. It is one of the best predictors of the survival for melanoma, together with the presence of ulceration and the mitotic rate [Azzola, M. F. et al., 2003; Balch, C. M. et al., 2009; Thompson, J. F. et al., 2011].

The level of invasion, or **Clark** level, which indicates the anatomic plane of invasion, is a significant predictor in patients with thin melanomas (1 mm or less) [Balch, C. M. et al., 2009]. Five anatomical levels are distinguished: Level 1, melanoma confined to the epidermis (melanoma in situ); level 2, invasion into the papillary dermis; level 3: invasion to the junction of the papillary and reticular dermis; level 4: invasion into the reticular dermis; level 5: invasion into the subcutaneous fat. A higher level has worse prognostic implications.

STAGE	SPREAD	5-YEAR SURVIVAL RATE	10-YEAR SURVIVAL RATE
IA	Localized Melanoma	97 %	95 %
IB		92 %	86 %
IIA		81 %	67 %
IIB		70 %	57 %
IIC		53 %	40 %
IIIA	Regional Metastatic Melanoma	78 %	68 %
IIIB		59 %	43 %
IIIC		40 %	24 %
IV	Distant Metastatic Melanoma	15 %	10 %

Table 2. Melanoma staging and associated survival rates (based on the 2008 American Joint Committee on Cancer database). Survival rates are calculated based upon outcomes of nearly 60 000 patients under proper treatment [Balch, C. M. et al., 2009]. Stages I and II correspond to localized melanomas, whereas stages III and IV correspond to a regional or distant metastatic melanoma, respectively.

Aside from stage, other factors also affect survival, as following indicated:

- Older people usually have shorter survival times, regardless of stage. The sharpest drop in survival begins at the age of about 70 [Balch, C. M. et al., 2013].
- Melanoma is uncommon amongst black people, but when it does occur, survival times tend to be shorter than in whites [Kabigting, F. D. et al., 2009; Lodder, J. V. et al., 2010].
- Some studies have found that melanoma tends to be more serious if it arises on the sole of the foot or palm of the hand, as well as in a nail bed [Bristow, I. R. et al., 2010]. Cancers in these areas represent a larger portion of melanomas in blacks than in whites.

- Immunodepressed population, such as people with transplanted organs or infected by HIV, also have a greater risk of dying of their melanoma [Rodrigues, L. K. et al., 2002; Hoffmann, C. et al., 2005].

1.3.8. MAIN DISEASE MECHANISMS OF CUTANEOUS MELANOMA.

Multiple cellular pathways involved in signal transduction, development, transcription and cell cycle regulation have been demonstrated to participate in melanomagenesis [Shtivelman, E. et al., 2014]. Likewise, a huge number of genes have been found mutated in this pathology, with a higher or lower frequency [Shtivelman, E. et al., 2014; The_Cancer_Genome_Atlas, 2015]. The high rate of mutations in melanoma makes it particularly difficult to distinguish between causative ("driver") mutations and bystander ("passenger") mutations. The most persistently implicated genes, as far as it is known, seems to be *BRAF*, *NRAS*, *KIT* and *NF1*, but the search for driver mutations in melanoma continues [Shtivelman, E. et al., 2014].

Next, some of the main disease mechanisms are described, as well as their genetic alterations and their possible role in melanomagenesis.

1.3.8.1. RAS GTPases.

RAS proteins are a superfamily of membrane-bound small (20-25 kDa) G proteins that act as molecular switches, being essential components of signaling networks that regulate cell proliferation, differentiation or survival. RAS GTPases starts a substantial number of signaling pathways, amongst which are MAPK (RAF-MEK-ERK1/2), PI3K (PI3K-AKT-mTOR) and PLC (PLC-DAG-PKC) [Rajalingam, K. et al., 2007], as represented in figure 12. Receptor tyrosine kinases (RTKs) and G-protein-coupled receptors (GPCRs) can activate RAS and, hence, trigger all these signaling pathways.

Somatic gain-of-function (GoF) mutations in *RAS* genes were the first specific genetic alterations identified in human cancer, about three decades ago [Cooper, G. M., 1982; Chang, E. H. et al., 1982; Santos, E. et al., 1982]. The oncogenic mutations of *HRAS, NRAS* or *KRAS* genes, frequently found in human tumors, are known to throw off balance the normal outcome of different signaling pathways, leading to tumor appearance [Stites, E. C. et al., 2009].

Mutations in *RAS* oncogenes have been described in about 31 % of cutaneous melanomas, most of them (28 %) affecting NRAS isoform and much fewer, affecting the

other three isoforms (HRAS, KRAS4A and KRAS4B) [The_Cancer_Genome_Atlas, 2015]. By contrast, other cancer types harbor KRAS mutations far more frequently than mutations in other isoforms. Each isoform seems to display preferential coupling to particular cancer types [Quinlan, M. P. et al., 2009]. Most NRAS mutations are located at the hotspot position Q61 of the protein [The_Cancer_Genome_Atlas, 2015]. Mutated RAS proteins have been found with higher frequency in primary tumors from skins continuously exposed to UV radiation in comparison with those from intermittently or non-sun exposed sites. They have been proposed as a feature of tumor progression [Ball, N. J. et al., 1994]. Mutations in *NRAS* seem to be involved in the development of melanomas deriving from giant congenital nevi [Shakhova, O. et al., 2012].

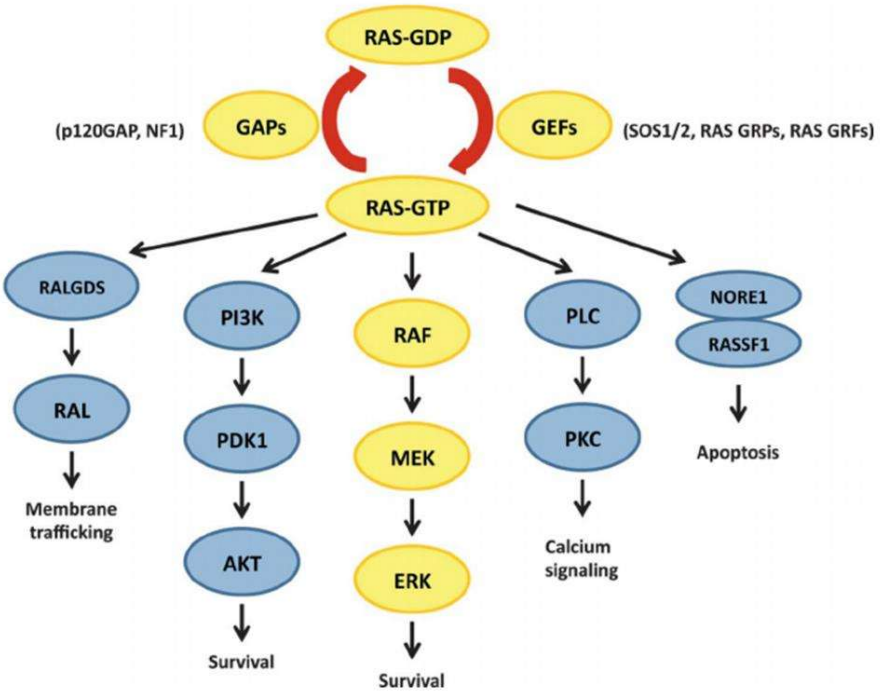

Figure 12. Effectors of RAS (adapted from [Lourenco, S. V. et al., 2014]). GTP–RAS binds to numerous effectors to trigger various signaling cascades, which in turn modulate different cell processes, such as growth, survival, migration, differentiation and death.

1.3.8.2. NF1.

Neurofibromin 1 (NF1) is a tumor suppressor protein that acts as a negative regulator of RAS, so its mutations can deregulate PI3K, MAPK and PLC pathways, among others. Germline mutations in this gene result in familial neurofibromatosis, and some patients with neurofibromatosis with inactivation of NF1 develop melanomas.

About 14 % of cutaneous melanoma samples harbor *NF1* mutations, most of which are predicted to be loss-of-function (LoF) events, including nonsense, splice-site and frame-shift

mutations. LoF mutations in *NF1* can be viewed as an alternative way to activate the canonical MAPK signaling pathway. Indeed, mutations in *NF1* are more frequently found in melanomas that have wild-type *BRAF* and *NRAS*, while they are anti-correlated with hotspot mutations in *BRAF* [Hodis, E. et al., 2012; The_Cancer_Genome_Atlas, 2015], hinting at *NF1* mutations as possibly being acting as driver mutations in a subset of cases.

An interesting detail is that *NF1* mutations are present in samples with very high mutational rates (39 mutations / Mb, on average) and with an age of melanoma accession higher than the mean [The_Cancer_Genome_Atlas, 2015].

1.3.8.3. MAPK – ERK1/2 PATHWAY.

Mitogen-activated protein kinase (MAPK) pathway is composed of three families of cytosolic serine/threonine kinases; RAF, MEK, and ERK, which form a tiered protein kinase cascade downstream of RAS.

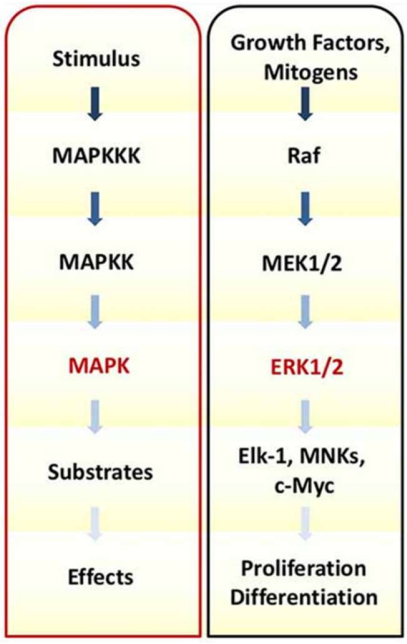

Figure 13. Schematic representation of MAPK-ERK1/2 signaling pathway (adapted from [Dinh, C. T. et al., 2015]) There are three tiers of protein kinases that comprise each family of MAPKs: (1) MAPK; (2) MAPK kinase (MAPKK); and (3) MAPKK kinase (MAPKKK). Following a specific stimulus, MAPKKK phosphorylates and activates MAPKK, which then phosphorylates and activates MAPK to then phosphorylate target substrates that can regulate cellular proliferation, survival, inflammation, and cell death. One of the MAPK clases include ERK1 and ERK2, which are phosphorylated by MEK1 and MEK2, which, in turn, are phosporylated by RAF.

This highly conserved cascade of protein kinases operates in sequential fashion (represented in figure 13), as follows:

1. Activated RAS binds to the N-terminal domain of RAF (MAPKKK or MAP3K), inducing a conformational change that enables its dimerization. Dimerization of RAF then leads to its phosphorylation and consequent activation [Wellbrock, C. et al., 2004].
2. Activated RAF binds to MEK1 and MEK2 (MAPKK or MAP2K), promoting its phosphorylation and activation.
3. Activated MEK1/2 phosphorylates and activates ERK1 and ERK2 (or MAPKs). Upon phosphorylation, ERK1 and 2 form homodimers that are able to phosphorylate a variety of substrates, distributed over different subcellular compartments, including the nucleus and the cytoplasm. Many of these substrates are transcriptional factors, such as c-MYC that modulate the expression of genes involved in cell growth, proliferation, differentiation, migration, and apoptosis [Khokhlatchev, A. V. et al., 1998; Raman, M. et al., 2007].

RAF.

There are three mammalian *RAF* genes (*ARAF, BRAF* and *RAF1*) that encode three RAF proteins (ARAF, BRAF, and CRAF, respectively). These three proteins perform distinct functions in the cell. BRAF is a more powerful activator of MEK compared with ARAF and CRAF [Mercer, K. E. et al., 2003; Tran, N. H. et al., 2005; Fischer, A. et al., 2007].

Approximately 50 % of melanomas of all clinical types harbor mutations in *BRAF* gene [Davies, H. et al., 2002; The_Cancer_Genome_Atlas, 2015]. Mutations in this gene are more frequently found in cutaneous melanomas developed in intermittently-sun-exposed skin. A noteworthy circumstance is that *NRAS* and *BRAF* mutations are almost always mutually exclusive [The_Cancer_Genome_Atlas, 2015]. It has been noticed that patients with *BRAF* mutations tend to be younger at the time of diagnosis than those wild-type *BRAF* [Pracht, M. et al., 2015; The_Cancer_Genome_Atlas, 2015]. In contrast to *BRAF*, mutations in *ARAF* and *CRAF* are very rare in cancer and, so far, they are not described in melanoma [Emuss, V. et al., 2005; The_Cancer_Genome_Atlas, 2015].

Mutations in *BRAF* are often located at position 1799 of the gene, affecting the protein position V600. The most frequent substitution at this position is T → A (T1799A transversion), which results in replacement of the amino acid valine (V) for the amino-acid glutamic (E). This mutation (V600E) represents 70 % – 80 % of all *BRAF* mutations in all cancers and encodes a constitutively active oncoprotein. Other mutations at this position cause alternate amino-acid substitutions (V600K, V600D or V600R) and are accounted in 5 % – 15 % of all *BRAF* mutations [Shtivelman, E. et al., 2014]. These V600 mutations are activating

as well, so confer to this kinase the ability to activate MEK (the only known substrate of BRAF) in an independent manner of RAS activity.

Aside these mutations, a number of BRAF proteins mutated in positions distinct to V600 have been identified in human cancers [Davies, H. et al., 2002], albeit some of them have a relatively low kinase activity in comparison with BRAF V600E [Wan, P. T. et al., 2004].

The overactivation of BRAF causes deregulation of cell proliferation by overcoming the G1 restriction point and causing cyclin D1 production in mid-G1 [Bhatt, K. V. et al., 2005]. $BRAF^{V600E}$ mutation predicts a high dependency of the melanoma on the MAPK signaling cascade [Wan, P. T. et al., 2004; Hoeflich, K. P. et al., 2009], an observation that has motivated the treatment of these melanoma cases with RAF and MEK inhibitors, as will be detailed below (section 1.3.9.1.1.).

Notably, acquisition of activating *BRAF* mutations seems to be an early event in melanomagenesis, but these mutations are also found with high frequency in benign nevi. However, few nevi develop into melanoma, fact which supports the deduction that mutations in the *BRAF* gene by themselves do not prompt melanoma [Pollock, P. M. et al., 2003]. Furthermore, the expression of the BRAF mutated protein in preclinical models has been associated with a phenomenon known as "**oncogene-induced senescence (OIS)**" [Michaloglou, C. et al., 2005], which results in a cell-cycle arrest. The pathways which mediate OIS are complex and incompletely elucidated, but the proliferative arrest involves activation of both the RB and p53 pathways. OIS brings about cessation of growth of some benign tumors, including melanocytic nevi and several other lesions, such as pituitary and thyroid adenomas. It protects against progression to cancer and, in this way, it complements oncogene-induced apoptosis [Chandeck, C. et al., 2010]. Consequently, and as it will be further discussed in next section, some additional genetic events in BRAF-mutant cells must be necessary to finally produce a cancerous phenotype [Flaherty, K. T. et al., 2012].

MEK.

Seven different genes encode the family of MEK proteins. Two of these genes (*MAP2K1* and *MAP2K2*) encode MEK1 and MEK2 (respectively) isoforms, which are the upstream activators of the extracellular signal-regulated kinases 1 and 2 (ERK1 and 2).

Although MEK mutations are rare in human cancer, melanoma included, GoF recurring somatic mutations in *MAP2K1* and *MAP2K2* have been found in 8 % of cutaneous melanoma lesions [Nikolaev, S. I. et al., 2012]. These somatic MEK mutations did not seem to correlate with *BRAF* or *NRAS* mutational status, albeit analysis of a set of melanoma metastases identified constitutively active mutated MEK protein in samples harboring *BRAF* mutations

other than V600E [Nikolaev, S. I. et al., 2012]. Activating MEK mutations result in constitutive ERK phosphorylation, with ensuing effects in cellular proliferation, apoptosis, differentiation and migration.

As it will be explained below (section 1.3.9.1.4.), mutational activation of MEK is one of the mechanisms involved in the appearance of resistances to therapies with BRAF inhibitors.

1.3.8.4. PI3K – AKT – mTOR PATHWAY.

The PI3K-AKT-mTOR cascade is triggered by receptor tyrosine kinases (RTKs) and G-protein-coupled receptors (GPCRs), situated at the cell surface.

Phosphoinositide 3 kinases (PI3Ks) are dimeric enzymes, consisting of a catalytic and a regulatory subunit. These kinases can be separated into three different classes (class I, class II and class III). When the RTK o the GPCR bind their extracellular ligand, they sequester the regulatory subunit of PI3K, allowing the catalytic subunit to catalyze phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP2) at the 3 position of the inositol ring (see figure 14). The product, Phosphatidylinositol 3,4,5-trisphosphate (PIP3), acts as a second messenger and controls a number of biological processes, like cellular growth, survival, proliferation, motility and morphology [Katso, R. et al., 2001].

PIP3 leads to phosphorylation and activation of Protein Kinase B (PKB, also known as AKT), positioned at the plasma membrane, both directly and through the 3-Phosphoinositide-Dependent Protein Kinase 1 (PDK1) (figure 14). Active AKT drives cell survival, proliferation and cellular metabolism through inhibitory phosphorylation of downstream proteins, including glycogen synthase kinase 3 (GSK3), forkhead box O (FOXO), peroxisome proliferator-activated receptor-γ (PPARγ), co-activator 1α (PGC1) and p27, and through activatory phosphorylation of ectonucleoside triphosphate diphosphohydrolase 5 (ENTPD5), sterol-responsive element-binding protein 1C (SREBP1C), AS160 and S phase kinase-associated protein 2 (SKP2).

AKT can also activate the mammalian target of Rapamycin (mTOR) complex 1 (mTORC1), composed of mTOR, DEPTOR, mLST8, PRAS40 and RAPTOR (figure 14), by mediating the inhibitory phosphorylation of its negative regulators TSC2 and PRAS40. Reciprocally, mTOR complex 2 (mTORC2), composed of mTOR, DEPTOR, mLST8, mSIN1, PROTOR and RICTOR (figure 14), promotes AKT activation [Song, M. S. et al., 2012].

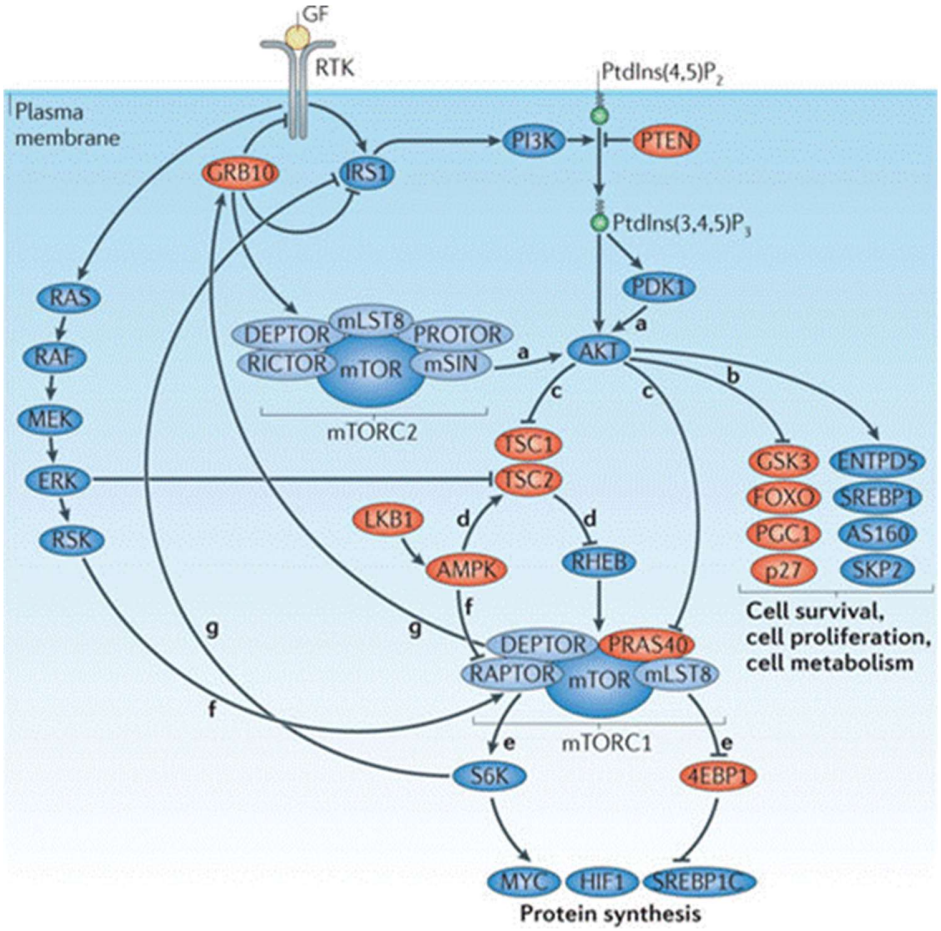

Figure 14. The PTEN–PI3K–AKT–mTOR pathway (from [Song, M. S. et al., 2012]). PTEN opposes PI3K function, leading to inactivation of AKT and mTOR signalling. Following PTEN loss, PIP3 accumulation recruits AKT and PDK1. Once positioned at the membrane, AKT is activated by PDK1-mediated phosphorylation at Thr308 and by mTOR complex 2 (mTORC2) phosphorylation at Ser473 (a). Active AKT drives cell survival, proliferation and cellular metabolism through inhibitory phosphorylation of downstream proteins, including GSK3, FOXO, PGC1 and p27, and through activatory phosphorylation of ENTPD5, SREBP1C, AS160 and SKP2 (b). AKT can also activate mTOR complex 2 (mTORC1) by mediating the inhibitory phosphorylation of its negative regulators TSC2 and PRAS40 (c). TSC2 is also phosphorylated by ERK, which inhibits the ability of TSC2 to function as a GTPase-activating protein (GAP) towards RHEB, whereas AMPK-mediated phosphorylation positively regulates the GAP activity of TSC2 (d). mTORC1 phosphorylates S6K and 4EBP1 to activate protein translation and cell survival (e). RSK-mediated phosphorylation of RAPTOR contributes the activation of mTORC1 signalling, whereas AMPK-mediated phosphorylation of RAPTOR results in the inhibition of mTORC1 signalling (f). Genetic inactivation or pharmacological inhibition of mTORC1 can activate AKT by preventing a negative feedback loop mediated by the mTORC1–S6K-induced phosphorylation of IRS) and GRB10 (g). Blue- and red-coloured molecules represent activators and repressors of the signalling pathway, respectively. GF, growth factor; HIF1, hypoxia-inducible factor 1; LKB1, liver kinase B1; MEK, mitogen-activated protein kinase kinase; RTK, receptor Tyr kinase.

mTOR is a serine/threonine kinase which signals to downstream effectors, either through direct phosphorylation or via the inhibition of the phosphatase PP2A. mTORC1 phosphorylates the p70 ribosomal protein S6 Kinase (S6K) and the eukaryotic translation-

initiation factor 4E (eIF4E)-binding protein 1 (4EBP1) [Song, M. S. et al., 2012]. Altogether, these effects of mTOR increase protein synthesis. In addition, mTOR reduces the degradation of proteins, by inhibiting their autophagy [Klionsky, D. J. et al., 2000]. Besides its preponderant role in the control of protein abundance, mTOR seems to have a pleiotropic function in the regulation of cellular death. This function appears to be dictated by the cellular context (cell type and activation state) as well as by multiple downstream targets, including well known apoptosis-regulatory proteins, such as p53, BAD and BCL2. Intriguingly, mTOR can act on a range of additional proteins with potential apoptosis-modulatory functions, like protein kinase C (PKC), retinoblastoma (RB), STAT3 and c-MYC [Castedo, M. et al., 2002].

On the other hand, the PI3K-AKT-MTOR pathway is antagonized by various factors, including PTEN, GSK3B, and HB9 (see figure 14).

The phosphatase and tensin homolog deleted on chromosome TEN (PTEN) is both a protein phosphatase and a lipid phosphatase. One of their multiple functions is removing the phosphate in the D3 position of PIP3. This major function antagonizes that of the PI3K and makes PTEN the major brake of PI3K pathway. However, the repertoire of PTEN functions does not end here, but include also phosphatase-independent activities within the nucleus [Song, M. S. et al., 2012].

The activation of the PI3K pathway serves to overcome the above mentioned oncogene-induced senescence (OIS,) associated with *RAF* and *RAS* mutants [Shtivelman, E. et al., 2014]. Consistently with this, *PTEN* aberrations have been often found together with the presence of $BRAF^{V600E}$, and seem to cooperate with mutant BRAF protein to induce malignant melanoma, most likely by overcoming such OIS [Dankort, D. et al., 2009]. In support of this notion, whereas *BRAF* mutations are present in both nevi and melanoma sections of contiguous nevi-melanoma biopsies, activation of PI3K pathway (either by loss of PTEN function or by activation of AKT) was detected only in the melanoma portions [Vredeveld, L. C. et al., 2012]. Findings like this hint at the activation of PI3K pathway during progression to malignant melanoma, most likely as a means of overcoming OIS, which can be an important aspect to keep in mind during the selection of a targeted therapy.

Increased activity of the PI3K-AKT-mTOR pathway occurs in approximately 70 % of sporadic melanomas [Kantrow, S. M. et al., 2007]. Several alterations in different components of this pathway have been described.

AKT.

Protein Kinase B (PKB), also known as AKT, belongs to the AGC family of serine/threonine kinases. Three mammalian isoforms this protein are kown; PKBα, PKBβ and

PKBγ (AKT1, AKT2 and AKT3, respectively), which are products of three distinct genes (*AKT1*, *AKT2* and *AKT3*) [Hanada, M. et al., 2004].

Alterations of AKT1 and AKT2 are rare in melanoma, but genetic amplifications of AKT3 have been seen in 25 % of cutaneous melanoma tumors. Moreover, deregulated AKT3 activity has been shown to promote the development of malignant melanoma [Stahl, J. M. et al., 2004]. A screen of 137 melanomas and 65 cell lines identified an activating mutation (E17A) in *AKT1* (in one patient) and *AKT3* (in one patient and two cell lines), all with concurrent *BRAF* mutations [Davies, M. A. et al., 2008].

As mentioned above, augmented activity of AKT3 has been reported to promote the progression of nevi harboring $BRAF^{V600E}$ mutation to melanoma [Cheung, M. et al., 2008; Vredeveld, L. C. et al., 2012]. $BRAF^{V600E}$ and AKT3 proteins activity have been demonstrated to cooperate in melanoma development. AKT3 phosphorylates mutant $BRAF^{V600E}$, reducing its (so MAP kinase pathway) activity to those levels that promote (rather than retard) melanocytic cell growth and transformation [Cheung, M. et al., 2008]. $BRAF^{V600E}$ mutation seems to initially promote the occurrence of nevi but, however, the resulting intense activation of the MAP kinase pathway inhibits their progression into cancer. AKT3 activation becomes then necessary to bypass this barrier and promote melanoma development.

PTEN.

The phosphatase and tensin homolog deleted on chromosome TEN (PTEN) was originally discovered as a candidate tumor suppressor mutated and lost in various cancers, such a prostate and brain [Li, J. et al., 1997]. It acts as a dual-specific protein phosphatase, dephosphorylating tyrosine-, serine- and threonine-phosphorylated proteins. As well, PTEN acts as a lipid phosphatase, which remove the phosphate in the D3 position of the inositol ring from, among others phosphoinositides, PIP3.

PTEN is altered in approximately 20 % of melanomas, most commonly via allelic loss and focal deletions [Tsao, H. et al., 1998; Hodis, E. et al., 2012; The_Cancer_Genome_Atlas, 2015]. PTEN is also deregulated in pre-clinical models of melanoma via loss of ZEB2, a competitive endogenous RNA (ceRNA) [Karreth, F. A. et al., 2011]. Moreover, genomic aberrations of MAGI2 (a protein that stabilizes PTEN) have been detected in melanoma [Berger, M. F. et al., 2012].

In a similar manner to that described for *AKT*, *PTEN* alterations are often associated with the presence of $BRAF^{V600E}$ mutations, and cooperate with mutant BRAF protein to induce metastasis in melanoma. Again, this effect appears to be due to provide an OIS inhibitory outcome by activating the PI3K pathway [Dankort, D. et al., 2009].

PTEN-inactivating aberrations are not associated with NRAS mutations, perhaps because the latter lead to activation of PI3K in the absence of mutations in this pathway [Shtivelman, E. et al., 2014; The_Cancer_Genome_Atlas, 2015].

Other PI3K pathway alterations involved in melanoma.

Mutations in genes like *mTOR*, *IRS4*, *PIK3R1*, *PIK3R4*, and *PIK3R5* have been described in a number of cutaneous melanoma tumors; 17 % of tumors carrying *BRAF* mutations and 9 % of that carrying *NRAS* mutations harbor mutation/s in one of more of these genes [Shull, A. Y. et al., 2012], although their biological impact remains unclear.

Increased expression of PDK1 was observed in a large cohort of melanoma samples compared with nevi. Furthermore, deletion of *PDK1* in mouse models of melanoma significantly retarded the emergence of tumors and metastases [Scortegagna, M. et al., 2014].

1.3.8.5. OTHER DISEASE MECHANISMS INVOLVED IN MELANOMA.

1.3.8.5.1. RECEPTOR TYROSINE KINASES (RTKs).

Receptor Tyrosine Kinases (RTKs) are a family of cell-surface receptors with similar structure, consisting of an extracellular ligand binding domain, a single transmembrane helix and a cytoplasmic region containing the protein tyrosine kinase activity (occasionally split into two domains by an insertion, termed the kinase insertion), with juxta-membrane and C-terminal regulatory regions. Binding of the appropriate agonist to the extracellular RTK domain evokes its dimerization (and sometimes oligomerization). A small subset of RTKs aggregates into multimers even in the absence of activating ligand. This leads to autophosphorylation in the tyrosine kinase domain in a trans orientation, becoming a site of assembly of protein complexes and stimulation of multiple signal transduction pathways, including MAPK, PI3K and PLC [Ullrich, A. et al., 1990].

A total of 58 RTKs have been identified in the human genome, falling into 20 subfamilies [Lemmon, M. A. et al., 2010]: type I (ERBB receptors), type II (insulin receptors), type III (PDGFR, CSFR, KIT, FLT3 receptors), type IV (VEGF receptor), type V (FGF receptor), type VI (PTK7/CCK4), type VII (Neurotrophin receptor/Trk family), type VIII (ROR family), type IX (MuSK), type X (HGF receptors), type XI (TAM receptors), type XII (TIE receptors), type XIII (ephrin receptors), type XIV (RET), type XV (RYK), type XVI (DDR receptors), type XVII (ROS receptors), type XVIII (LMR receptors), type XIX (LTK receptors) and type XX (STYK1).

RTKs transduce signals from hormones, cytokines and growth factors, being key regulators of critical cellular processes, such as proliferation, differentiation, cell survival, metabolism, cell migration and cell cycle control [Ullrich, A. et al., 1990; Blume-Jensen, P. et al., 2001].

In normal cells, the activity of RTKs is strictly regulated; dysregulation or constitutive activation of RTKs has been found in a wide range of cancers [Zwick, E. et al., 2001; Chen, Y. et al., 2014; Regad, T., 2015]. The deregulated activation can be owing to GoF mutations, gene rearrangement, gene amplification, overexpression or abnormal stimulation (of either receptor or ligand).

KIT.

The v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog, commonly known as KIT, is a type III Receptor Tyrosine Kinase activated by binding of the cytokine stem cell factor (SCF), as already mentioned in section 1.3.4.2., related to disease mechanisms of MCC.

Activating *KIT* mutations have been detected in roughly 13 % of melanoma samples [The_Cancer_Genome_Atlas, 2015]. Most of these mutations are located in two positions: L576P position, in exon 11, and K642E position, in exon 13 (affecting the kinase domain) [Woodman, S. E. et al., 2010].

Focal amplifications containing *KIT* gene are much frequent in a subtype of melanomas that lacks of mutations in *BRAF*, *NRAS* and *NF1* genes. Consistently with that, tumors with wild-type *BRAF*, *NRAS* and *NF1* show an augmented abundance of KIT in comparison with those with mutations in any of these genes [The_Cancer_Genome_Atlas, 2015].

ERBB4.

The ERBB family of receptors is a set of type I RTK consisting of four members in vertebrates: ERBB1 (also known as EGFR and HER1), ERBB2 (or HER2), ERBB3 (or HER3) and ERBB4 (or HER4). Ligands of these receptors can display specificity (like, EGF, TGF-α, AR and epigen, which bind to EGFR) or, by contrast, bind to more than one of the four receptors (as happens with neuregulins 1–4, which bind ERBB2, ERBB3 and ERBB4, or with HB-EGF, epiregulin, and β-cellulin, which activate EGFR and ERBB4) [Chang, H. et al., 1997; Stein, R. A. et al., 2006].

ERBB4 receptor has been proposed as a major oncogenic "driver" in melanoma. Alterations involving the *ERBB4* gene are usually GoF mutations and predominantly involve the extracellular domain of the receptor, although sequencing efforts have additionally

identified recurrent mutational clusters affecting the ligand binding, extracellular domain alignment, or intramolecular tether formation [Prickett, T. D. et al., 2009; Lau, C. et al., 2014]. Most of these alterations leads the receptor to adopt a tethered conformation in the absence of ligand. Ligands of ERBB4 are neuregulins, heparin-binding EGF-like growth factor (HB-EGF) and betacellulin.

The role for ERBB4 in cancer had been uncertain for a long time. Several studies had concluded that ERBB4 may function as a tumor suppressor in prostate cancer [Williams, E. E. et al., 2003], possibly by antagonizing ERBB2 signaling, but this remains controversial and it seems that, unless in melanoma, ERBB4 plays a pivotal role in tumorogenesis. Reported somatic mutations, resulting in hyperactivation of this RTK, in 19 % of malignant melanoma specimens reassert this theory [Prickett, T. D. et al., 2009].

MET.

The hepatocyte growth factor receptor (HGFR), also called c-Met, or MET, is a type X RTK encoded in humans by a single gene (*MET*, or *c-Met*) [Bottaro, D. P. et al., 1991]. Its only known ligand is the hepatocyte growth factor (HGF).

This receptor is aberrantly activated in many human cancers via mutation, amplification or protein overexpression. Mutations in MET have not been described in melanoma, but there is strong evidence that this RTK is involved in melanoma growth and metastases. A study published in *Nature* [Straussman, R. et al., 2012] demonstrated the production of HGF by stromal cells in patients with melanoma, which resulted in activation of MET and MAPK and PI3K pathways. Furthermore, copy number gains involving MET locus have been documented in melanoma, circumstance that agrees with the high level of MET expression detected in this disease [Puri, N. et al., 2007; Moore, S. R. et al., 2008] .

1.3.8.5.2. RHO GTPases AND JAK-STAT PATHWAY.

The Rho family of GTPases consists of 22 small (of approximately 21 kDa) signaling G proteins. It is a subfamily enclosed within the Ras superfamily. Rho family controls a wide range of essential signaling pathways, orchestrating cellular processes as diverse as cell migration, cell cycle progression, cytokinesis or agonist-regulated gene transcription [Li, J. et al., 1993; Debidda, M. et al., 2005; Villalonga, P. et al., 2006; Saito, K. et al., 2012]. It is well known their role in melanoma (and other cancers) metastasis through managing cell movement [Sanz-Moreno, V. et al., 2008; Kidera, Y. et al., 2010; Wilhelm, I. et al., 2014; Sadok, A. et al., 2015].

Rho proteins are activated by guanine-nucleotide exchange factors (GEFs) through the exchange of GDP for GTP. In turn, GTPase activating proteins (GAPs) help the hydrolysis of GTP to GDP, leading Rho proteins to their inactive conformation [Sanz-Moreno, V. et al., 2009].

Amongst Rho GTPases, there are four that have been found mutated in melanoma, albeit not very frequently; RAC1, RAC2, RHOT1 and CDC42 [Hodis, E. et al., 2012; Shtivelman, E. et al., 2014; Watson, I. R. et al., 2014]. RAC1 plays a role in conveying oncogenic signaling from mutant NRAS in melanoma [Li, A. et al., 2012]. Hotspots mutations at P29S position of RAC1 protein, affecting the conserved switch domain, have been described in 5-10 % of melanoma tumors and, moreover, their functional role *ex vivo* have been confirmed [Hodis, E. et al., 2012; Krauthammer, M. et al., 2012]. $RAC1^{P29S}$ is a recurrent UV-signature mutation less frequently found, although not in a mutually exclusive manner, in NRAS or BRAF mutated melanomas.

However, activity of RAC pathway is affected not only by mutations, but it is regulated, as well, by the available cellular pool of GTP, which is in turn controlled by several enzymes, including GMPR (guanosine monophosphate reductase), which ultimately depletes cellular GTP pools. Thus, high levels of GMPR downregulates Rho-GTPase levels. Conspicuously, the expression of GMPR is lost in invasive melanoma, where activity of RHO pathway contributes to this invasiveness [Wawrzyniak, J. A. et al., 2013].

Rho GTPases have been recurrently proposed to participate in metastasis owing to their control over cell movement. While ago it was reported that overexpression of RhoA increases tumor metastasis in human melanomas [Collisson, E. A. et al., 2002]. Afterwards, two members of Rho family, RhoA and RAC, appeared to have opposing effects on different models of tumor cells motility. Thus, a change in the activity of one of them resulted in the change of the mode of movement [Sanz-Moreno, V. et al., 2008]. It is possible that an efficient metastasis needs each form of movement, depending on the cellular microenvironment.

Recent studies suggest that Rho signaling plays a role in brain metastasis and transmigration of melanoma cells through the blood–brain barrier. Accordingly, the inhibition of Rho-associated protein kinase (ROCK), an effector of Rho proteins, increases the number of melanoma cells attached to the brain endothelium and strengthens the adhesion between melanoma and endothelial cells [Wilhelm, I. et al., 2014]. In a similar way, ROCK inhibition reduces the ability of melanoma cells to migrate and efficiently colonize the lungs [Sadok, A. et al., 2015].

ROCK-dependent cell migration and invasion is regulated, unless in part, by a signaling cascade involving Janus Kinase (JAK) and started by interleukin 6 (IL6) family [Sanz-

Moreno, V. et al., 2011]. There has been reported a positive feedback involving JAK-STAT pathway that would result in a prolonged activation of ROCK. In this network, the transcriptional factor STAT3 would prompt the expression of interleukin 6 signal transducer (IL6ST) and, thereby, the activity of JAK, resulting in ROCK activation and enhanced STAT3 phosphorylation (activation). Such an organization may provide the basis for sustained signaling responses required for the process of invasion, which takes place over a long time scale [Sanz-Moreno, V. et al., 2011]. Accordingly, proinflammatory cytokines, like those of the IL6 family, are frequently observed in the tumor microenvironment. Moreover, some works have demonstrated a correlation between IL6 expression and poor prognosis in melanoma [Rose-John, S. et al., 2007; Melnikova, V. O. et al., 2009; Erez, N. et al., 2010].

Finally, some studies have shown that the balance between phosphorylated STAT1 and STAT3 could serve as a biomarker for melanoma progression [Wang, W. et al., 2008]. Results from these studies showed that the percentage of P-STAT3-positive melanocytes was positively associated with the atypical degree of nevi and that the relative balance of pSTAT1/pSTAT3 may be associated with melanocyte differentiation *in vivo*.

1.3.9. CURRENT TREATMENT OPTIONS IN CUTANEOUS MELANOMA.

Early-stage primary melanoma is curable by surgical excision, but the treatment of late-stage metastatic melanoma remains a formidable challenge and it is associated with poor survival [Balch, C. M. et al., 2009; Erdei, E. et al., 2010]. Malignant melanoma demonstrates a strong capacity for invasion and metastasis, and high rates of recurrence and mortality, as well as a limited response to currently available treatments [Boyle, G. M., 2011; Shore, R. N. et al., 2011].

In the last decade, basic and translational research in melanoma has led to the development of therapies which have allowed to yield improved disease responses and prolonged survival [Smyth, E. C. et al., 2015]. From a simplified point of view, two main strategies of therapy are now being used to treat advanced melanoma; targeted therapies, which tries to block the effect of specific driver mutations, and immunotherapy, based on stimulating the immune system activity [F.D.A., 2011; F.D.A., 2011; F.D.A., 2011; F.D.A., 2013; F.D.A., 2014; F.D.A., 2014; F.D.A., 2014; Smyth, E. C. et al., 2015].

1.3.9.1. TARGETED THERAPIES.

One of the most compelling examples of targeted therapy in cancer is the use of BRAF inhibitors for the treatment of advanced-stage BRAF-mutant melanoma [Bollag, G. et al.,

2010; Flaherty, K. T. et al., 2010]. Current targeted therapies in melanoma are based on inhibiting MAPK-ERK1/2 pathway by means of $BRAF^{V600E}$-selective inhibitors (Vemurafenib, or Dabrafenib), either alone or in combination with MEK inhibitors (Trametinib or Selumetinib). This therapy is currently being used to treat melanoma cases that harbor activating mutations in *BRAF* gene, circumnstance that occurs in approximately 50 - 60 % of cases, depending on the study and examined population [Curtin, J. A. et al., 2005; Lee, J. H. et al., 2011]. Association of BRAF and MEK inhibitors has enabled to reach an overall survival sate at 12 months of 72 % and a median progression-free survival of 11,4 months [Robert, C. et al., 2015].

Contrary to results obtained in *BRAF* mutant melanomas, BRAF inhibitors can activate MAP kinase pathway in cells with wild-type *BRAF*. Notably, in *KRAS* mutant and *RAS/RAF* wild-type tumors, BRAF inhibitors activate the RAF-MEK-ERK pathway in a RAS-dependent manner, enhancing thus the tumor growth in some xenograft models. Inhibitor binding activates wild-type RAF isoforms by inducing dimerization, membrane localization and interaction with RAS-GTP. These events occur independently of kinase inhibition and are, instead, linked to direct conformational effects of inhibitors on the RAF kinase domain [Hatzivassiliou, G. et al., 2010].

1.3.9.1.1. MAPK-ERK1/2 PATHWAY INHIBITION.

Vemurafenib (RG7204, PLX4032 or RO5185426) is an orally available, small molecule designed to specifically inhibit signaling from the BRAF mutant protein [Bollag, G. et al., 2010]. It is also able to inhibit CRAF and ARAF, but in much higher concentrations. This drug was licensed in 2011 and approved by the National Institute for Health and Clinical Excellence (NICE) in 2012. Firstly, in *in vivo* and *ex vivo* melanoma models, Vemurafenib inhibited phosphorylation of MEK and, consequently, of ERK, leading to G1 phase cell-cycle arrest and apoptosis [Joseph, E. W. et al., 2010]. Next, phase I clinical studies showed that Vemurafenib treatment caused significant tumor regressions in a majority of metastatic melanoma patients with activating mutations in *BRAF*; complete or partial tumor regression was observed in 81 % of patients whose melanoma had the $BRAF^{V600E}$ mutation [Flaherty, K. T. et al., 2010]. Importantly, tumor regressions were highly dependent on pathway blockade, with a high threshold required. For example, 60 % inhibition was insufficient for tumor regression, whereas 90 % inhibition often correlated with robust regression. Near the threshold relatively modest differences in pathway blockade had large consequences on tumor response [Bollag, G. et al., 2010]. Subsequent trials reported an overal survival (OS) rate of 65 % at 12 months and a median progression-free survival (PFS) of 7.3 months [Robert, C. et al., 2015].

However, despite the rapid respones of *BRAF*-mutant tumors to Vemurafenib, responsive tumors developed resistance to treatment after few months. Amongst the patients who had a response to treatment, the duration of such response ranged from 2 to 18 months, with most patients relapsing within 6-7 months [Flaherty, K. T. et al., 2010].

Dabrafenib (Tafinlar) is another inhibitor of the BRAF-mutant protein, approved by the US Food and Drug Administration (FDA) in 2013 for the treatment of patients with advanced melanoma that contains the V600E mutation. In the pivotal phase III trial, Dabrafenib produced a median PFS of 5.1 months and an OS (updated at the 2015 ASCO meeting [Daud, A. et al., 2015]) of 70 % at 12 months.

Unfortunately, as happened with Vemurafenib, relapses appeard after a short initial response to the treatment with this drug.

Sorafenib (BAY 43-9006, or Nexavar), is an inhibitor of RAF that, unlike Vemurafenib and Dabrafenib, acts over both wild-type and mutant BRAF protein, as weel as over CRAF protein. In clinical trials, Sorafenib, used either alone or in combination with chemotherapy, has not had significant antimelanoma effects. It is possible that the non-BRAF effects of Sorafenib mediate side effects that limit the likelihood of achieving a drug concentration that is high enough to counteract the effects of $BRAF^{V600E}$ mutation [Eisen, T. et al., 2006].

Resistance to BRAF inhibitors seems to be due, at least in part, to the ability of tumors to activate MEK (mechanisms of resistance will be addressed in section 1.3.9.1.4.). Therefore, researchers tried to prevent tumors from using this escape mechanism by combining BRAF inhibitors with MEK inhibitors, like Trametinib or Selumetinib.

Selumetinib (AZD6244,or ARRY-142886) is a non-ATP-competitive and highly selective inhibitor of both MEK1 and MEK2 kinases. Results obtained with cutaneous melanoma in phase II trials suggest that, in carefully selected patients, Selumetinib can induce tumor regression and prolong OS. In contrast, treating unselected patients result in a low response rate and reduce OS [Kirkwood, J. M. et al., 2012; Catalanotti, F. et al., 2013]. For example, significant tumor regression has been described specially in tumors with low pAKT expression (this fact is further discussed below).

Trametinib (GSK1120212) is an ATP-competitive inhibitor of MEK1 and MEK2. In 2013 it became the first MEK inhibitor licensed in the US as monotherapy for $BRAF^{V600E}$ or $BRAF^{V600K}$ advanced melanoma, due to its evident survival benefit in a phase III clinical trial (METRIC, NCT01245062) [Flaherty, K. T. et al., 2012]. Trametinib produced an objective

response rate of 22 %, a PFS of 4.8 months and a OS rate of 81 % at 6 months (data updated at the Society for Melanoma Research (SMR) Meetin, in November, 2013 [Shapiro, G. et al., 2011]).

Cobimetinib (GDC-0973, XL-518) is a noncompetitive inhibitor highly specific for MEK1 and MEK2. A Phase III, randomized study has been recently conducted in order to compare the efficacy of the combination of Vemurafenib and Cobimetinib versus Vemurafenib alone. This study showed an increased PFS in the combination group when compared to the control group, with 9.9 months for Cobimetinib plus Vemurafenib versus 6.2 months for Vemurafenib plus placebo [Larkin, J. et al., 2014]. Several other studies with Vemurafenib plus Cobimetinib in various clinical conditions, such as brain metastases, as an adjuvant therapy or with other agents like bevacizumab, are now underway (NCT01495988, NCT02230306).

The use of BRAF and MEK inhibitors in combination has provided the best results, so far, of targeted therapies in melanoma. Combining Dabrafenib and Trametinib, Robert et al. [Robert, C. et al., 2015] reported a OS rate at 12 months of 72 % (this endpoint is not much higher than those for Dabrafenib in monotherapy), a median PFS of 11.4 months and a objective response rate of 64 % (the last two endpoints are considerably higher than those achieved with the idividual use of any of this drugs).

1.3.9.1.2. PARALLEL CO-TARGETING OF MAPK AND PI3K PATHWAYS.

As previously expounded in this thesis, activation of PI3K pathway is frequently found in melanomas, but not in benign nevi from which them arise. It makes this pathway an excellent candidate to be a target of anticancer drugs, perhaps primarily in those melanomas that evolve from nevi.

Furthermore, the enhanced activity of PI3K-AKT pathway related to chronic BRAF inhibition suggests the possible existence of a negative cross-talk between the two pathways. Such cross-talk has been reported in several cancer systems [Carracedo, A. et al., 2008; Carracedo, A. et al., 2008; Mirzoeva, O. K. et al., 2009], but not much is yet known in melanoma, so this issue deserves further exploration.

Under conditions of continuous BRAF inhibition, melanomas rely on survival pathways mediated by the insuline receptor (IR) and the insulin-like growth factor 1 receptor (IGF-1R) in order to circumvent the adverse conditions. Consistently with this notion, inhibitors of IGF-1R (like Linsitinib, currently on phase III clinical trials) or PI3K (like Idelalisib), possibly in

combination with MEK inhibitors (like Trametinib), could induce death more effectively in cells resistant to BRAF inhibitors. Based on this idea, a large set of combinatorial preclinical studies co-targeting RAS-MEK-ERK and PI3K-AKT-mTOR pathways has been developed in melanoma.

For example, Selumetinib was combined, both *ex vivo* and *in vivo,* with different PI3K-mTOR inhibitors, such as the PI3K inhibitor BEZ235 or the mTOR kinase blocker AZD8055, leading to a synergistic reduction of cell viability, an enhanced apoptosis [Aziz, S. A. et al., 2010; Shi, H. et al., 2011], a tumor regression and an extension of median survival [Gopal, Y. N. et al., 2010; Roberts, P. J. et al., 2012]. Mechanistically, the modulation of several markers of proliferation (decreased pAKT and GSK3α/β) and apoptosis (increased BIM protein, cleavage of PARP and caspase-7 and reduced Mcl-1) was described. Furthermore, knockdown of AKT confirmed that the inhibition of the PI3K/AKT pathway sensitizes melanoma cells to Selumetinib [Gopal, Y. N. et al., 2010]. Selumetinib has also been associated with other AKT/mTOR inhibitors (MK-2206, Rapamycin or AKTi), as described by many groups that confirmed a reduction in cell viability, the blockade of cell cycle progression and concomitant enhanced apoptosis in different melanoma models (human, murine and canine) [Atefi, M. et al., 2011; Shi, H. et al., 2011].

As well, supporting these anti-proliferative and pro-apoptotic effects of dual targeting MAPK and PI3K pathways, Posch et al [Posch, C. et al., 2013] described how different combinations of several inhibitors of MEK and PI3K/mTOR eventually affected genes involved in cell division. Furthermore, such combinations induced a substantial decrease of cyclin D1 and an upregulation not only of tumor suppressor genes but also of pro-apoptotic genes.

In addition, *ex vivo* studies done with melanomas resistant to BRAF inhibitors showed that simultaneous MEK and IGF-1R/PI3K inhibition leads to cytotoxicity specifically in those cases which had stopped to respond to anti-MAPK therapy [Villanueva, J. et al., 2010].

In the light of these evidences, several clinical trials of combinatorial treatment targeting these two signaling cascades have been done.

For example, a phase II trial identified as NCT01519427 (https://clinicaltrials.gov) studied how well Selumetinib and the AKT inhibitor MK2206 works in treating patients with stage III or stage IV $BRAF^{V600E}$-mutant melanoma, whose disease had progressed on prior therapy with a selective BRAF inhibitor (i.e., Vemurafenib, Dabrafenib, LGX818). Patients given the combinatorial therapy responded to the treatment during a period of time, after which, the disease progressed again. The mean PFS was of 105 days.

In the same line, preliminary data from a phase II clinical trial (NCT01616199) with BRAF-mutant patients recibing Vemurafenib plus PX-866, a nonreversible pan-PI3K inhibitor, were presented at the 10th International Congress of the SMR (Philadelphia, 2013). The reported overall response was 53 % and such response was seen both in patients who had not been previously treated with a BRAF or MEK inhibitor and in patients whose cancer progressed following either a BRAF or MEK inhibitor. This trial is still unconcluded, so no much more information is still available. In addition, two more clinical trials (NCT01363232 and NCT01337765), in which both BRAF- and NRAS-mutant melanomas are included, are curently ongoing, with still unreported results.

However, despite the promissing preliminary results of *ex vivo*, *in vivo* and clinical inhibition of the PI3K pathway, these expounded data come from the joint use of MAPK pathway and PI3K pathway inhibitors, whereas not much is known about effects of inhibiting just IR, IGF-1R or PI3K signaling, keeping a raised MAPK signaling. As already explained in the section 1.3.8.4., activation of the PI3K pathway seems to help tumor cells to overcome the oncogene-induced senescence (OIS) associated with an excessive MAPK activity. Therefore, it might be feasible that the use of PI3K signaling inhibitors in monotherapy could lead melanocytes with activacing mutations in MAPK pathway to die.

1.3.9.1.3. TARGETED THERAPIES AGAINST OTHER PATHWAYS.

As many different signaling pathways have been found altered in melanoma, it can be reasonable to contemplate the possibility that, apart from PI3K, some other mechanism can be acting in a cooperative manner with MAPK pathway in cancer promoting. Involved mecanisms can be different across each particular cancer case, hence making advantageous to add a personalized nature to targeted therapies.

JAK/STAT:

An example of this kind of cooperative pathways could be the JAK signal transducer and associated STAT3 transcriptional factor, which are thought to play a central role in melanoma cell biology, as previously discussed in this thesis.

To date, a number of strategies for inhibiting the STAT3 pathway have been evaluated in melanoma and other cancer types in a preclinical setting. Some approaches have focused on inhibiting upstream kinases, such as JAK2, while others have focused on targeting the STAT3 protein by means of siRNAs, shRNAs, and also by some drugs intended to target other key oncogenic pathways or processes, like Sunitinib [Xin, H. et al., 2009; Yu, H. et al., 2009; Yue, P. et al., 2009].

Regardless of the approach used, inhibiting STAT3 leaded to a growth inhibition and proapoptotic effects on malignant cells and, in addition, appeared to be an effective means for augmenting immune-mediated tumor recognition. Particularly relevant are some data demonstrating that therapies tarteting STAT3 were effective in melanoma cells that had acquired resistance to the Vemurafenib [Liu, F. et al., 2013].

As a single-agent inhibition of JAK2 (an indirect way of targeting the STAT3 pathway) has been well tolerated in clinical trials of hematologic malignancies and it is in an early phase clinical trials for patients with solid tumors [Quintas-Cardama, A. et al., 2013]. It could be practical to evaluate the effects of combined inhibition of JAK2 in preclinical studies, in order to generate data in support of clinical trials with these agents.

c-KIT:

Also aforementioned in this thesis, c-KIT is a RTK which activates signaling pathways with proliferative and prosurvival effects.

A Phase II trial in patients with metastatic melanoma harboring c-KIT mutations or amplifications was conducted in order to test the efficacy of Imatinib, a tyrosine kinase inhibitor. The study yielded a median PFS of 3.5 months and a regression of tumor mass was noticed in 41.9 % of the participants. The 1 year OS was 51 % [Guo, J. et al., 2011]. Trials with other c-KIT inhibitors such as Nilotinib and Masatinib in melanoma are currently underway (NCT01099514, NCT01280565).

1.3.9.1.4. MECHANISMS OF RESISTANCE TO TARGETED THERAPIES.

Despite the tremendous progress made in developing therapies for treating melanoma, resistances remain a major problem that limits the long-term responsiveness of the majority of patients to these drugs. Indeed, as already mentioned, despite of the rapid response of most *BRAF*-mutant melanomas to MAPK inhibitors, such response is not durable and most patients relapse within 2 to 18 months from the beginning of the treatment. In addition, about 15 % of patients do not achieve tumor regression at all [Flaherty, K. T. et al., 2010; Sosman, J. A. et al., 2012; Robert, C. et al., 2015].

Most melanomas show an **acquired resistance**, characterized by a progressive disease following an initial response to a therapy. However, **primary** (or **intrinsic**, or ***de novo***) resistance, characterized by a lack of response since the beginning of the treatment, has been observed in about 15 % of melanomas [Flaherty, K. T. et al., 2010].

Drug resistance that leads to clinical relapse is acquired by virtually all patients treated with MAPK inhibitors. It involves a dynamic process of subclonal competition that eventually dictates a multifactorial resistance, which is currently believed to promote the reactivation of MAPK signaling or other proliferative or pro-survival pathways [Campbell, P. J. et al., 2008; Campbell, P. J. et al., 2008; Shi, H. et al., 2014]. A key arised question is whether resistances appear through drug-induced selecting pressure that drives the mutational landscape, perhaps facilitating the proliferation of pre-existing *BRAF* wild-type clones. Evidences in favour of this possibility came from a whole exome sequencing study of multiple progressing lesions from one patient failing dabrafenib therapy after 383 days [Shi, H. et al., 2014]. Of the nine distinct progressing lesions analysed in this study, at least five co-existent mechanisms of resistance were identified, including an acquired *KRAS* mutation, a *BRAF* splice mutation, a *BRAF* amplification and a *PTEN* indel. At the same time, the mutational spectra of the progressing tumours was significantly altered, with a reduction in the frequency of C → T transitions compared with the pre-treatment tumours.

A convergent event at the time of resistance seems to be the reactivation of ERK1/2. Melanomas harboring activating *BRAF* mutations are initially addicted to MAPK signaling. When BRAF is repressed, melanomas trigger alternative signaling programs which allows the addicted tumor to continue relying on ERK1 and ERK2 activation for the maintenance of the neoplastic phenotype. However, private mechanisms responsibles of this remain still unclear [Paraiso, K. H. et al., 2010; Lito, P. et al., 2012].

Multiple different mechanisms have been proposed to confer resistance to MAPK inhibitors. Following list is an attempt to brieftly summarize the main ones:

1. Deregulation of receptors tyrosine kinase (RTKs) activity, like platelet-derived growth factor receptor (PDGR) or insulin-like frowth Factor 1 receptor (IGF-1R) [Nazarian, R. et al., 2010; Villanueva, J. et al., 2010]. As already mentioned, RTKs can activate MAPK and PI3K pathways, amongst others.

2. Activation of NRAS and MEK, which can be due to mutations or to the reduction of RAS repression exerted by P-ERK (owing to the decreased level of activated ERK during the treatment with MAPK inhibitors). Researches hoped to avoid resistant related with RAS and MEK activation by combining BRAF and MEK inhibitors [Emery, C. M. et al., 2009]. However, although this approach yielded better results than monotherapy, resistences appear anyway.

3. Enhanced IGF-1R and PI3K/AKT activity that has been associated with chronic BRAF inhibition. Augmented IGF-1R expression and AKT phoshorylation correlate with resistance to BRAF inhibitors in samples from relapsed patients with cutaneous melanoma [Villanueva, J. et al., 2010]. In the same work, Villanueva et al. also demonstrated that, under conditions of chronic BRAF inhibition, melanomas rely on

IR/IGF-1R pathways to support drug resistance and promote cell proliferation. Consistently with this notion, inhibitors of MEK and IGF-1R or PI3K in combination were more effective inducing cell death of BRAF-inhibitor resistant cells than when used as single agents.This implies the role of PI3K pathway in both acquired and *de novo* resistances to MAPK inhibitors. In addition, several preclinical data from a number of independent studies associated mutations that activate the PI3K/AKT pathway, including alterations in PTEN, with diminished sensitivity to MEK and BRAF inhibition in BRAF-mutant cell lines [Gopal, Y. N. et al., 2010; Xing, F. et al., 2012]. In the light of this data, drug combinations co-targeting MEK and IGF-1R/PI3K were propossed as an alternative to overcome resistance to BRAF inhibitors [Villanueva, J. et al., 2010], as recently aforesaid in section 1.3.9.1.2. There are clear evidences of multiple levels of cross-talk between MAPK and PI3K/AKT pathways. Furthermore, it has been shown that ERK1/2 can be phosphorylated by the PI3K-AKT-mTOR pathway [Grammer, T. C. et al., 1997; Jiang, C. C. et al., 2011]. In addition, cells with resistance to MAPK inhibitors have MEK-independent survival mechanisms that can be blocked by inhibitors of the PI3K-AKT-mTOR pathway [Jiang, C. C. et al., 2011].

4. Switching amongst the three different RAF isoforms [Villanueva, J. et al., 2010]. Upon chronic BRAF inhibition, $BRAF^{V600E}$ melanomas would rewire their signaling circuitries, by a yet unidentified mechanisms, in order to utilize one of the other two RAF isoforms; ARAF or CRAF. It has been often reported that CRAF reduce sensitivity to Vemurafenib in $BRAF^{V600E}$ cell lines, suggesting an ability of these kinases to mediate resistance to RAF inhibition [Johannessen, C. M. et al., 2010]. In this regard, utilizing Sorafenib (which inhibits both BRAF and CRAF) could be more effective but, actually, and as mentioned above, survival do not increase when this drug is used.

5. Expression of $BRAF^{V600E}$ splicing variants [Poulikakos, P. I. et al., 2011]. Alternative splicing forms of $BRAF^{V600E}$ lacking RAS-binding domain have been identified in cell lines and patients resistant to Vemurafenib. Acquired resistance mediated by this alternative splicing is due to insensitivity of the enzyme to inhibitors. These tumors should retain sensitivity to inhibitors of downstream components of the pathway, such as MEK, so, again, MEK inhibitors, used in combination with those of BRAF, could be a solution to prevent resistance by this mechanism.

6. Activation of ERK through MEK-dependent and MEK-independent mecanisms mediated by COT (*MAP3K8*) [Johannessen, C. M. et al., 2010]. COT (cancer Osaka thyroid) is a serine/threonine protein kinase that can activate both MAPK and JNK kinase pathways, and whose expression has been associated with both intrinsic and acquired resistance. This kinase have been found amplified in a few cell lines with *BRAF* mutations that show intrinsic PLX4032 resistance [Johannessen, C. M. et al.,

2010]. COT activates ERK1/2, primarily through MEK-dependent mechanisms that do not require RAF signalling. Therefore, scientist reasoned that combined RAF and MEK inhibition might circunvent resistance mediated by COT. However, Johanesen et al. saw that ectopic COT expression in melanoma cell lines decreased their sensitivity to MEK inhibitors. Furthermore, MEK1/2 knockdown only modestly suppressed COT-mediated ERK phosphorylation *ex vivo*. In the light of this results, they raised the possibility that COT activate ERK also through MEK-independent mechanisms, besides its MEK-dependend effects. This could be the reason why COT is related with primary resistances to MAPK inhibitors; cells with COT mutations have mechanisms independent of MAPK pathway to activate ERK1 and 2, before a sustained exposure to such inhibitors [Johannessen, C. M. et al., 2010].

7. Cyclin D1 amplification and RB inactivation, which have been signaling by preclinical evidences as related to resistance [Smalley, K. S. et al., 2008; Xing, F. et al., 2012].

8. GNAQ signaling, which has been shown to activate two independent downstream mechanistic subsets: A) calcium and MAPK signaling and B) p38MAPK, JNK, MAPK7, NFKB and YAP, via TRIO/RHO-GTPases [Vaque, J. P. et al., 2013; Feng, X. et al., 2014]. In addition, GNAQ has been shown to reduce *ex vivo* the sensibility of cutaneous melanoma cells to BRAF inhibitors [Mitsiades, N. et al., 2011; Turajlic, S. et al., 2014].

The evaluation of mechanisms of resistance in a specific clinical setting could help to address the adaptability of melanoma cells to current therapies. Understanding the multiple case-specific mechanisims linked to the development of biological resistant to treatments could promote the development of alternative and efficient therapeutic strategies.

1.3.9.1.5. OTHER INCONVENIENT CONSEQUENCES OF TARGETED THERAPIES.

Aside from relapse, targeted agents have also revealed an unexpected consequence; the frequent emergence of secondary malignancies, such a quamous-cell carcinomas, leukemias or new primary melanomas [Flaherty, K. T. et al., 2010; Robert, C. et al., 2011; Callahan, M. K. et al., 2012; Su, F. et al., 2012; Zimmer, L. et al., 2012].

Both relapse and appearance of secondary diseases could be sometimes related with an unintended or "**paradoxical activation**" of MAPK signalling that occurs in cells with an increased RAS activity (and either wild-type or low-activity mutant BRAF protein) when they are treated with BRAF inhibitors. In these cases, the inhibition of BRAF protein can lead to

an hiperactivation of ERK1/2, resulting in a tumorigenic transformation [Gibney, G. T. et al., 2013]. Two alike mechanistic explanations for this phenomenon have been offered. The first one postulates that BRAF is stucked in the cytosol in an autoinhibited state and that their binding to the BRAF inhibitor activates the protein, enabling it to dimerize with CRAF [Heidorn, S. J. et al., 2010]. The second one is focused on potential conformational changes in BRAF or CRAF caused by their physical binding to the inhibitor. Acording to thes second model, if the BRAF inhibitor is present at low concentrations it binds to only one of the RAF protomers, changing the protein conformation and allowing, thereby, the dimerization and transactivation of the inhibitor-free protomer. By contrast, at high concentrations the drug binds and inhibits both RAF members of the dimer, blocking the signalling [Hatzivassiliou, G. et al., 2010; Poulikakos, P. I. et al., 2010]. In both models, the paradoxical ERK activation that follows BRAF inhibition depends on the upstream activity at the level of RAS, that can arise from either increased RTK signalling, reduced activity of RAS inhibitor, or directly as a result of activating mutations in RAS [Halaban, R. et al., 2010; Heidorn, S. J. et al., 2010; Joseph, E. W. et al., 2010; Poulikakos, P. I. et al., 2010].

A next-generation of “paradox-breakers” RAF inhibitors are now under developed, showing an inhibition of RAF signaling that overcome several known mechanisms of resistance associated to first-generation RAF inhibitors, including paradoxical effects [Zhang, C. et al., 2015].

1.3.9.2. IMMUNOTHERAPY.

Although the idea of using the immune system to treat cancer is an old concept, the better understanding reached during the last years of the important role that it plays in controlling and eradicating cancer have encouraged the development of different strategies aimed to exploit such capacity. Even though responses against autologous cells are difficult to provoke, the immune system is still able to orchestrate a response against cancer cells. Mutations in genes may create new gene products, including new antigenic epitopes (neoepitopes) which would make a cell to become immunogenic. Hence, antibodies and T-cells can recognize carbohydrate motifs and protein entities expressed only by cancer cells [Lloyd, K. O., 1991; Disis, M. L. et al., 1994]. Therefore, a greater immunogenicity and consequent immune response would be expected in those tumors with a higher mutational burden, as aforementioned in section 1.2.1.4.2.

On the other hand, tumor cells are able to exploit some mechanisms that, during the normal functioning of immune system, contribute to maintain the immunologic homeostasis, acting as negative regulators of immune cells after an immune response. By means of activating these checkpoints, the malignancy can evade the immune response which may

eliminate the tumor [Krummel, M. F. et al., 1995; Tivol, E. A. et al., 1995; Dong, H. et al., 2002; Keir, M. E. et al., 2006]. Two of these mechanisms starts when the cytotoxic T-lymphocyte antigen 4 (CTLA-4) and the programmed cell death 1 (PD-1) –two membrane receptors expressed by activated T-cells, B-cells and Natural Killers– binds to their ligands. This originate a signaling cascade that prompt, in the end, a downregulation of cell activity through arresting cell cycle or inducing apoptosis [Dong, H. et al., 2002; Sage, P. T. et al., 2014]. It has been described that tumors often express one of the ligands of PD-1, the PD-L1, having thus the capacity of inhibit T-cell activity [Dong, H. et al., 2002]. In patients with different types of cancer, including melanoma, high levels of PD-1 expression have been detected in tumor-infiltration T cells (TILs), presumably due to chronic antigen stimulation [Ahmadzadeh, M. et al., 2009].

On the basis of that, antibodies targeting CTLA-4, PD-1 and PD-L1, have been developed to avoid the ligand-receptor binding and, hence, blockade these immune checkpoints [Hodi, F. S. et al., 2008; Hodi, F. S. et al., 2014; Powles, T. et al., 2014; Topalian, S. L. et al., 2014]. The use of these antibodies enhances the immune cells activation and proliferation.

The overall mutation load has been correlated with clinical responses to immunotherapy in different cancer types, including melanoma [Le, D. T. et al., 2015; Rizvi, N. A. et al., 2015; Van Allen, E. M. et al., 2015]. However, it is important to note that low mutational loads not always preclude clinical responses, and conversely, high mutational loads do not always correlate with responses [Hugo, W. et al., 2016]. As well, the extent of pretreatment and, especially, treatment-induced intra-tumor T cell infiltration correlates with clinical responses [Tumeh, P. C. et al., 2014], supporting the unleashing of tumor-specific T cells as the primary mechanistic base of immunotherapy agents.

Different strategies aimed to increase the activity of immune cells infiltrated in the tumor have been −and are being− developed. From a broad point of view, these strategies can be separated into three categories:

1. Nonspecific stimulation of the immune system, which can be achieved by using interleukin 2 (IL-2) and/or immune checkpoint blockade agents.
2. Active immunization, managed by means of agents like peptide or whole tumor cell vaccines, recombinant viruses encoding tumor-associated antigens or dendritic cells.
3. Adoptive cell transfer, consisting in reinfusing autologous tumor-infiltrating lymphocytes, together or not with dendritic cells.

Immune checkpoint blockade.

Therapies designed to avoid the inhibition of T-cells, B-cells and Natural Killers have reported successful results in improving the survival of patients with advanced melanoma. These therapies are based on antibodies that recognize and bind CTLA-4 (like Ipilimumab), PD-1 (like Nivolumab and Pembrolizumab) or PD-L1 (like Atezolizumab (or MPDL3280A)), preventing the signaling cascade that ends in arresting the cell cycle or inducing apoptosis of immune cells.

Responses to these agents are often durable in time (reviewed by [Sharma, P. et al., 2015]), but, unfortunately, most patients (approximately 70 %) do not derive benefit from them, not showing any response [Hodi, F. S. et al., 2010; Topalian, S. L. et al., 2012; Hamid, O. et al., 2013; Delyon, J. et al., 2015; Sharma, P. et al., 2015]. Therefore, the effective clinical use of immune checkpoint agents is encumbered by a high rate of innate resistance. The mechanistic basis for the variation in response patterns remains mainly unknown. Thus, future work about this issue should be very helpful. As well, developing diagnostic tools enabling the previous discrimination between responders and non-responders should have an enormous importance, for both medical and financial reasons.

Vaccines.

Several vaccines have been progressively developed to prevent or treat different cancer types, including melanoma. Albeit just a few of them have been licensed for their use in the clinical practice, there are more in ongoing clinical trials [Schlom, J., 2012]. However, vaccines are not yet a major type of cancer treatment.

In general, anti-cancer vaccines can be divided into two types: **therapeutic**, used to treat patients who already have cancer, and **preventive**, used to prevent cancer to appear, and sometimes designed against any etiological agent that cause cancer (such as the HPV vaccine) [Finn, O. J., 2003].

Focusing on melanoma treatment, one of the most advanced vaccines is the "**gp100 melanoma vaccine**". This is a therapeutic vaccine containing an enhanced version of a tumor-associated antigen, the glycoprotein 100 (gp100), which is expressed on the surface of melanoma cells. The enhanced version of this vaccine consisted of the amino acids 280−288 of this glycoprotein with a valine substitution at amino acid position 288 (gp100:280-288(288V)) to improve its immunogenicity. Vaccination with this peptide may stimulate the host immune system to mount a cytotoxic T lymphocyte (CTL) response against tumor cells positive for the gp100 antigen, resulting in decreased tumor growth.

A phase III clinical trial (NCT00019682) [Schwartzentruber, D. J. et al., 2011] with advanced melanoma (stage III and IV) patients who received the gp100 vaccine, along with standard Interleukin 2 (IL-2) therapy, returned a significantly improved clinical response rate and a longer progression-free survival, compared with patients who were given the IL-2 therapy only.

Currently, a new melanoma vaccine belonging to the preventive type is in an ongoing clinical trial (NCT01898039) [2013]. This study is designed for patients who had malignant melanoma and, following tumor removal, are now free of disease, or have only very minor residual disease, but they have a very high risk of recurrences. These patients will be treated with an allogeneic melanoma cell vaccine, derived from a cell line that highly express melanoma associated antigens, which has been genetically modified to express both the human leukocyte antigen (HLA)-A2 and the 4-1BB ligand, with potential immunostimulating and antineoplastic activities. Upon administration, the 4-1BB ligand binds to 4-1BB on activated T-cells, which induces a strong immune response against HLA-A2 positive melanoma cells.

Adoptive cell transfer with autologous tumor-infiltrating lymphocytes.

A promising advance in immunotherapy is a new approach that lies in taking the T cells that are in the tumor, trying but failing to kill it, and proliferating them to large numbers outside the body of the patient, to be then reinfused into the body in combination with IL-2. To qualify for adoptive T cell transfer plus IL-2, patients must have CD8-positive T cells that are highly reactive and tumor specific, a tumor that can be surgically accessed, and the ability to tolerate high-dose IL-2.

Contrary to immunomodulatory drugs, such as IL-2 or Ipilimumab, adoptive cell transfer can theoretically beat the immunosuppressive tumor microenvironment because activation and expansion of the antitumor lymphocytes occur *ex vivo*. Furthermore, the previous manipulation of the host patient with lymphodepletion may create an environment conducive for further cell expansion and activation, and may also prevent immunosuppressive regulatory T cells from interfering with the full capacity of the transferred cells [Phan, G. Q. et al., 2013].

So far, adoptive cell transfer has reported OR rates from 40 % to 72% and long-term response rates of up to 40 % [Rosenberg, S. A. et al., 2011]. An active research is ongoing to bring this to fruition for all patients. T-cell engineering technology may someday lead to “off-the-shelf” reagents, personalized to a private HLA and tumor antigen status [Phan, G. Q. et al., 2013].

However, without detracting the fact that more than half of patients responded to this therapy, the main weakness can be that its use is limited by the technical expertise involved in isolation and expansion of these cells, as well as the infrastructure required for this therapeutic approach [Svane, I. M. et al., 2014].

1.3.9.3. COMBINATION OF TARGETED THERAPY AND IMMUNOTHERAPY.

The combination of targeted therapy and immunotherapy was initially hypothesized to promote better outcomes in melanoma than either modality separately. This idea was based, unless in part, on the finding that chemotherapy and BRAF inhibitors not only brake tumor growth during a period of time, but also make tumors more recognizable to the immune system [Knight, D. A. et al., 2013; Ilieva, K. M. et al., 2014]. However, later analyses of clinical data hinted at prior failure of MAPK-targeted therapy as being a negative factor for subsequent response to immune checkpoint blockade in melanoma [al., R. e., 2015; al., S. e., 2015; Puzanov et al., 2015]. In addition, acquired resistance to MAPK-targeted therapy has been correlated with depletion of intra-tumor T cells, exhaustion of CD8 T cells and loss of antigen presentation [Hugo, W. et al., 2015]. More recently, a study with melanoma patients treated with anti-PD-1 revealed the presence of transcriptomic signatures induced by MAPK inhibitors in nearly all non-responding while in only 1 of 15 responding samples [Hugo, W. et al., 2016].

However, the BRAF inhibitor Vemurafenib plus adoptive T-cell transfer resulted in superior antitumor effects in a mouse melanoma model [Koya, R. C. et al., 2012]. Inhibition of BRAF was also found to increase the expression of melanoma differentiation antigens and induce infiltration of CD8+ T cells in posttreatment melanoma tumor samples [Boni, A. et al., 2010]. Additionally, inhibition of BRAF and MEK in melanoma cells leads to augmented tumor-specific T cell function, as well as dendritic cell function, *ex vivo* [Ott, P. A. et al., 2013]. Similarly, the c-KIT inhibitor Imatinib, was shown to synergize with anti-CTLA-4 antibodies in another mouse model [Balachandran, V. P. et al., 2011]. In addition, the combination of Imatinib with IFNα also generated promising results in patients [Chen, L. L. et al., 2012]. In addition, a superior antitumor effect was demonstrated *in vivo* when dual targeted therapies, using BRAF and MEK inhibitors, were combined with immunotherapeutic approaches (including adoptive cell transfer and immune checkpoint blockade with anti-PD-1) in a murine model of $BRAF^{V600E}$ mutant metastatic melanoma [Hu-Lieskovan, S. et al., 2015].

All these evidences have encouraged the launch of clinical trials combining BRAF or MEK inhibitors with immunotherapies, such as high-dose IL-2, Ipilimumab or Atezolizumab. These studies (for example, and just to name a few, those identified as NCT01673854,

NCT01767454 or NCT01656642) are testing different possibilities of combining, either simultaneously or sequentially, targeted therapies and immunotherapy in melanoma.

2. Hypotheses and objectives

2.1. HYPOTHESIS.

Advanced melanoma and Merkel cell carcinoma (MCC) are the most aggressive tumors originated in the skin. Effective therapies for them are still required.

Upon molecular characterization of individual cancer cases we can detect specific disease mechanisms. This can allow the identification of precise therapeutic targets, as well as predictive biomarkers to support diagnoses and prognoses.

Mutational signatures harbored by tumor cells can originate aberrant signaling networks, which could determine the clinical evolution of a cancer and also its response to a given treatment.

Molecular characterization of cancer can be the key to achieving a rational selection of the appropriate treatment for each cancer case.

2.1.1. HYPOTHESIS SPECIFIC OF ARTICLE 1: Shared oncogenic pathways implicated in both virus-positive and UV-induced Merkel Cell Carcinomas.

Genomic characterization of Merkel cell carcinomas could unveil unknown disease mechanisms, as well as mechanistic similarities and differences between MCPyV-positive and MCPyV-negative tumors.

2.1.2. HYPOTHESIS SPECIFIC OF ARTICLE 2: Individualized strategies to target specific mechanisms of disease in malignant melanoma patients displaying unique mutational signatures.

Mutational characterization of advanced melanoma lesions may allow the identification of case-specific disease mechanisms, which could be targeted by specific inhibitors.

Melanoma tumors develop aberrant signaling mechanisms of transformation consisting of MAPK-dependent and MAPK-independent mechanisms, independently of the BRAF mutational status.

2.2. OBJECTIVES.

The aim of this thesis is the characterization of tumor lesions from two types of aggressive skin cancer at a molecular level and the later use of the obtained information to identify specific disease mechanisms and/or biomarkers.

2.2.1. OBJETIVES SPECIFIC OF ARTICLE 1: Shared oncogenic pathways implicated in both virus-positive and UV-induced Merkel Cell Carcinomas.

Four different objectives have driven de development of this work:

1. Search for somatic mutations in the whole exome of a series of clinically characterized MCC cases, including both MCPyV-positive and -negative cases.
2. Search for altered biological and mechanistic processes through the analysis and interpretation of the mutational data.
3. Explore the potential of immunohistochemical (IHC) surrogate markers to be used as readouts for the activity of deregulated mechanisms in MCC cases.
4. Study the biological implications and the potential prognostic value of these biomarkers.

2.2.2. OBJETIVES SPECIFIC OF ARTICLE 2: Individualized strategies to target specific mechanisms of disease in malignant melanoma patients displaying unique mutational signatures.

We focused on four specific objectives during the progression of this work:

1. Develop a platform to prospectively study somatic mutations affecting a selection of genes in a time compatible with the clinic.
2. Analyze advanced melanoma lesions obtained from patients at diagnosis (non-treated).
3. Explore potential mechanisms that could be useful as targets for specific therapies, independently of *BRAF* mutational status.
4. Study the biological and mechanistic effects of targeted therapies, selected based on case-specific mutational profiles:
   a. *Ex vivo*,
      a.1. using commercial melanoma cell lines.
      a.2. using cell lines directly established from tumor biopsies.
   b. *In vivo*,
      b.1. using cell-line-derived xenografts (CDX).
      b.2. using patient-derived xenografts (PDX).

3. Publication 1

TITLE:

Shared oncogenic pathways implicated in both virus-positive and UV-induced Merkel Cell Carcinomas.

AUTHORS:

M.C. González-Vela *, S. Curiel-Olmo *, S. Derdak, S. Beltrán, M. Santibañez, N. Martínez, A. Castillo-Trujillo, M.Gut, R. Sánchez-Pacheco, C. Almaraz, L. Cereceda, B. Llombart, A. Agraz-Doblas, J. Revert-Arce, J.A. López Guerrero, M. Mollejo, P.I Marrón, P. Ortiz-Romero, L. Fernández-Cuesta, I. Varela, I. Gut, L. Cerroni, M.A. Piris[#] and J.P. Vaqué[#$].

* These authors contributed equally to this work.
[#] Senior authors.
[$] Corresponding author.

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Shared oncogenic pathways implicated in both virus-positive and UV-induced Merkel Cell Carcinomas

González-Vela M.C.[1,2]*, Curiel-Olmo S.[2]*, Derdak S.[3,14], Beltran S.[3,14], Santibañez M[4]., Martínez N.[2], Castillo-Trujillo A.[5], Gut M.[3,14], Sánchez-Pacheco R.[1], Almaraz C.[2], Cereceda L.[2], Llombart B.[6], Agraz-Doblas A.[7,16], Revert-Arce J.[2], López Guerrero J.A.[8], Mollejo M.[9], Marrón, P.I.[10], Ortiz-Romero P.[11], Fernandez-Cuesta L.[12, 15], Varela I.[7], Gut I.[3,14], Cerroni L.[13], Piris M.A.[1,2,#], Vaqué J.P.[2,7,#,$]

[1]Pathology Department, Hospital Universitario Marqués de Valdecilla, Santander, Spain.
[2]Cancer Genomics Laboratory, Instituto de Investigación Marqués de Valdecilla, IDIVAL, Santander, Spain.
[3]CNAG-CRG, Centre for Genomic Regulation (CRG), Barcelona Institute of Science and Technology (BIST), Baldiri i Reixac 4, 08028, Barcelona, Spain.
[4]Universidad de Cantabria-IDIVAL, Santander, Spain.
[5]Oncology Department, Hospital Universitario Marqués de Valdecilla, Santander, Spain.
[6]Department of Dermatology, Instituto Valenciano de Oncología, Valencia, Spain.
[7]IBBTEC-UC-CSIC-SODERCAN Instituto de Biomedicina y Biotecnología de Cantabria, Santander, Spain.
[8]Biobanco -Fundación Instituto Valenciano de Oncología, Valencia, Spain.
[9]Hospital Universitario Virgen de la Salud, Toledo, Spain.
[10]Biobanco-Hospital Universitario Central de Asturias, Oviedo, Spain.
[11]Dermatology Service, Instituto I+12, Hospital Universitario 12 de Octubre, Madrid, Spain
[12]International Agency for Research on Cancer (IARC-WHO).
[13]Department of Dermatology Medical University of Graz, Austria.
[14]Universitat Pompeu Fabra (UPF), Barcelona, Spain.
[15]Department of Translational Genomics, Center of Integrated Oncology Cologne–Bonn, Medical Faculty, University of Cologne, 50931 Cologne, Germany.
[16]Josep Carreras Leukemia Research Institute and School of Medicine. University of Barcelona. Barcelona. Spain.
*These authors contributed equally to this work; [#]Senior author, [$]Corresponding author.

SHORT TITLE: Shared mechanisms of disease in MCC

KEYWORDS: Merkel cell carcinoma, biomarker, survival, polyoma, mutations, signaling, NFAT, JAK-STAT, cAMP-CREB, CALCIUM, YAP, C-MYC

CORRESPONDING AUTHOR: José Pedro Vaqué, PhD

Instituto de Biomedicina y Biotecnología de Cantabria, PCTCAN,

C/ Albert Einstein 22, 39011 Santander, Spain

Tel: +34 674 009195; Fax: +34 942 266399; e-mail: vaquej@unican.es

ABSTRACT

Merkel cell carcinoma (MCC) is a highly malignant neuroendocrine tumor of the skin whose molecular pathogenesis is not completely understood, despite the role that Merkel cell polyomavirus (MCPyV) can play in 55% to 90% of cases. To study potential mechanisms driving this disease in clinically characterized cases, we searched for somatic mutations using whole exome sequencing, and extrapolated our findings to study functional biomarkers reporting on the activity of the mutated pathways. Confirming previous results, MCPyV-negative tumors had higher mutational loads with UV signatures and more frequent mutations in *TP53* and *RB* compared with their MCPyV-positive counterparts. Surprisingly, despite important genetic differences, the two MCC etiologies both exhibited nuclear accumulation of oncogenic transcription factors such as NFAT, P-CREB and P-STAT3, indicating commonly deregulated pathogenic mechanisms with the potential to serve as targets for therapy. A multivariable analysis identified P-CREB as an independent survival factor with respect to clinical variables and MCPyV status in our cohort of MCC patients.

INTRODUCTION

Merkel cell carcinoma (MCC) is a highly malignant neuroendocrine tumor of the skin, with an increasing number of diagnosed cases and deaths attributable to the tumor. MCC has a relatively low incidence, but the mortality rate of diagnosed cases is 33%, which exceeds that of melanoma in the skin (reviewed in (Hughes et al, 2014)). MCC primarily affects older and immunosuppressed patients who, at the time of diagnosis, frequently have advanced clinical stage. This dramatically affects 5-year survival, which

is 64% in patients with localized tumors, 39% in those with loco-regional metastasis, and 18% in those with distant metastasis (Sarnaik et al, 2010).

The origin of MCC remains uncertain. The role of Merkel and/or pluripotent epidermal cells is currently under discussion (Ratner et al, 1993, McCardle et al, 2010). Recent evidence suggests that clonal integration of Merkel cell polyoma virus (MCPyV) is one of the main etiological mechanisms by which MCC develops. It has been found in 55% to 90% of the cases analyzed (Feng et al, 2008, Becker et al, 2009, Duncavage et al, 2009, Garneski et al, 2009, Bhatia et al, 2010). MCPyV viruses express large, small and 57 kDa T antigens that have the potential to inhibit retinoblastoma (RB) activity (by large T antigen) and promote MCC tumorigenesis, although the mechanisms responsible are poorly understood (Stakaityte et al, 2014). Along with MCPyV, there is increasing evidence that sun exposure may also be a major independent etiological factor. MCC most commonly develops in areas of the skin exposed to the sun, such as the head and neck, as in other malignant skin tumors such as melanoma (Lunder and Stern 1998, Davies et al, 2002, group 2009). In this regard, recent work by independent laboratories has revealed important genetic differences between MCPyV-positive (MCPyV+ hereon) and MCPyV-negative (MCPyV- hereon) MCC tumors, the latter harboring higher mutational burdens with UV signatures (Goh et al, 2015, Harms et al, 2015, Wong et al, 2015).

From a molecular perspective, the main mechanisms of transformation promoting MCC are still partially unknown. Mutations in *TP53* have been observed in 14% to 33% of MCC cases. They are considered a rare event in MCCs that are mostly confined to MCPyV-negative cases (Lassacher et al, 2008, Harms et al, 2015). Moreover, mutations affecting *PIK3CA* and deregulated PI3K-mTOR activity have been observed in patient

samples and cell lines (Hafner et al, 2012, Nardi et al, 2012), and alterations in NOTCH and RAS/MAPK signaling pathways (Harms et al, 2015) have been detected in MCCs. However, these advances are not yet used to diagnose or treat the disease. Current treatment options mainly rely on surgical excision combined with loco-regional adjuvant radiation (Harring et al, 2011).

In this study, we have examined a cohort of clinically characterized MCC patients using a combination of whole exome sequencing approaches and a selection of biomarkers, chosen on the basis of the mechanistic interpretation of the genomic data. We characterized two main MCC etiologies in our cohort: MCPyV-positive tumors, which had few mutations and a high survival rate, and MCPyV-negative tumors, which had more genomic alterations and a worse clinical outcome. We detected a number of signaling mechanisms with the potential to participate in the development of MCC, and studied these using specific end-point immunohistochemical biomarkers to examine their activity. We found that MCPyV+ and MCPyV- tumors can share specific disease mechanisms, as implied by the detection of positive NFAT, P-CREB and P-STAT immunostaining in MCC samples. In addition, MCPyV- tumors almost exclusively developed alternative mechanisms of disease, as indicated by C-MYC and LEF1 expression in our sample series with respect to their MCPyV+ counterparts. Finally, we found a clinical correlation between P-CREB nuclear expression and a worse prognosis in MCC patients that was independent of sex, age, clinical stage and MCPyV status.

RESULTS

Inverse correlation between MCPyV and UV mutational signature in MCC

We initially characterized the genomic variants in a series of 15 patients with MCC (henceforth referred to as the 'discovery cohort'; see the clinical data in Table 1 and Supplementary Table S1). The mean age of patients was 75.5 years, and 53% of the study population were male. All samples corresponded to primary tumors located in sun-exposed areas in eight out of 15 cases. We generated two paired-end 101-bp whole-exome sequencing (WES) libraries and sequenced paired tumoral and non-tumoral genomic DNA for each case. This allowed us to uniquely map an average of ~86 million reads per sample, which were analyzed for the presence of somatic mutations as previously described (Martinez et al, 2014, Vaque et al, 2015). Under these conditions, the mean coverage of the target sequence was 105x (53x-170x), with an average of 92% (71.25-97.07) of the targeted bases having at least 15x coverage (see Supplementary Table S2 for a detailed description of the genomic analysis). Finally, our somatic mutation analyses detected 16,198 somatic single-base mutations (SSMs) in tumor samples (range, 29-3644 in the discovery cohort of samples), of which 4913 (range, 5-1275) are responsible for amino-acid changes (Table 1 and Supplementary Tables S3 and S4, respectively).

MCPyV status was determined by immunohistochemistry (IHC) for MCPyV T antigen protein expression in all patients (except patients 8 and 13) followed by a confirmatory PCR in a subset of cases that showed no discrepancies (Supp. Methods). From a genomic perspective, we detected two clearly different subsets of patients. On the one hand, those MCC lesions that were positive for the Merkel polyoma virus (MCPyV+) had a low number of somatic mutations, with an average mutational index of 0.75 amino-acid-changing SSMs/megabase (muts/Mb). On the other hand, lesions that were negative for this virus (MCPyV-) showed higher mutational indexes, averaging 19.81

muts/Mb, which is comparable to values observed in cutaneous melanomas (Berger et al, 2012). We found that all MCPyV- lesions, except that from patient 13, harbored a UV signature defined by the detection of more than 60% C>T transitions at dipyrimidine sites or more than 5% CC>TT mutations of the total number of SSMs in each case (Table 1 and Figure 1a) (Brash 2015, Network 2015). In contrast, this UV signature was not detected in the MCPyV+ cases. Thus, we can conclude that the presence of MCPyV in our discovery cohort was inversely correlated with mutational indexes and the presence of UV signatures.

Common and divergent mechanisms of disease in MCPyV+ and MCPyV- MCCs

Turning our attention to those somatic mutations that cause amino-acid changes, we detected a number of recurrently mutated genes in our discovery cohort, including *FAT4*, *TP53*, *RYR2*, *RPTOR, APC* and *RB1*, amongst others, that were found almost exclusively in MCPyV- MCC lesions. We performed a validation panel over 279 positions, which rendered 98% of validated SSMs (Supplementary Table S5). A list of the most recurrently detected genes with mutations is provided in Supplementary Table S6. To gain a deeper insight into the functional relevance of the mutations found in this study, we performed an unbiased OncodriveFM analysis (described in Supplementary Information and in (Gonzalez-Perez and Lopez-Bigas 2012)) using all the SSMs detected in this work (Supplementary Table S3). This enabled us to detect potential driver genes like *TP53*, *CDK5RAP1*, *FAT4*, *ADAM8*, *GLB1L2*, *OGG1, HIVEP2* and *RB1* with a significant q value (Supplementary Table S7). Moreover, using this approach we were also able to detect a number of significant gene modules from the KEGG pathway that included TP53, small cell lung carcinoma (SCLC), non-small cell lung cancer (NSCLC), melanoma, and basal cell carcinoma, to name but a few

(Supplementary Table S8). We searched for the main biological and biochemical mechanisms/signaling pathways associated with each significant gene found in our analysis alongside those included in the specific KEGG gene modules detected with OncodriveFM (Supplementary Tables S7 and S8). To simplify the presentation of our findings, we grouped these into functional categories. These included specific subsets of KEGG gene sets (Supplementary Table S8) with redundant biological activities (A-F below), or with shared mechanisms (G-I below), as follows: A) focal adhesion and extracellular matrix: including actin cytoskeleton, extracellular matrix, axon guidance, cell adhesion proteins, focal adhesion and tight junction KEGG gene sets; B) metabolism: valine isoleucine degradation, ether lipid metabolism and propanoate metabolism; C) transcriptional regulation: transcriptional misregulation in cancer; D) RAS/MAPK: MAPK signaling; E) PI3K/mTOR: PI3K-AKT and mTOR; F) WNT; G) receptors with tyrosine kinase activity (RTKs): with many RTKs and associated downstream signaling included in multiple KEGG gene sets like for example, neurotrophin signaling, pathways in cancer, non-small cell lung cancer or ERBB signaling, to name but a few; H) cAMP/CREB: included in alcoholism and amphetamine addiction; I) calcium: vascular smooth muscle contraction or glutaminergic signaling and J) other. According to our data, these might act as disease mechanisms in MCC (Figure 1c and Supplementary Table S8).

We next studied the expression of specific MCC biomarkers, such as MCPyV, p63, RB and TP53, in conjunction with specific transcription factors as activated surrogate IHC markers corresponding to the signaling pathways described above. For this purpose, we considered 48 clinically characterized MCC patients (henceforth referred to as the 'validation cohort'), of whom 15 had been included in the genomic study (see the

clinical characteristics in Supplementary Table S1). Along with the aforementioned biomarkers, we analyzed the nuclear expression of B-CATENIN and LEF-1 (WNT pathway), P-CREB (cAMP/CREB), NFAT (calcium), C-MYC and P-STAT (RTKs), in our validation cohort of samples (Table 2). Our results revealed that MCPyV+ lesions had a homogeneous expression landscape, with a uniform staining pattern for specific markers like B-CATENIN (positive in the membrane) and RB (positive) and C-MYC and LEF-1 (negative), and heterogeneous staining for TP53, p63, P-CREB and NFAT. P-STAT was detected in only four of the 26 MCPyV+ cases. On the other hand, MCPyV- lesions showed heterogeneous biomarker expression. 50% of the cases had a loss of RB expression while for C-MYC and LEF-1, expression was detected almost exclusively in MCPyV- cases, but was limited to about half of these cases (Table 2). A representation of IHC markers for a representative MCPyV+ and MCPyV- patient is shown in Figure 2. Despite the different genomic characteristics observed between MCPyV+ and MCPyV- lesions, it is possible that the two MCC etiologies share common disease mechanisms (TP53, RB, NFAT, P-STAT and P-CREB), with MCPyV- lesions developing additional molecular features.

Biological relevance derived from the characterization of MCC tumors

To explore the clinical implications of the molecular mechanisms (biomarkers) identified in this work, we studied their association with the clinical outcome of the patients in our validation cohort. The clinical data for each patient are summarized in Supplementary Table S1. We analyzed the death events caused by MCC in different sets of patient tumors characterized with respect to the positive or negative expression of each biomarker depicted as follows: A) biomarkers already studied by other groups in different cohorts of patients: MCPyV, TP53, RB and p63; and B) biomarkers

expressed in both MCPyV+ and MCPyV- tumors: NFAT, P-CREB and P-STAT. We could not include biomarkers in this study that were mainly expressed in MCPyV- tumors (C-MYC and LEF1) alone, due to the limited cohort size. As expected, and in accordance with previous observations by other laboratories (Carson et al, 1998, Sihto et al, 2011, Waltari et al, 2011, Nardi et al, 2012), MCC patients with tumors that were negative for the expression of MCPyV and RB tended to have a poorer clinical outcome, whereas, at least in our hands, TP53, p63 and NFAT did not show this tendency (Supplementary Figure 1).

Interestingly, those cancers that were positive for the expression of P-CREB or P-STAT showed significantly shorter survival than their negative counterparts (p=0.011 and p=0.024, respectively), with a crude hazard ratio (HRc) of 3.89 for P-CREB and 3.37 for P-STAT (Figure 3). We decided to perform multivariable analyses of P-CREB and P-STAT including clinical data (age, sex and stage) with MCPyV status and detected stage as a confounding variable (not shown). After including all the above variables in the same multivariable model, only P-CREB-positive status remained as an independent predictor of mortality: adjusted HR (HRa) P-CREB 5.56; 95%CI (1.22-25.33) (Figure 3).

DISCUSSION

MCC is a type of cancer with increasing incidence and a high mortality rate. Despite recent advances in our understanding of the main biological mechanisms involved in its clinical evolution, we still know little about the molecular biology of this disease, as is reflected by the current lack of any specific therapy (Becker 2010, Hughes et al, 2014).

In this study, we used a combination of genomic techniques and clinical data to mechanistically and biologically characterize MCC tumors. These methods included deep-sequencing approaches (WES) in samples from 15 patients (the discovery cohort) and a selection of immunohistochemical markers in 48 samples from MCC patients (the validation cohort).

First, we found different genetic patterns of disease mechanisms in our cohort of patients with MCPyV+ and MCPyV- MCC tumors. Consistent with previous observations, our cohort of MCPyV- tumors accumulated most of the detected somatic mutations, giving an average mutational index (MI) of 20 amino-acid-changing mutations/Mb, and a UV signature comparable to that of malignant melanoma (C>T at dipyrimidine sites above 60% of the total SSMs) (Goh et al, 2015, Harms et al, 2015, Network 2015, Wong et al, 2015). By contrast, MCPyV+ tumors had lower mutational indexes (average MI=0.75 amino-acid-changing mutations/Mb) that can be similar to those observed for small B-cell lymphomas (Vaque et al, 2014). To provide our mutational data with a functional interpretation we used an unbiased approach (OncodriveFM), which enabled us to identify specific genes and pathways that were significantly altered in our MCC samples, presumably with the potential to participate as disease mechanisms in MCC. We explored this possibility by focusing on the expression of a group of transcription factors as endpoint surrogates for the activity of the genes and signaling pathways identified in this study. These included B-Catenin and LEF-1 (WNT pathway (Duchartre et al, 2016)), NFAT (calcium, reviewed in (Mognol et al, 2016)), P-CREB (cAMP/CREB signaling; (Rodriguez and Setaluri 2014)), P-STAT (RTKs signaling, reviewed in (Yu et al, 2014)) and C-MYC (RAS/MAPK, PI3K and RTKs signaling pathways; (Kress et al, 2015)), along with MCPyV, TP53, p63 and

RB (previously identified MCC biomarkers; (Zager et al, 2011)) (Figures 1 and 4). Interestingly, our data enabled a number of MCC mutations (with a previous COSMIC-ID, as shown in Supplementary Table S3) to be associated with specific biomarker expression in MCC samples from the validation cohort (Table 2 and Figure 1). For example, in patient 5 we detected two potentially inactivating *TP53* mutations provoking amino-acid changes, R280K (COSM129830) and H47Y (COSM129851), and a truncating *RB1* mutation (W195*, COSM214151) with positive (TP53) and negative (RB) protein expression. These can be due to a direct effect on a specific gene but we cannot rule out potential indirect effects of specific gene mutations on the activity of its associated signaling pathway (see Figure 4). In this regard and to serve as an example, *HIVEP2* (also known as Myc Intron Binding Protein 1, MIBP1) is a significantly mutated gene in our analysis, detected in patients 1, 3, 7 (missense mutations) and in patient 6 (a truncating mutation). In our work, IHC data were positive for C-MYC in patients 1, 3 and 6, suggesting a potential correlation between MIBP1 mutations (presumably inactivating) and C-MYC expression. Furthermore, it has been shown that MIBP1 can inhibit transcription of *C-MYC* (Iwashita et al, 2012) and the expression of both genes has been shown to be inversely correlated in human cells (Zajac-Kaye et al, 2000). Also, *ERBB4* mutations in patients 1 (two missense mutations) and 6 (R711C, COSM160827) could contribute to activate c-MYC and STAT (see also Figure 4). On the other hand, we detected a large number of mutations in adenylate cyclases and GPCRs that could participate in cAMP/CREB activation, for example ADCY10-P241H (COSM899133) and GRM3-E538K (COSM229505), which were found in patient 1 (P-CREB-positive). In our study, perhaps with the exception of TP53, we did not detect hotspot mutations with functional validation to predict activation or repression of a specific signaling pathway. It is possible that pursuing the study of

inactivating mutations in *MIBP1* or presumably activating mutations such as ERBB4-R711C could improve our ability to understand important MCC oncogenic mechanisms. Nevertheless, it is conceivable that by analyzing the expression of a specific subset of biomarkers in MCC samples we could detect deregulated mechanisms of disease, as might be expected given the mutational data. From a mechanistic perspective, and despite substantial differences in the number of mutations, the MCPyV+ and MCPyV- tumors examined here unexpectedly shared several deregulated signaling mechanisms, as indicated by the detection of specific biomarkers such as P-STAT, P-CREB and NFAT in samples from our validation cohort of MCC patients. On the other hand, we also found that C-MYC and LEF1 were expressed almost exclusively in MCPyV- cases. At first glance, this could be due to nothing more than the accumulation of somatic mutations in this type of MCC tumor. However, MCPyV+ cases showed activated P-STAT-, P-CREB- and NFAT-associated signaling in the absence of specific mutations, resulting in the mechanistic mimicry of MCPyV- tumors. In our opinion, these findings suggest that MCPyV- tumors with high mutational loads can acquire specific biological properties through the activation of alternative oncogenic pathways including, but not restricted to, those detected in this work: C-MYC and LEF1. Our results strongly suggest a need for further studies that would determine their contribution to the biology and targeted therapy of this disease.

From a biological perspective, the ability of a range of markers to predict a worse clinical evolution of MCC patients has been examined. Of these, MCPyV- tumors (Nardi et al, 2012), p63 expression (Asioli et al, 2007) and the percentage of MCC cells expressing Ki67 (Llombart et al, 2005) have been proposed as factors associated with bad prognosis. We found a tendency towards worse survival in patients characterized

with specific biomarkers used individually: MCPyV (negative), and RB (negative). Under these circumstances, it is possible that more significant results could be obtained from additional methods that attempt to better determine the status of specific biomarkers such as TP53, or from increasing the cohort size. Notably, two mechanisms shared by MCPyV+ and MCPyV- MCC tumors were highly significant predictors of survival: P-CREB and P-STAT. A multivariable analysis detected P-CREB as being a strong predictor of mortality independent of sex, age, stage, MCPyV status and P-STAT. Thus, our findings argue for a potential role for these disease mechanisms in the biological evolution of both types of MCC lesions. Further efforts should be made to explore the biological role of these markers in larger series of samples from clinically characterized MCC patients. Our results also suggest that a better characterization of the molecular mechanisms that can control CREB activation in MCC cancers with different MCPyV status could be useful for developing novel tools for diagnosis, prognosis and treatment of this disease.

In summary, we characterized a cohort of MCC patients using an original combination of genomic and IHC approaches applied to this field. Confirming previous data, we found two distinct etiologies with clearly divergent mutational signatures. Despite these differences, the data arising from our work show that MCPyV+ and MCPyV- MCC tumors can develop similar mechanisms of disease with clinical implications for patient survival as well as the potential to serve as targets for therapy. Moreover, a subset of MCC patients with CREB and/or STAT activation developed a more aggressive disease that was associated with worse survival. In fact, within our patient cohort, we found P-CREB to be a strong independent survival factor for this disease. Thus, in the current MCC clinical setting, upon mechanistic characterization of MCC tumors at diagnosis,

we may be able to predict poorer outcomes and explore novel approaches for specific therapy.

MATERIALS AND METHODS

Ethics statement. All human samples used in this study were collected following the Declaration of Helsinki protocols after obtaining written informed consent from each patient and the doctors involved, as required by the CEIC (Comité Ético de Investigación Clínica, Cantabria). We kept the original records under specific restricted conditions to fulfill the current legal requirements. All processes were approved and conducted in adherence with the specific recommendations of the CEIC.

Patient samples. Samples from 48 clinically characterized MCC patients were used (validation cohort; Supplementary Table S1). From these, 15 patients (discovery cohort; Table 1 and Supplementary Table S1) with a total of 30 paired (non-tumoral and tumoral) FFPE and freshly frozen samples were selected for whole exome sequencing analysis. Patient cases were included consecutively.

CONFLICTS OF INTERESTS

None of the authors declares any conflicts of interest.

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Table 1. Clinical and genomic characteristics of patients in the discovery cohort.

PATIENT	SEX	AGE	LESION LOCALIZ.	STAGE	MCPyV	SAMPLE TYPE	C>T DIPYRIMIDINE	% C>T DIPYRIMIDINE	CC>TT	% CC>TT	OTHER SUBSTITUTIONS	% OTHER SUBSTITUTIONS	TOTAL SSMs	TOTAL SSMs AA change	M.I. (aa chang. SSMs/Mb)
1	M	83	Leg	IIIA	Negative	FF	1875	83.71	6	0.27	365	16.03	2240	722	24.07
2	M	94	Ear	IV	Negative	FFPE	1562	79.17	16	0.81	411	20.02	1973	622	20.73
3	M	74	Nose	IA	Negative	FFPE	296	70.14	3	0.71	126	29.15	422	132	4.40
4	M	81	Face	IIA	Negative	FF	1875	75.88	12	0.49	596	23.63	2471	666	22.20
5	F	78	Face	IV	Negative	FF	1995	75.45	16	0.61	649	23.94	2644	680	22.67
6	F	92	Leg	IV	Negative	FF	1306	72.72	8	0.45	490	26.84	1796	588	19.60
7	F	75	Face	IIA	Negative	FFPE	2562	70.31	11	0.30	1082	29.39	3644	1275	42.50
8	F	80	Leg	IIIA	Positive	FF	11	37.93	0	0.00	18	62.07	29	7	0.23
9	M	72	Arm	IIIA	Positive	FFPE	143	33.57	0	0.00	283	66.43	426	106	3.53
10	F	88	Thigh	IIA	Positive	FF	12	21.05	0	0.00	45	78.95	57	13	0.43
11	M	86	Leg	IIIB	Positive	FF	17	26.98	0	0.00	46	73.02	63	9	0.30
12	F	80	Face	IV	Positive	FF	9	36.00	0	0.00	16	64.00	25	8	0.27
13	M	69	Lip	IV	Negative	FFPE	71	21.91	0	0.00	253	78.09	324	71	2.37
14	F	80	Face	IIA	Positive	FF	6	16.22	0	0.00	31	83.78	37	5	0.17
15	F	81	Leg	IIB	Positive	FF	8	17.02	0	0.00	39	82.98	47	9	0.30

Table 2. Immunohistochemistry detection of specific MCC biomarkers in the validation cohort characterized patients.

PATIENT	B-CATENIN	p63	NF-ATC1	P-CREB	P-STAT3	p53	RB	MYC	LEF1-L	POLYOMA
9	Membrane	Positive	Positive	Positive	Negative	Negative	Positive	Negative	Negative	Positive
10	Membrane	Negative	Positive	Positive	Positive	Negative	loss	Negative	Negative	Positive
11	Membrane	Negative	Positive	Negative	Negative	Positive	Positive	Negative	Negative	Positive
12	Membrane	Positive	Positive	Positive	Negative	Negative	Positive	Negative	Negative	Positive
14	Membrane	Positive	Positive	Negative	Negative	Negative	Positive	Negative	Negative	Positive
15	Membrane	Positive	Negative	Negative	Negative	Positive	Positive	Positive	Negative	Positive
16	Membrane	Negative	Negative	Negative	Negative	Negative	Positive	Negative	Negative	Positive
17	Negative	Negative	Negative	Negative	Negative	Negative	Positive	Negative	Negative	Positive
18	Membrane	Negative	Negative	Negative	Negative	Negative	loss	Negative	Negative	Positive
19	Membrane	Positive	Negative	Negative	Positive	Negative	Positive	Negative	Negative	Positive
20	Membrane	Positive	Positive	Negative	Negative	Positive	Positive	Negative	Negative	Positive
21	Membrane	Positive	Negative	Positive	Negative	Positive	Positive	Negative	Negative	Positive
22	Membrane	Negative	Negative	Negative	Negative	Negative	Positive	Negative	Negative	Positive
23	Membrane	Negative	Negative	Positive	Negative	Negative	Positive	Negative	Negative	Positive
24	Membrane	Positive	Positive	Negative	Positive	Negative	Positive	Negative	Negative	Positive
25	Membrane	Negative	Negative	Negative	Negative	Negative	Positive	Negative	Negative	Positive
26	Negative	Negative	Negative	Negative	Negative	Negative	Positive	Negative	Negative	Positive
27	Membrane	Negative	Negative	Negative	Negative	Negative	Positive	Negative	Negative	Positive
8	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	Positive
28	Membrane	N/D	Negative	Positive	Negative	Positive	Positive	Negative	Negative	Positive
29	Membrane	Negative	Negative	Negative	Negative	Positive	Positive	Negative	Negative	Positive
30	Membrane	Negative	Positive	Negative	Negative	Negative	Positive	Negative	Negative	Positive
31	Membrane	Positive	Positive	Negative	Negative	Negative	Positive	Negative	Negative	Positive
32	Membrane	Positive	Positive	Positive	Negative	Positive	Positive	Negative	Positive	Positive
33	Membrane	Positive	Negative	Positive	Negative	Positive	Positive	Negative	Negative	Positive
34	Membrane	Positive	Positive	Positive	Negative	Positive	Positive	Negative	Negative	Positive
35	Membrane	Positive	Positive	Negative	Positive	Positive	Positive	Negative	Negative	Positive
1	Negative	Positive	Negative	Positive	Positive	Negative	Positive	Positive	Positive	Negative
2	Negative	Negative	Negative	Negative	Negative	Positive	loss	Negative	Positive	Negative
3	Membrane	Positive	Negative	Positive	Positive	Positive	Positive	Positive	Negative	Negative
4	Negative	Positive	Negative	Positive	Negative	Positive	loss	Negative	Negative	Negative
5	Membrane	Positive	Positive	Negative	Negative	Positive	loss	Positive	Negative	Negative
6	Membrane	Positive	Negative	Negative	Positive	Positive	Positive	Positive	Negative	Negative
7	Negative	Positive	Negative	Positive	Negative	Positive	Positive	Negative	Negative	Negative
13	N/D	N/D	Negative	N/D	N/D	N/D	N/D	N/D	N/D	Negative
36	Membrane	Positive	Positive	Negative	Negative	Positive	loss	Negative	Negative	Negative
37	Membrane	Positive	Negative	Positive	Positive	Positive	loss	Positive	Positive	Negative
38	Membrane	Positive	Negative	Positive	Positive	Positive	loss	Negative	Negative	Negative
39	Membrane	Positive	Positive	Positive	Negative	Positive	loss	Negative	Negative	Negative
40	Negative	Positive	Negative	Positive	Positive	Negative	loss	Negative	Negative	Negative
41	Membrane	Negative	Negative	Negative	Positive	Negative	Positive	Positive	Negative	Negative
42	Membrane	Positive	Negative	Positive	Negative	Negative	Positive	Negative	Positive	Negative
43	Membrane	Negative	Negative	Negative	Negative	Positive	loss	Positive	Negative	Negative
44	Negative	Negative	Positive	Negative	Positive	Positive	loss	Negative	Negative	Negative
45	Membrane	Negative	Negative	Negative	Negative	Negative	loss	Negative	Negative	Negative
46	Membrane	Positive	Positive	Positive	Positive	Negative	Positive	Positive	Negative	Negative
47	Negative	Negative	Positive	Positive	Positive	Negative	Positive	Negative	Positive	Negative
48	Membrane	Positive	Positive	Positive	Positive	Negative	Positive	Positive	Positive	Negative

FIGURE LEGENDS

Figure 1. Inverse correlation between MCPyV expression and UV mutational signature in MCC. Tumor samples from the discovery cohort are presented from left to right. A) Mutation spectra for each sample, showing the percentage of C>T transitions in dipyrimidine sites (violet), the percentage of CC>TT mutations (orange) and other substitutions (green); B) mutational index (MI) calculated as the number of amino-acid-changing mutations per megabase and MCPyV presence (black boxes) or absence (white boxes). C) Mutated genes (written vertically on the left side and grouped into gene-sets) are represented for each sample (colored box). Mutation type is highlighted in a colored box: missense (blue), nonsense (yellow) and UTR region (pink).

Figure 2. Pathway characterization of MCPyV+ and MCPyV- MCC tumors. Immunohistochemical analyses of two representative cases of MCPyV+ (*left;* Patient 19), and MCPyV- (*right;* Patient 37). The IHCs show staining for the indicated marker in each case. In Patient 37 staining for NFAT is negative in the nucleus of the tumoral cell while there are some positive lymphocytes. Staining for P-CREB shows nuclear positivity in tumor cells. Scale bar=100µM.

Figure 3. Association of survival of MCC patients with specific characterization. Kaplan-Meier curves showing percentage of survival from patients with positive (red) or negative (black) expression of the indicated biomarkers and their respective p values: P-CREB (p=0.011) and P-STAT (p=0.024). The table below shows the crude hazard ratio (HRc), and hazard ratio adjusted for P-STAT3, P-CREB, MCPyV status, sex, age and stage (HRa), with 95% confidence intervals (95% CIs).

Figure 4. Deregulated mechanisms of disease in MCC. Schematic representation of the main signaling pathways found to be altered in MCC. Pink boxes indicate recurrently mutated genes in our discovery cohort. Percentage of expression for each biomarker in our validation cohort is represented in maroon (MCPyV+) or yellow (MCPyV-) boxes. EM: extracellular matrix.

Table 1. Clinical and genomic data from discovery cohort patients. The table shows clinical data of 15 MCC cases, including sex, age at diagnosis, location of the lesion and stage. The genomic data from each patient are also represented. MCPyV: detection of MCPyV; SAMPLE TYPE: types of samples from where genomic DNA was extracted, FF: freshly frozen, FFPE: formaldehyde-fixed paraffin-embedded; C>T DYPIRIMIDINE: number of C>T transitions found at dipyrimidine sites; %: percentage; OTHER SUBSTITUTIONS: number of other single somatic single-base mutations; TOTAL SSMs: total number of single somatic mutations; TOTAL SSMs AA change: single somatic nucleotide variants that provoke amino-acid changes; MI (aa change. SSMs/Mb): the mutational index, calculated as the total number of SSNVs that cause an amino acid change per megabase. PA.: Patient, LOC.: Localization, DIPYR.:Dipyrimidine, SUBST.: Substitution.

Table 2. Immunohistochemical detection of specific MCC biomarkers in the validation cohort. Assessment of the expression of specific markers by immunohistochemistry. All these markers are related to pathways detected by an unbiased approach as being especially altered in tumor samples. The table shows the results for the study of B-CATENIN, p63, NF-ATC1, P-CREB, P-STAT-3, TP53, RB1, C-MYC and LEF1-L, as well as Polyomavirus: MCPyV+ (red) or MCPyV- (green). Grey boxes indicate potentially pathogenic markers.

Figure 1. Inverse correlation between MCPyV expression and UV mutational signature in MCC.

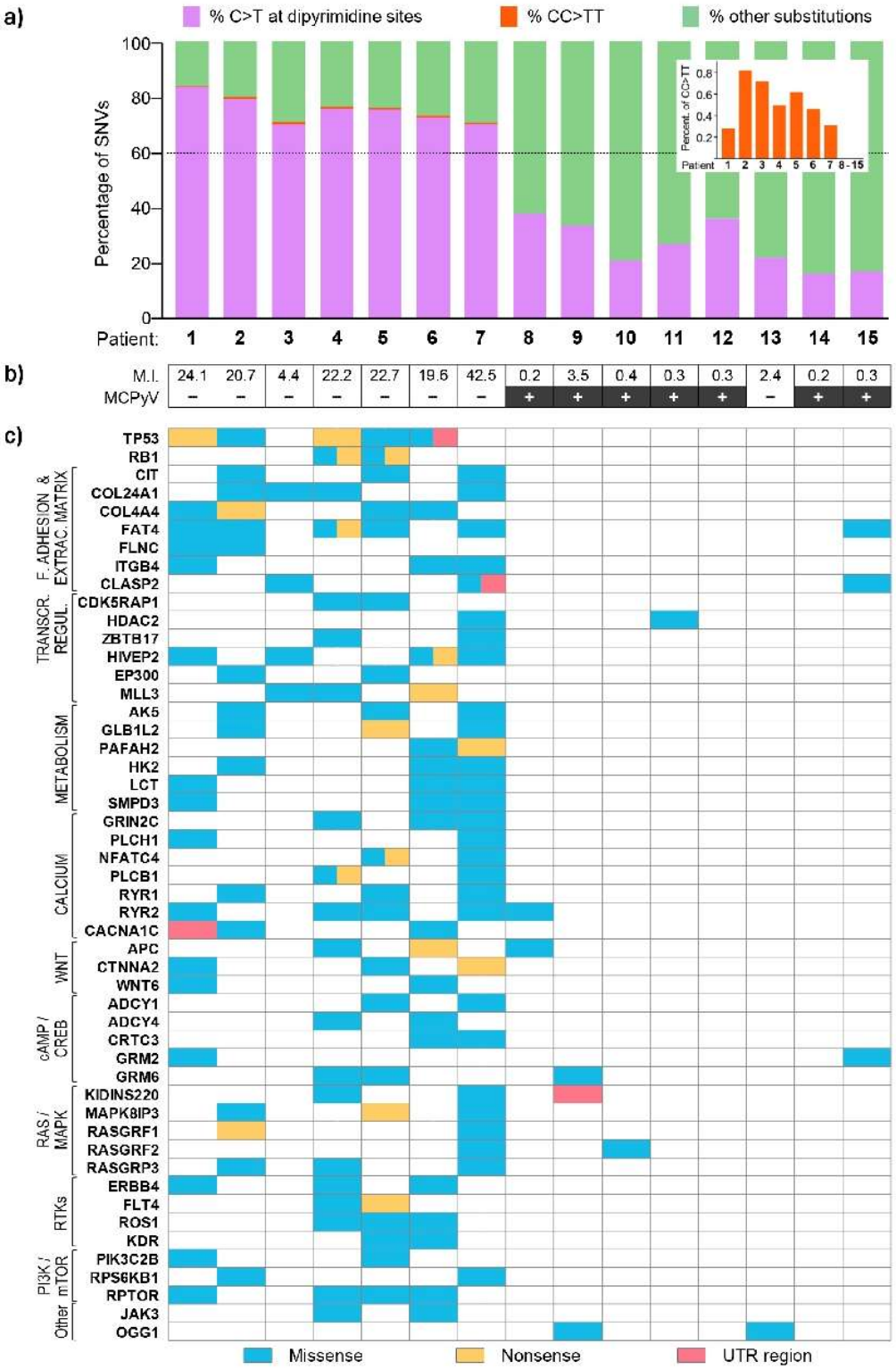


Figure 2. Pathway characterization of MCPyV-positive and MCPyV-negative MCC tumors.

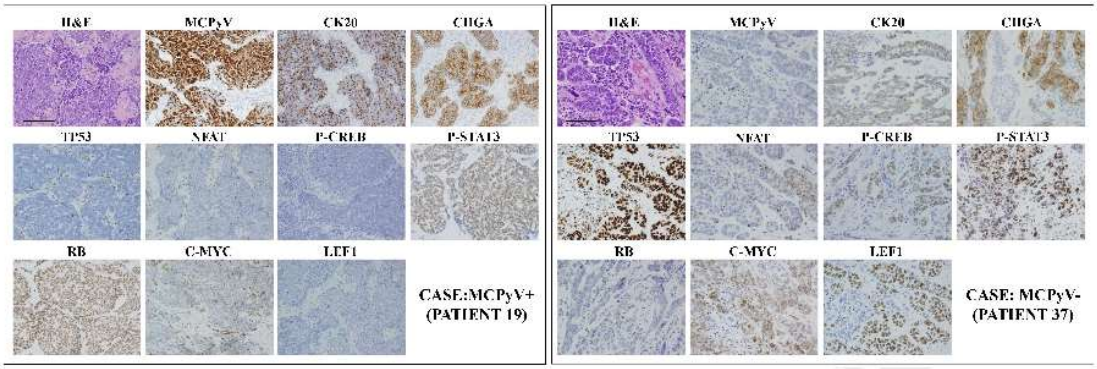
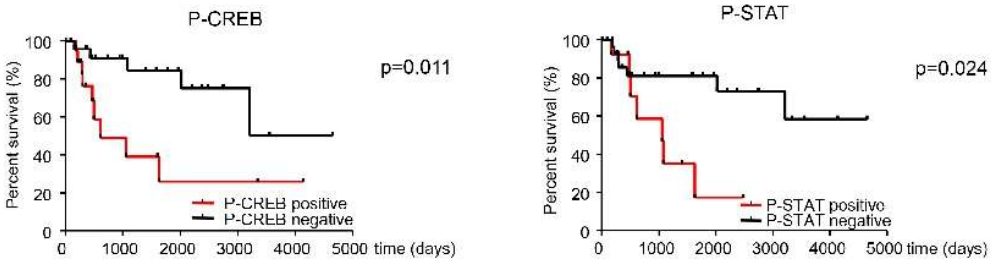


Figure 3. Association of survival of MCC patients with specific characterization.

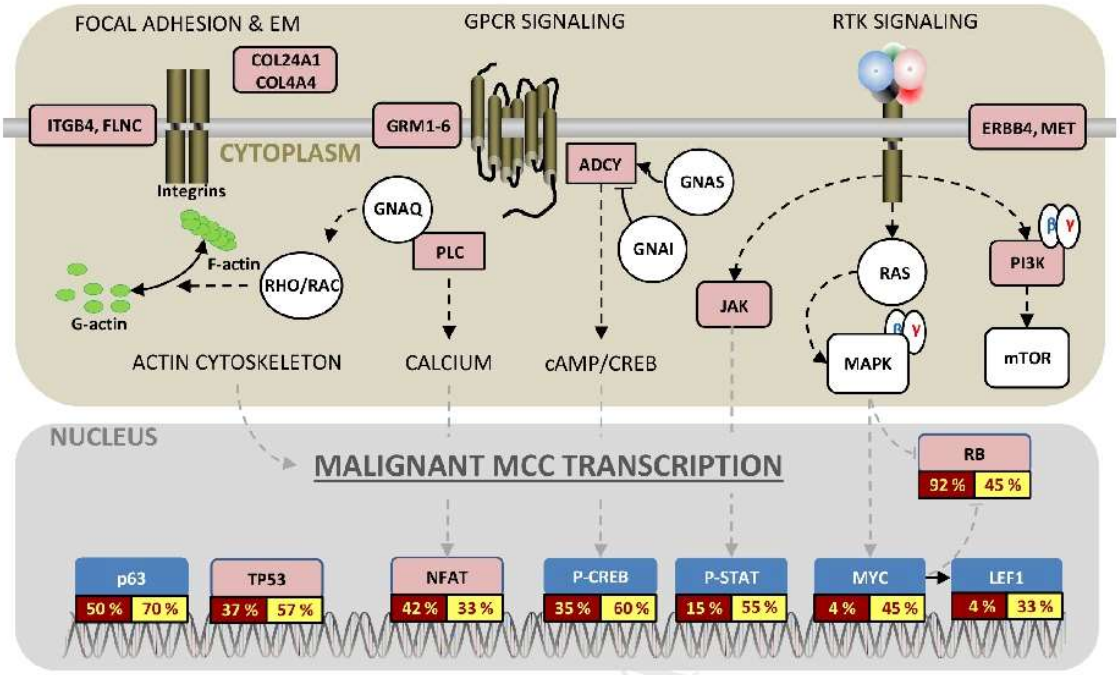


Hazard Ratios for P-CREB and STAT markers, in relation to mortality

	Vital status Death N=15	Survival N=33	HRc[a]	(95%	CI)	HRa[b]	(95%	CI)
P-CREB								
Negative	5	20	1	--		1	--	
Positive	9	12	3.89	1.28	11.87	5.56	1.22	25.33
P-STAT								
Negative	7	24	1	--		1	--	
Positive	7	8	3.37	1.1	10.3	1.65	0.22	12.35

a HRc = Crude Hazard Ratio.
b HRa=Hazard Ratio adjusted for P-CREB, P-STAT, MCPyV status, sex, age and stage.

Figure 4. Deregulated mechanisms of disease in MCC.



This article has supplementary material. It is available in an attached CD, located in the back cover of this thesis.

4. Publication 2

TITLE:

Individualized strategies to target specific mechanisms of disease in malignant melanoma patients displaying unique mutational signatures.

AUTHORS:

S. Curiel-Olmo*, A. García-Castaño*, R. Vidal, H. Pisonero, I. Varela, A. León-Castillo, E. Trillo, C. González-Vela, N. García-Díaz, C. Almaraz, T. Moreno, L. Cereceda, R. Madureira, N. Martínez, P. Ortiz-Romero, E. Valdizán, M.A. Piris[#] and J.P. Vaqué[#$].

* These authors contributed equally to this work.
[#] Senior authors.
[$] Corresponding author.

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Individualized strategies to target specific mechanisms of disease in malignant melanoma patients displaying unique mutational signatures

Soraya Curiel-Olmo[1,*], Almudena García-Castaño[2,*], Rebeca Vidal[3,4,8], Helena Pisonero[1], Ignacio Varela[4], Alicia León-Castillo[1,5], Eugenio Trillo[6], Carmen González-Vela[1,5], Nuria García-Diaz[1], Carmen Almaraz[1], Thaidy Moreno[4], Laura Cereceda[1], Rebeca Madureira[1], Nerea Martinez[1], Pablo Ortiz-Romero[7], Elsa Valdizán[3,4], Miguel Angel Piris[1,5,#], José Pedro Vaqué[1,4,#]

[1]Cancer Genomics Group, IDIVAL, Instituto de Investigación Marqués de Valdecilla, Santander, Spain

[2]Oncology Service, Hospital Universitario Marqués de Valdecilla, Santander, Spain

[3]Department of Pharmacology, University of Cantabria (UC), Santander, Spain, and Centro de Investigación Biomédica en Red de Salud Mental (CIBERSAM), ISCIII, Madrid, Spain

[4]Instituto de Biomedicina y Biotecnología de Cantabria (IBBTEC), CSIC, Universidad de Cantabria, Departamento de Biología Molecular, Universidad de Cantabria, Santander, Spain

[5]Pathology Service, Hospital Universitario Marqués de Valdecilla, Santander, Spain

[6]Plastic Surgery Service Hospital Universitario Marqués de Valdecilla, Santander, Spain

[7]Dermatology Service, Instituto I+12, Hospital Universitario 12 de Octubre, Madrid, Spain

[8]Department of Pharmacology, Medicine School, Complutense University, Madrid, Spain

[*]These authors have contributed equally to this work

[#]Senior author

Correspondence to:
José Pedro Vaqué, **e-mail:** vaquej@unican.es

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ABSTRACT

Targeted treatment of advanced melanoma could benefit from the precise molecular characterization of melanoma samples. Using a melanoma-specific selection of 217 genes, we performed targeted deep sequencing of a series of biopsies, from advanced melanoma cases, with a Breslow index of ≥4 mm, and/or with a loco-regional infiltration in lymph nodes or presenting distant metastasis, as well of a collection of human cell lines. This approach detected 3–4 mutations per case, constituting unique mutational signatures associated with specific inhibitor sensitivity. Functionally, case-specific combinations of inhibitors that simultaneously targeted MAPK-dependent and MAPK-independent mechanisms were most effective at inhibiting melanoma growth, against each specific mutational background. These observations were challenged by characterizing a freshly resected biopsy from a metastatic lesion located in the skin and soft tissue and by testing its associated therapy *ex vivo* and *in vivo* using melanocytes and patient-derived xenografted mice, respectively.

The results show that upon mutational characterization of advanced melanoma patients, specific mutational profiles can be used for selecting drugs that simultaneously target several deregulated genes/pathways involved in tumor generation or progression.

INTRODUCTION

Melanoma is a form of cancer whose incidence is rising each year in the developed world, and is second to leukemia in terms of loss of years of potential life from cancers [1]. Despite recent improvements in mortality rates, current deaths from melanoma are estimated to comprise 85% of all cancers affecting the skin. This is corroborated by the poor survival associated with melanoma when diagnosed at an advanced stage [2]. Therefore, the development of effective therapies is a major challenge in this field.

Molecular diagnostics of cancer have proved that targeted therapies can be effective in many cancer settings, as measured by the recent improvement in cancer survival statistics (World Cancer Statistics 2008; ICD-10 C18–21). The use of EGFR inhibitors in lung cancer [3, 4] and Imatinib in chronic myeloid leukemia (CML) patients [5] are two relevant examples. Targeted therapies in melanoma are mostly directed towards inhibiting MAPK-ERK1/2 signaling (MAPK hereafter), [6]. Mutational analyses have recently enabled the detection of up to 50% of malignant melanomas carrying an activating mutation in *BRAF* [7], and these can now be treated with specific B-RAF inhibitors [8]. In the clinic, this targeted approach, even when used in combination with MEK inhibitors, is of limited benefit to patient survival and, after a period, the cancer reappears aggressively [9–11].

From a molecular perspective, data from Next Generation Sequencing (NGS) show that more mutated genes than initially expected participate in tumorigenesis, including that of melanoma [12–14]. This involves a dynamic process of subclonal competition that eventually dictates multifactorial clinical resistance to B-RAF inhibitors, which is dependent on reactivation of MAPK signaling or other proliferative and/or pro-survival pathways [15–17].

Taking advantage of available melanoma NGS data, we characterized biopsies from advanced melanoma patients and cell lines by studying the presence of somatic mutations in a selected group of genes. We thereby detected unique signatures of mutated genes that are potentially associated with specific inhibitors, and explored the effects of case-specific combinations of the latter *ex vivo* and *in vivo*. Guided by individual mutational profiles, tailored combinations of inhibitors simultaneously targeting MAPK-dependent and MAPK-independent signaling were very efficient at inhibiting aberrant melanoma growth assessed in multiple cell lines, and xenografted tumors and biopsies grown in mice. Thus, specific mutational signatures could guide the design of personalized therapies based on the use of specific combinations of drugs that target case-specific pathogenic signaling mechanisms.

RESULTS

A targeted approach to characterizing the mutational status of lesions of advanced melanoma patients

To better understand the molecular character of specific melanoma lesions, we set up a targeted mutational study followed by functional analyses (described in Supplementary Figure 1). The genomic design of this study focused on the coding regions of a specific group of 217 genes that had previously been shown to be mutated in melanoma and selected mainly on the basis of their relevance in melanoma and their association with inhibitors of potential clinical use (see Materials and Methods for further explanation). To test this approach our selection of genes was compared *in silico* with the whole genome/exome sequencing (WGS/WES respectively) data already available for 11 advanced melanoma cell lines and 158 human melanomas (see Materials and Methods, [13, 14, 18, 19]). This comparison revealed an average of 3.74 mutated genes that can participate in multiple targetable signaling pathways, including PLC, MAPK, RTKs (receptors with tyrosine kinase activity), PI3K-mTOR and JAK-STAT (Figure 1 and Supplementary Table I). These results prompted us to study advanced melanoma cases (Breslow index ≥4 mm or metastasis) in 18 clinically characterized patients (clinical characteristics summarized in Supplementary Table II) using a targeted primary ultrasequencing approach, followed by secondary validation analysis (see Materials and Methods for further details). By these methods, an average of 3.4 mutated genes were identified in 11 of the 18 patients, enabled the detection of lesion-specific genes such as *BRAF*, *RAC1*, *KRAS*, *HGF* and *MAPK7*, amongst others. Interestingly, there was a wide range of mutation frequencies and combinations, which perhaps reflects the rich and heterogeneous microclonal composition expected in melanoma tumors (400X average depth/mutation; Table I) [20]. Furthermore, actionable mutations such as $BRAF^{V600E}$ that can guide targeted therapy (using B-RAF inhibitors) were detected in the same melanoma alongside other mutated genes that may also guide therapy (Table I). It is significant that mutations in four patients could not be validated due to limitations of the tissue sample (see Materials and Methods), and that no mutations were identified in three other patients. Thus, this targeted approach could be adopted to identify genomic alterations affecting one or several genes. These may be explored as potential targets for therapy in specific cases of melanoma.

Effects of specific targeted therapy guided by mutational signature

To explore how to use mutational data to design targeted therapies based on specific mutational

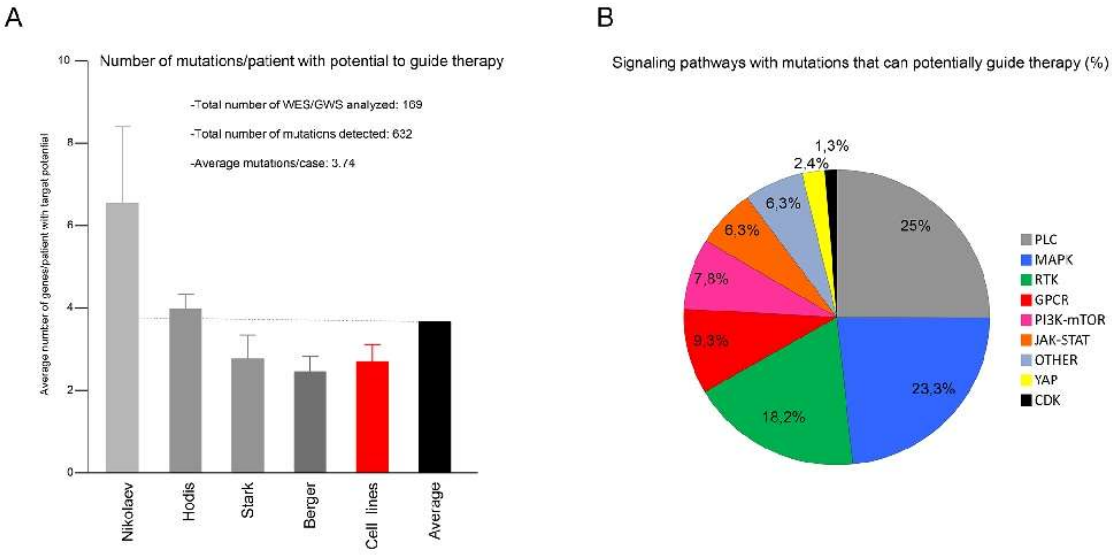

Figure 1: *In silico*-targeted mutational profiling of advanced melanoma patients. A. Meta-analysis showing the average number of mutated genes per case with the potential to guide targeted therapy. Original mutational data from cell lines (red bar) were obtained from the Cancer Cell Line Encyclopedia website (see Material and Methods); mutational data from patients (grey bars) were obtained from Nilolaev [19], Hodis [18], Stark [14] and Berger [13]; Black bar, shows the average frequency of mutations amongst all data sets. **B.** Percentage of hits in A) involved in the indicated signaling pathway.

characterizations, the functional effects of specific combination therapies were studied in advanced melanoma cell lines with known mutational profiles (Supplementary Table III). Taking A375 advanced melanoma cells as an example, we detected and validated mutations in *BRAF*, *FGFR2* and *mTOR* that could reasonably be expected to associate with Vemurafenib (BRAFi (V), hereafter), Vargatef (FGFR2i (Va)) and Everolimus (mTORi (E)). Exponentially growing A375 cells were incubated with increasing concentrations of each inhibitor. This caused a concentration-dependent reduction in cell proliferation from which the IC_{50} of each inhibitor was calculated (Figure 2A and Supplementary Table III). These concentrations were used for subsequent experiments. Next, the mechanistic effects of treatment with each inhibitor (using IC_{50} values in each case) were analyzed in A375 cells that had been serum-starved to provoke the inhibition of the intended mutation-associated downstream signaling. These were assessed by western blot using P-ERK1/2, P-p38 and P-S6 antibodies (Figure 2B).

To discover more about the biological effects of multiple combinations of these inhibitors on proliferation, A375 cells were incubated with IC_{50} concentrations of BRAFi, FGFR2i and mTORi in single, double or triple combinations (blue, green and red lines, respectively, in Figure 2C). The combinatorial treatments were more effective at reducing melanoma cell growth than the monotherapies. The triple combination was the most efficient, and had no non-specific cytotoxic effects (Figure 2C and 2E). These results were confirmed using DNA synthesis as an alternative read-out (Figure 2D and 2E). Thus, under these conditions, a combination of inhibitors guided by a specific mutational signature, simultaneously targeted multiple signaling mechanisms controlling the growth of A375 cells. Analyzing the mechanistic effects of these drug combinations on their associated signaling pathways in this system, showed that treatment with BRAFi inhibited MAPK signaling. However, treatment of A375 cells with the inhibitors mTORi and FGFR2i, alone or in combination (E+Va), had no such effect (Figure 2F and 2G), despite being very effective at inhibiting cell proliferation and DNA synthesis (Figures 2C, and 2E). Thus, using genetically defined inhibitors in this system we can specifically target a combination of MAPK-dependent (V) and MAPK-independent (E+Va) signaling mechanisms that control the malignant growth of A375 melanoma cells. This observation was not confined to these cells and more examples of specific mutational signatures guiding effective combinatorial therapies comprising MAPK-dependent and MAPK-independent mechanisms in other human advanced melanoma cell models are shown in Supplementary Figures 2, 3 and 5.

Increased effects of targeted therapy against an appropriate mutational background

As part of a heterogeneous network of aberrant intracellular signaling, multiple deregulated pathways can participate in the mechanistic control of melanoma growth (Figure 1). We examined whether a combination therapy designed for a specific mutational signature could

Table 1: Validated mutations found in advanced melanoma patients

Patient	Chrom.	Position	Ref_base	Mut_base	Total_Cov	Observed_Freq	Gene_ID	p.Annot
2	Chr4	126370467	G	A	59	0.58	**FAT4**	p.E2766K
2	Chr2	141986902	C	T	83	0.18	**LRP1B**	p.E234K
4	Chr5	167881030	GGA	-	464	0, 53	**WWC1**	p.V861 VE > V
4	Chr5	150923714	T	C	724	0.39	**FAT2**	p.N2325S
4	Chr17	7578490	A	C	884	0.08	**TP53**	p.V147G
5	Chr12	25380269	C	G	177	0, 16	**KRAS**	p.E63D
5	Chr1	9781272	G	C	424	0, 07	**PIK3CD**	p.G593R
6	Chr7	140453136	A	T	39	0, 69	**BRAF**	p.V600E
6	Chr3	3134041	T	A	100	0, 3	**IL5RA**	p.E287D
6	Chr19	15290897	C	T	1200	0, 27	**NOTCH3**	p.G1105S
8	Chr7	6426892	C	T	233	0, 67	**RAC1**	p.P29S
8	Chr7	81346551	G	A	243	0, 61	**HGF**	p.R468C
8	Chr7	140453136	AC	CT	61	0, 54	**BRAF**	p.V600R
8	Chr3	155311800	C	T	145	0, 49	**PLCH1**	p.G104S
8	Chr18	51053024	CC	TT	119	0, 48	**DCC**	p.S1383F
8	Chr5	89910783	C	T	60	0, 4	**GPR98**	p.R52C
8	Chr1	23233289	T	G	76	0, 39	**EPHB2**	p.Y659D
8	Chr13	28886195	C	T	190	0, 32	**FLT1**	p.E1143K
8	Chr18	50432552	C	T	163	0, 37	**DCC**	p.P184L
8	Chr2	170136083	C	T	38	0, 29	**LRP2**	p.G455D
8	Chr11	46406865	G	A	1048	0, 27	**CHRM4**	p.P415S
9	Chr7	31855742	C	T	66	0, 24	**PDE1C**	p.E597K
9	Chr7	140453135	CA	TT	55	0, 11	**BRAF**	p.V600E
12	Chr7	140453136	A	T	142	0, 1	**BRAF**	p.V600E
12	Chr6	32170007	C	T	302	0, 06	**NOTCH4**	p.G1201R
13	Chr2	166905414	C	T	321	0, 15	**SCN1A**	p.G337E
13	Chr5	55247832	G	A	362	0, 12	**IL6ST**	p.L542F
16	Chr16	9858517	C	T	450	0, 06	**GRIN2A**	p.E962K
17	Chr7	140453136	A	T	326	0, 44	**BRAF**	p.V600E
17	Chr4	126239082	C	T	1417	0, 27	**FAT4**	p.L506F
17	Chr17	19285669	C	T	1646	0, 23	**MAPK7**	p.P546S
17	Chr18	50450115	C	T	371	0, 2	**DCC**	p.P246S
18	Chr10	96014751	C	T	651	0, 13	**PLCE1**	p.P1167S
18	Chr2	21233091	G	A	167	0, 08	**APOB**	p.H2217Y
18	Chr13	29001438	C	T	824	0, 08	**FLT1**	p.E432K
18	Chr7	151851165	CC	TT	876	0, 08	**KMT2C**	p.G4069N
18	ChrX	112035176	C	T	420	0, 07	**AMOT**	p.E195K

Patient: Patient number; Chrom: Chromosome number; Position: Genomic location of the mutation in the chromosome; Ref_base: normal nucleotide; Mut_base: mutated nucleotide; Total Cov: Number of reads analyzed at each position; Observed_Freq: Frequency of mutation; Gene_ID: Gene name; pAnnot: Aminoacid change.

be more effective when used against a genetically appropriate background. A group of melanoma cell lines harboring unique mutational signatures (Supplementary Table III) was treated in parallel with the genetically defined inhibitors for A375 cells, BRAFi, FGFR2i and mTORi, alone or in combination. In general, each treatment was more effective in A375 cells than in the other melanoma cell lines, with the possible exception of mTORi used alone (Figure 3A). The triple drug combination treatment (V+E+Va) simultaneously targeting MAPK-dependent and MAPK-independent proliferation mechanisms produced greater inhibition of A375 cell growth than the others (Figure 3A and 3B). Likewise, other specific treatments based on the combination of genetically defined inhibitors in other melanoma cell lines showed a stronger effect than that of A375 cells (Supplementary Figure 4A and 4B). More examples of specific mutational signatures guiding more effective combination therapies when used against an appropriate mutational background are shown in Figure 5E and Supplementary Figure 5C. In summary, based on specific mutational signatures, a specific treatment consisting of a combination of genetically defined inhibitors may have stronger anti-melanoma activity when used against an appropriate genomic background.

In vivo effects of a targeted therapy that combines MAPK-ERK-dependent and MAPK-ERK-independent mechanisms of inhibition

To study the *in vivo* effects of targeted therapy oriented by a specific mutational profile, xenografted tumors from A375 melanoma cells were generated in BALB/C mice ($nu^{-/-}/nu^{-/-}$). Once grown to a volume of approximately 100 mm^3, tumors were assigned to four comparable groups and treated daily with vehicle, a MAPK-dependent inhibitor (BRAFi), a MAPK-independent combination of inhibitors (FGFR2i+ mTORi), or a triple combination of the latter (BRAFi+FGFR2i+mTORi). As shown in Figure 4A and 4B, both treatments used independently reduced tumor growth to a similar extent. However, the triple combination (V+Va+E) proved most effective at reducing melanoma growth in this system. Once the experiment was finished, the remaining growth potential of these tumors was characterized by studying Ki67 and the mitotic index in tumor sections. As might be expected, a marked decrease in both proliferation markers in those tumors treated with the combination of MAPK-dependent and MAPK-independent inhibitors (Figure 4C-4G) was observed, thereby confirming *in vivo* our previous findings in cultured cells (Figures 2D, 2E, and 5).

A pre-clinical example of targeted therapy guided by a specific mutational signature in patient 17

These findings were challenged by integrating the study of a freshly resected biopsy from patient 17 (Table I and Supplementary Table II) in the working pipeline (illustrated in Figure 5A and Supplementary Figure 1). First, a fragment of the biopsy was characterized which enabled the detection of somatic mutations in the *BRAF* and *MAPK7* (*ERK5* hereafter) genes (Table I and Figure 5A), which were associated with specific inhibitors like Vemurafenib (BRAFi (V)) and XMD-8–92 (ERK5i (X)).

Second, freshly isolated melanocytes (MELANOMA17 cells hereafter) were also inspected for the presence of mutations of *BRAF* and *ERK5* (Figure 5B) and for the expression of well-known melanoma markers such as S100A and MCSP (Supplementary Figure 6A and 6B). Once characterized, MELANOMA17 cells were incubated with increasing concentrations of BRAFi and ERK5i, and the IC_{50} of each was calculated (Supplementary Table III). Treatment with BRAFi and ERK5i inhibited B-RAF and ERK5-dependent signaling, assessed by western blot, in starved MELANOMA17 cells (Figure 5C). Simultaneous treatment with both inhibitors was more effective at reducing MELANOMA17 cell proliferation than either inhibitor alone (Figure 5D). Furthermore, and consistent with our previous observations in multiple cell lines, the combination treatment (BRAFi+ERK5i) was also more effective in cells with an appropriate mutational signature (MELANOMA17 cells) when compared with a panel of other melanoma cell lines (Figure 5E), with the possible exception of A375 cells, which were highly sensitive to treatment with the BRAFi dose used (compare the IC_{50} values for MELANOMA17 with those of A375 cells in Supplementary Table III). This combination therapy also consisted of MAPK-dependent (BRAFi) and MAPK-independent (ERK5i) mechanisms, which successfully suppressed the aberrant growth of MELANOMA17 cells (Figure 5F).

Third, another fragment of the biopsy was implanted in NSG mice and allowed to grow until four tumor-comparable groups of mice could be established. The groups of mice were then treated with vehicle, BRAFi, ERK5i, or a combination of the two (V+X). When used separately, the two inhibitors were able to suppress xenografted MELANOMA17 tumor growth to a similar extent, but growth inhibition was more effective when used in combination (Figure 5G-5I).

Thus, it is possible to study the effects of personalized therapies, guided by targeted mutational profiling of advanced melanoma patients, in pre-clinical *ex vivo* and *in vivo* models using freshly resected material from each lesion.

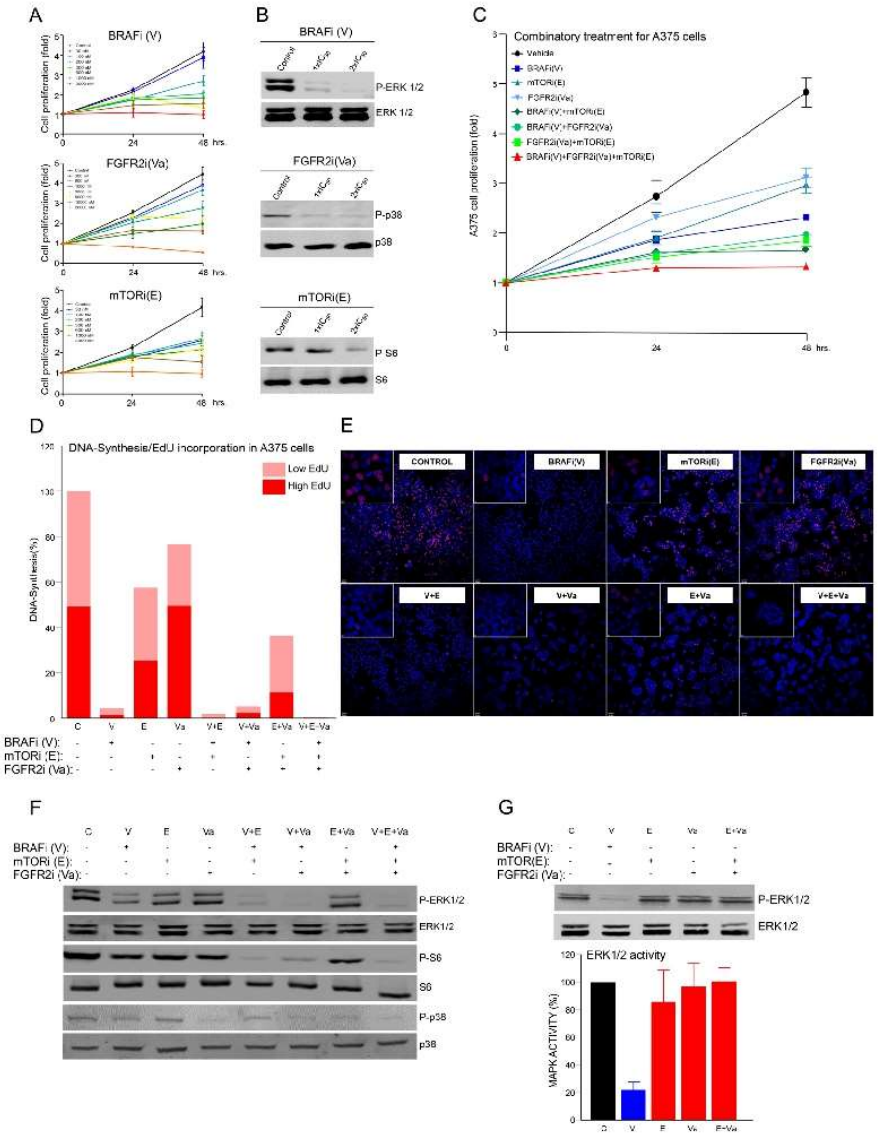

Figure 2: Effects of specific targeted therapy guided by mutational signature. A. Proliferation analysis of A375 cells at 0, 24 and 48 h. Cells were seeded in 96-well plates and treated with the indicated concentrations of each inhibitor: B-RAFi (V: Vemurafenib), FGFR2i (Va: Vargatef), and mTORi (E: Everolimus). **B.** Western blots using whole cell lysates from starved A375 cells incubated for 1 h with control vehicle (DMSO) or the indicated concentration of each inhibitor. The figure shows a representative experiment using P-ERK1/2, ERK1/2, P-p38, p38, P-S6 and S6 antibodies, as indicated. **C.** Proliferation analysis of A375 cells in the same conditions as in A), but incubated with control vehicle (DMSO) or the IC_{50} concentration of the indicated inhibitor alone (blue lines), or in a double (green lines) or triple combination (red line). $N = 6$. Error bars show the SEM. **D.** DNA synthesis using Click-iT® EdU in exponentially growing A375 cells seeded in an 8-well glass and incubated for 48 h with control vehicle (DMSO) or the indicated inhibitor or combination of inhibitors, as in C). Graph bars show percentage of low (clear red) or high (intense red) EdU-stained cells in three photographic fields from a representative experiment. **E.** Representative pictures of each treatment condition showing the nucleus of the total number of cells (blue dots) and EdU-positive cells (red dots). **F** and **G.** Western blots of whole cell lysates of the indicated cells. Cells were starved overnight and incubated for 1 h with control vehicle (DMSO), or the indicated inhibitor, or a combination of inhibitors under the same conditions as in C). Figures show representative experiments using P-ERK1/2, ERK1/2, P-p38, p38, P-S6 and S6 antibodies, as indicated. Bar graphs show the values of three independent experiments in G). Error bars indicate the SEM.

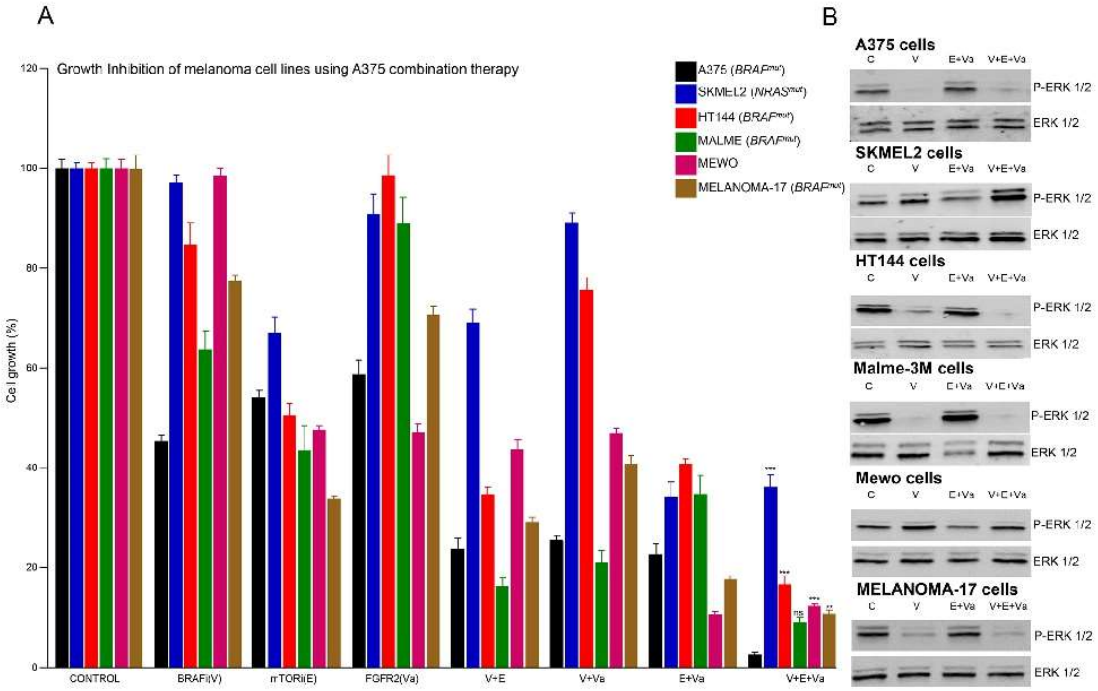

Figure 3: Increased effects of targeted therapy against an appropriate mutational background. A. Proliferation analysis of exponentially growing A375 (BRAF+), SKMEL2 (NRAS+), HT144 (BRAF+), MALME (BRAF+), MEWO and MELANOMA17 (BRAF+) cells treated with vehicle (DMSO) or the IC_{50} concentration (calculated for A375 cells) of the indicated inhibitor alone, in a double or triple combination for 48 h. $N = 6$. Error bars show the SEM. **B.** Western blots of whole cell lysates of the indicated cells. Cells were starved overnight and incubated for 1 h with control vehicle (DMSO), or the indicated inhibitor, or a combination of inhibitors under the same conditions as in A). Figure shows a representative experiment.

DISCUSSION

Metastatic melanoma provides an instructive example of the development of rationalized therapies guided by molecular diagnostics. Mechanistically, targeted therapy mainly involves the inhibition of the MAPK signaling pathway by using BRAF or MEK inhibitors, alone or in combination [21], as suggested by: A) activating mutations in BRAF and NRAS oncogenes in 48% and 15% of all diagnosed melanomas, respectively [22]; and B) multiple MAPK reactivation mechanisms that confer resistance to BRAF inhibitors [23–25]. This has improved the clinical management of those patients with mutated *BRAF*, whereby targeted inhibition of aberrant MAPK signaling can increase their OS by up to 11.4 months, although, from a different perspective, it still offers a limited benefit to these patients [10, 26, 27]. To explain this, we can hypothesize that, as part of an intricate network of transforming mechanisms in melanoma, this disease simultaneously uses multiple oncogenic mechanisms, such as, for example, PI3K, MET and GNAQ [28, 29], that, along with MAPK signaling, act as mechanistic drivers of the disease and promote progression or resistance to therapy. In this regard, data from genetically defined melanoma models now show that a rational combination of MAPK and PI3K inhibitors can improve the effects of therapy when used against specific genomic backgrounds [30]. Moreover, patients with specific mutations gained greater benefits when treated with immunotherapy [31, 32]. Thus, better characterization of advanced melanoma lesions could improve our ability to treat this disease through the use of specific therapies that simultaneously target multiple signaling mechanisms.

From a molecular perspective, melanoma is a very heterogeneous disease in which up to 1, 500 somatic mutations may be harbored in the coding exons of a single lesion [13]. This work studies mutations in 217 genes previously shown to be mutated in melanoma [13, 14, 18, 19, 33, 34] *in silico* by comparing them with mutations in 11 cell lines and 158 human melanomas, and *ex vivo* by characterizing 18 lesions from advanced melanoma patients. Under all conditions, genes like *BRAF*, *RAC1*, *FGFR2* and *IL6R* were mutated at varying frequencies, occurring as part of unique mutational signatures comprising specific combinations of mutated genes that have the potential to participate in multiple signaling

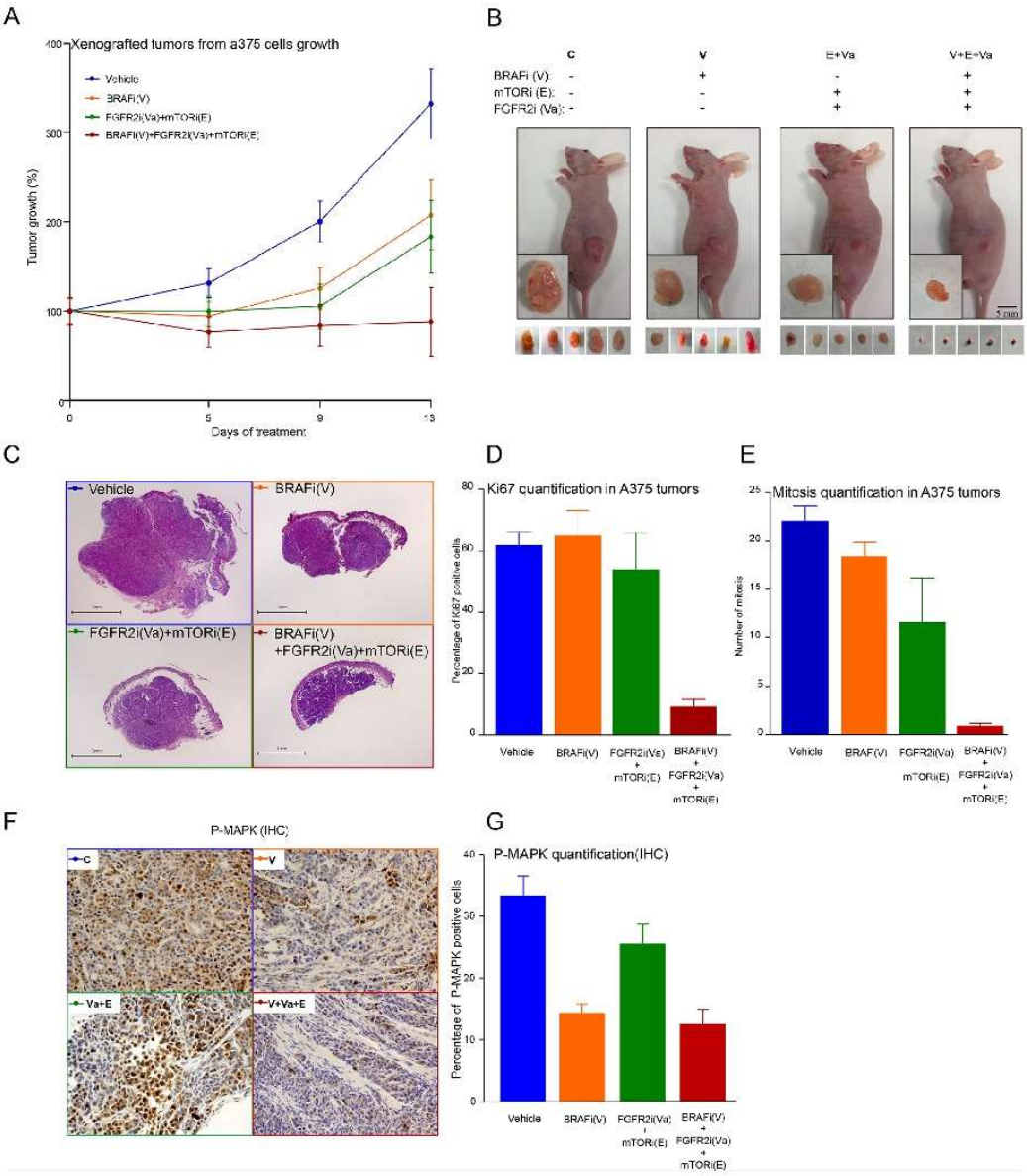

Figure 4: *In vivo* effects of a targeted therapy combining MAPK-ERK-dependent and MAPK-ERK-independent mechanisms of inhibition. A. Xenografted tumor growth-derived A375 cells injected subcutaneously in 48 BALB/C nude mice. Tumor size was monitored until a volume of 100 mm^3 was obtained, whereupon mice were assigned to four treatment groups: 1) Control (DMSO, blue line); 2) BRAFi (V) (orange line); 3) FGFR2i (Va) + mTORi (E) (green line); and 4) BRAFi (V) + FGFRi (Va) + mTORi (E) (red line). Mice were treated daily as indicated (see Materials and Methods for further details) and tumor volumes were measured until day 13, at which point the experiment was ended. Data were obtained from the 12 control, 11 (V), 7 (Va+E) and 10 (V+Va+E) mice that survived the entire process. Error bars indicate the SEM. **B.** Representative pictures illustrating the effects of the indicated treatment on the xenografted tumors that had been resected or were still in the mice. **C.** H&E staining of representative tumor sections from five representative mice for each treatment condition. Tumor sections were analyzed for Ki67-positive staining **D.** or by the number of mitoses **E.** Data are averages of five section cuts in each mouse. Error bars indicate the SEM. **F.** Immunohistochemical (IHC) analysis in tumors corresponding to the indicated treatment, as in C), using an anti-phospho ERK antibody stain. **G.** Tumor sections were analyzed for phosphor-ERK-positive staining. Results are the averages of five section cuts per mouse. Error bars indicate the SEM.

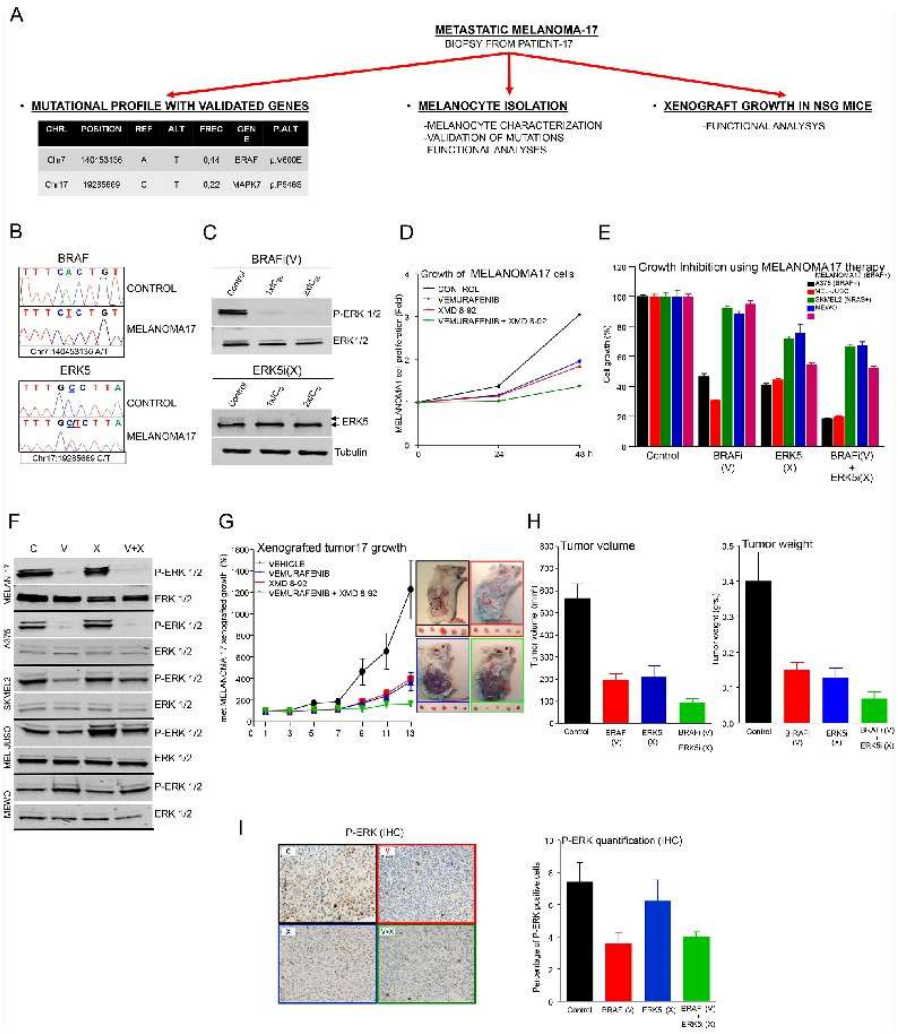

Figure 5: A pre-clinical example of targeted therapy guided by a specific mutational signature in melanoma patient 17. A. Schematic representation of the work performed with a freshly resected biopsy from patient 17. **B.** Sanger sequencing of *BRAF* (above) and *MAPK7* (*ERK5*; below) oncogenes in genomic DNA from control cells or isolated melanocytes from patient 17 (MELANOMA17 cells). **C.** Western blots of whole-cell lysates from starved MELANOMA17 cells incubated for 1 h with control vehicle (DMSO) or the indicated concentration of each inhibitor BRAFi (V: Vemurafenib) or ERK5i (X: XMD-8–92). The figure shows a representative experiment using P-ERK1/2, ERK1/2, ERK5 and tubulin antibodies, as indicated. **D.** Proliferation analysis of MELANOMA17 cells at 0, 24 and 48 h. 3×10^3 cells/well were seeded in 96-well plates and treated with control (DMSO) (black line), or an IC_{50} concentration of B-RAFi (V) (blue line) or ERK5i (X) (red line), alone or in combination (green line). $N = 6$. Error bars show SEM. **E.** Proliferation analysis of MELANOMA17, A375, MEL JUSO, SKMEL2, and MEWO cells at 0, 24 and 48 h, under the same conditions as in D). $N = 6$. Error bars show the SEM. **F.** Western blots using whole cell lysates of the indicated cells. Cells were starved overnight and incubated for 1 h with control vehicle (DMSO), or the indicated inhibitor, or a combination of inhibitors using the same concentrations as in E). Representative experiment using anti-P-ERK1/2 and anti-ERK1/2 antibodies. **G.** Tumor growth derived from 2-mm^3 MELANOMA17-derived tumor fragments implanted subcutaneously in 30 NSG mice (Jackson Laboratories). Tumors were monitored until they attained a volume of 100 mm^3, whereupon mice were assigned to four comparable treatment groups: 1: Control (DMSO, black line), 2: BRAFi (V) (blue line), 3: ERK5i (X) (red line) and 4: BRAFi (V) + ERK5i (X) (green line). Mice were treated daily as indicated (see Materials and Methods for further details) and tumor volumes were monitored until day 13, at which point the experiment was ended. Data were obtained from five survivor mice from each treatment group. Error bars indicate the SEM. The figure shows a representative image from treated tumors that were still in the mice (above) or had been freshly resected (below). **H.** Bar graph of average changes in tumor volume (left) and mass (right) for each treatment condition. $N = 5$. Error bars indicate the SEM. **I.** Examples of IHC analysis of tumors corresponding to the treatment indicated in D) using anti-phospho ERK antibody staining. Bar graphs show results for tumor sections analyzed for phosphor-ERK-positive staining. Data are the averages of five section cuts per mouse. Error bars indicate the SEM.

pathways and to be associated with specific inhibitors. Functionally, the effects of combination therapies guided by specific mutational signatures were analyzed in multiple melanoma cells harboring unique mutational signatures. Those treatments that simultaneously targeted MAPK-dependent and MAPK-independent signaling were most effective at reducing melanoma growth both *ex vivo* and *in vivo*. These observations can be aligned with work from other laboratories, showing that to promote transformation in melanocytes, aberrant MAPK-signaling elicited by BRAF or NRAS oncoproteins requires the active collaboration of other oncogenes, such as *PI3K*, *RAL-GDS*, *GNAS* or *C-MYC*, that can participate in alternative signaling pathways [35–38]. Thus, a combination of genetically defined inhibitors targeting multiple signaling pathways could be more effective against specific cases of malignant melanoma. We might expect targeting well-known melanoma mechanisms to affect the growth of melanoma cells in general, and this can indeed be observed in our data. Nevertheless, combination therapies guided by specific mutational signatures were most effective when used against an appropriate mutational background. Furthermore, different *BRAF*-mutated cell lines, each with an individual mutational signature, had different sensitivities to BRAF inhibition (Supplementary Table III). Finally, our data strongly suggest that combining several mutationally selected inhibitors can specifically block important mechanisms that participate in the control of aberrant melanoma cell growth and in the finely tuned cellular decision to activate DNA synthesis (Figures 2B, 2C, 2E, 4A, 4D and 4E). This rules out the possibility that the results were a consequence of nonspecific cytotoxic activities.

Starting with freshly resected material from a metastatic lesion (patient 17) and trying to match the timing with the clinic, a validated mutational profile was obtained within two weeks of resection. This data enabled the study of a combination therapy based on inhibitors with MAPK-dependent (BRAFi) and MAPK-independent (ERK5i) mechanisms of action in isolated MELANOMA17 cells and in xenografted tumors grown in mice. These gave the best results when combinatorial approaches were used. Of course, this study provides just one example of what targeted characterization of specific lesions might offer by way of diagnostic possibilities for human melanoma in the near future. Considering its potential applicability in routine clinical practice, this approach would require several limitations to be overcome. This would entail: 1) establishing efficient protocols to collect, manipulate and characterize specific lesions that are representative of the various steps of the disease; 2) managing the toxicity due to drug combinations; and 3) dealing with tumor heterogeneity and interactions with the immune system that may be responsible for the eventual resistance acquired after combination treatments. However, there is much scope for studying novel strategies for targeted therapy following a molecular rationale, particularly in a disease like advanced melanoma that offers a limited prospect of survival to patients who are suffering from it (Supplementary Figure 7).

In summary, by adopting targeted approaches we can envisage working with specific signatures of mutated genes that can: 1) help characterize individual lesions in advanced melanoma patients; 2) guide the use of specific inhibitors rationally combined in individualized therapies to target case-specific mechanisms of melanocytic transformation. In this work, a rational combination of genetically defined inhibitors simultaneously targeting MAPK-dependent and MAPK-independent signaling mechanisms showed improved biological outcomes with respect to the malignant growth of specific advanced melanomas.

MATERIALS AND METHODS

Cells and reagents for tissue culture

Eight human advanced melanoma cell lines were used. A375 (CRL-1619™), SK-MEL-28 (HTB-72™), SK-MEL-2 (HTB-68™), MALME-3M (HTB-64™), MEWO (HTB-65™) and HT-144 (HTB-63™) cells were obtained from the American Type Cell Collection (ATCC, Rockville, MD). MEL-JUSO (ACC 74) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Genomic data from these cells, including those of the somatic mutations detected in this study, are publicly available at the Broad-Novartis Cancer Cell Line Encyclopedia website (CCLE:http://www.broadinstitute.org). MELANOMA17 cells were established from a primary biopsy sample, as explained in the Supplementary Methods. Commercial cell lines were cultured as recommended by ATCC or DSMZ and incubated with inhibitors, as described in the Supplementary Methods.

Cell proliferation and DNA synthesis assays

Cells growing exponentially to approximately 50% confluence in T96 well plates were incubated with the specific inhibitors while keeping the total amount of DMSO (0.5%) constant. Cellular proliferation was evaluated using AlamarBlue reagent (Life Technologies) and colorimetric changes were quantified using the Synergy™ HTX Multi-Mode Microplate Reader (Biotek). To assess the effects on DNA synthesis, cells were grown in a Millicell EZ SLIDE 8-well glass (Merck Millipore, PEZGS0816) and after treatment with specific inhibitors were incubated for a further 2 h with Click-iT® EdU (Alexa Fluor® 594 Imaging Kit; Life Technologies, C10339). Immediately afterwards, cells were prepared for microscopy following the manufacturer's specifications (see Supplementary Methods for further explanation).

Cell images were captured with a Nikon A1R confocal microscope with Plan Apo 10x/0.45NA and Plan ApoVC 60x/1.40NA objectives.

Genomic DNA samples

Matched tumoral and non-tumoral material was obtained from 18 patients diagnosed with advanced melanoma and who were being monitored by the Oncology Department of the Hospital Universitario Marqués de Valdecilla (HUMV; see clinical characteristics in Table I). Tumoral DNA samples were obtained from freshly frozen tissue samples taken at the time of diagnosis, and matched non-tumoral DNA was extracted from saliva or peripheral blood neutrophils. We designed an intra-subject observational study of patients diagnosed with advanced melanoma and with a Breslow index of ≥4 mm, and with a loco-regional infiltration in lymph nodes or presenting distant metastasis. The study, the patient information sheet, and the informed consent form were approved by the Ethics Committee of the HUMV.

Enrichment library design, preparation, sequencing and variant calling

Genomic DNA samples were processed using the Qubit® dsDNA BR Assay Kit (Life Technologies) and quantified using Qubit 2.0 apparatus (Life Technologies). The DNA enrichment library was prepared using a specifically designed HaloPlex Target Enrichment System Kit for this melanoma study (Design ID: 00912-1339502780, Agilent Technologies) following the manufacturer's instructions. The design focused on the coding regions of a group 217 genes known to be mutated in melanoma, and which were selected because they were: A) genes of known relevance in melanoma, including BRAF, NRAS [7], and EGFR [18, 19]; B) genes that may be associated with pharmacological inhibitors of potential clinical use, such as FGFR2, KIT and ERBB4 [18, 20, 21]; and C) genes that may be involved in chromatin architecture (ARID1A and DNMT3A [14]), intracellular signaling (MEK1 [22]), or transcription (NFATC2 [22]). Briefly, 400 ng of genomic DNA was digested with the specific cocktail of restriction enzymes provided in the kit. Digested DNA was then hybridized to a probe for target enrichment, indexed and captured. Each DNA was then amplified by PCR at Tm = 60°C, for 18 cycles, using a Herculase II Fusion Enzyme kit (Agilent Technologies). Next, amplified target libraries were purified using an Agencourt AMPure XP Kit (Beckman Coulter Genomics), following the manufacturer's guidelines, and quantified with Qubit 2.0 apparatus (Life Technologies), using the Qubit® dsDNA HS Assay Kit (Life Technologies). They were also analyzed in parallel by capillary electrophoresis in a 2100 Bioanalyzer (Agilent Technologies), using High Sensitivity DNA reagents and chip Kits (Agilent Technologies). Libraries were sequenced at the Instituto de Medicina Genómica (IMEGEN, Valencia University, Spain) with a MiSeq Personal Sequencer (Illumina). The process of somatic mutation identification described in the Supplementary Methods.

Somatic mutation identification

Sequencing data were aligned against the human reference genome (hg19) using the BWA aligner [39]. The alignment was refined using SAMTOOLS fixmate (PMID: 19505943) and PICARD TOOLS cleanSam tools (http://broadinstitute.github.io/picard.). Local realignment of insertions and deletions (indels) was then performed using the GATK suite [40] before final sorting and indexing. The RAMSES application (PMID: 24296945), written in-house, was used to detect nucleotide substitutions. Small indels were identified using Pindel [41] in paired tumor-normal mode. For greater specificity, only simple insertion and deletion events of fewer than 10 bp were selected. An in-house perl script filter was used to extract high-quality indels: considering the high sequence coverage obtained in these samples, only those indels with a minimum coverage of 20 reads in both tumor and normal samples, and with a minimum frequency of 10% of the reads and a minimum of five independent reads supporting the event in the tumor sample, and with no evidence in the normal sample, were considered. All potential somatic mutations were filtered using the dbSNP132 and 1000 Genomes Project mutation databases and the functional consequence at the protein level was annotated according to the Ensembl database using an in-house perl script based on the Ensembl database API.

Validation analysis

Genomic DNA was amplified using the specific oligonucleotides described in Supplementary Table IV. All amplicons from the same patient were mixed in a tube and each sample was quantified by Qubit 2.0 (Life Technologies), using the Qubit® dsDNA BR Assay Kit (Life Technologies). MELANOMA17 cells were monitored by Sanger sequencing for the presence of mutations in *BRAF* and *MAPK7* (see the supplementary methods for further details).

Statistics

Unless otherwise specified, all experiments were done in independent triplicates and all numerical data were summarized as the average of the values ± the standard error of the mean (SEM) using GraphPad PRISM. Levels of statistical significance are indicated as follows: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

Western blot

Cells growing exponentially at approximately 70% confluence were treated under the desired conditions. Cells were starved overnight (unless otherwise stated), treated with the appropriate inhibitor and lysed as described in [42]. Whole cell lysates were subjected to acrylamide SDS-PAGE, using standard procedures, then transferred onto a nitrocellulose support membrane (Immobilon, Millipore) and western blotted. The primary and secondary antibodies and the data collection method are described in the Supplementary Methods.

Mice and reagents for *in vivo* studies

BALB/c Nude mice CAnN.Cg-Foxn1nu/Crl (Charles River) were injected with 6×10^6 A375 melanoma cells in the subcutaneous dorsal area. Approximately one week later, the tumor reached a volume of about 100 mm^3, at which point mice were assigned to four tumor size-comparable groups of 12 animals and treated as described in the Supplementary Methods.

Fresh tissue from patient 17, who had been diagnosed with metastatic melanoma (Table I and Figure 5), was minced and xeno-injected into NOD. Cg-Prkdcscid Il2rgtm1Wjl/SzJ mice (commonly known as NOD scid gamma (NSG) mice) (Charles River). Briefly, the animals were anesthetized using ketamine (75 mg/kg) and medetomidine (1.0 mg/kg) and a piece of tumor was inserted in the subcutaneous dorsal area through a small incision in the skin and allowed to grow. Next, mice were sacrificed (as described in supplementary methods) and tumors were collected and minced into pieces of about 2 mm^3 and reimplanted into the experimental group of mice. When these mice had grown tumors of an approximate volume of 100 mm^3, they were distributed among four groups of six mice, each with comparable tumor volumes and treatments were started, as described in Figure 5 and in the Supplementary Methods.

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CONFLICTS OF INTEREST

All authors declare no conflict of interest except MAP.

MAP has the following conflicts of interest: Takeda-advisory board. Novartis, Amgen and Roche: Speaker bureau.

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This article has supplementary material. It is available in an attached CD, located in the back cover of this thesis.

5. Discussion

Cutaneous melanoma and Merkel cell carcinoma (MCC) are two aggressive types of skin cancer which currently lack a therapy able to produce sustained responses in all patients, especially when detected in advanced stages or under inoperable circumstances. In such cases, possible responses to treatments are usually short lived, or detected only in a proportion of patients, whereas others do not show any response [Flaherty, K. T. et al., 2010; Hodi, F. S. et al., 2010; Sosman, J. A. et al., 2012; Topalian, S. L. et al., 2012; Robert, C. et al., 2015]. The standard approach to the initial management of primary tumors in both cancer types is the surgical resection [Lebbe, C. et al., 2015; Teng, J. et al., 2015], but its effectiveness depends on the spread of the cancer, amongst other factors. Therefore, survival is low in those cases with regional or distant metastases [Robert, C. et al., 2015], as well as in those showing high depth [Vollmer, R. T. et al., 2001; Santonocito, C. et al., 2007; Smith, F. O. et al., 2015] or area in the case of MCC [American_Joint_Committee_on_Cancer, 2010; Haymerle, G. et al., 2016].

Ultraviolet (UV) radiation appears to be an etiological factor in both cancer types [Dasgupta, A. et al., 2015; Harms, P. W. et al., 2015; Kaskel, P. et al., 2015]. In fact, the presence of UV genomic signatures has been described in most melanoma samples [Alexandrov, L. B. et al., 2013] and in a considerable proportion of MCC lesions [Harms, P. W. et al., 2015], which, in addition, are often found on sun-exposed sites, such as head and neck region or upper extremities [Lunder, E. J. et al., 1998]. However, Merkel cell polyomavirus (MCPyV) integration seems to play also a role in the development of MCC [Engels, E. A. et al., 2002; Feng, H. et al., 2008; Nghiem, P., 2015].

We started the study of these two cancer types by means of sequencing techniques, in order to identify mutations harbored by tumors, in an effort to unravel the molecular basis of carcinogenesis. A different sequencing approach was selected for the study of each disease, depending on the global knowledge and the available information about molecular alterations driving each cancer type. That means that an exploratory approach, with a global mutational analysis, was preferred to study a poorly understood disease, like MCC, whereas a more targeted and applied approach was considered advantageous to study a widely documented cancer type, like melanoma. Therefore, given the existing shortage of literature about genomic alterations in MCC, we chose the whole exome sequencing (WES) as a first approach, in order to achieve a broad mutational analysis. The obtained genomic data were then used to perform a wide analysis of signaling pathways and biological processes frequently altered in our cohort. By contrast, to study genomic alterations of cutaneous melanoma lesions, we selected a set of genes to be analyzed, supporting our choice of genes on data available in the literature. During the selection, we took into account the biological processes to which each gene is associated and, at the same time, we enriched our set in genes which have associated pharmacological inhibitors already approved, or in process of being approved, to be used in the clinic. Thus, we studied melanoma tumors by means of a targeted

sequencing approach, which allowed the analysis of a considerable number of samples under specific conditions that enabled us to detect mutations present in clonal and even subclonal proportions in our samples.

In both projects, the information recovered from sequencing approaches was used to elucidate signaling pathways, biological processes and/or biomarkers with a likely role in the development of each disease. Some of this pathways and markers could perhaps be used in the near future to establish more accurate prognosis and/or diagnosis, or even to guide targeted therapies.

5.1. CURRENT STATUS OF MERKEL CELL CARCINOMA DIAGNOSIS AND TREATMENT.

Merkel cell carcinoma could be considered a poorly known disease, since little is clear about its origin, etiology, disease mechanisms, diagnosis and treatment. This can be due, unless in part, to the relative rarity of this disease [Becker, S., 2007].

Diagnosis of MCC is sometimes tricky because of its histopathologic similarity with other cancer types, especially with metastatic small cell lung carcinoma (SCLC) [Pulitzer, M. P. et al., 2009], which can have nearly identical morphologic features and aggressive clinical behavior. The primary lesion of MCC is distinguished by its absence of distinctive clinical characteristics (reviewed by [Wang, T. S. et al., 2011]). Cytokeratin 20 (CK20) is expressed in roughly 95 % of Merkel cell carcinomas, so it is often used for its differentiation from morphologically similar entities [Moll, R. et al., 1992]. Lacking CK20 expression can make diagnosis of Merkel cell carcinoma more challenging.

With regard to treatment options for MCC, not many are available, apart from the primary standard approach of surgical resection followed by adjuvant radiotherapy [O'Connor, W. J. et al., 1997; Boyer, J. D. et al., 2002]. The infrequency of this neoplasm is a major cause for the concerning shortage of clinical trials, fact which is hindering the improvement of available therapies [Eng, T. Y. et al., 2007].

As for the etiology of MCC, it has been controversial for a long time. The discovery of the Merkel cell polyomavirus (MCPyV) DNA integrated in the genome of MCC tumor cells led to the proposal of a causal relationship between virus and cancer, providing thus the possible link between MCC and its association with immune suppression [Feng, H. et al., 2008]. However, MCPyV antigens are not detected in all MCC tumors, but this virus has been detected a cohort-dependent proportion ranging from 50 % to 90 % of analyzed cases [Feng, H. et al., 2008; Becker, J. C. et al., 2009; Duncavage, E. J. et al., 2009; Garneski, K. M. et al., 2009; Bhatia, K. et al., 2010]. On the other hand, UV radiation seemed to be involved in the

appearance of the disease, as many primary tumors occurred in sun-exposed areas of skin and as Caucasian population had a greater risk of suffering this disease [Lunder, E. J. et al., 1998; Harms, P. W. et al., 2015].

In this context, we collected tumor and normal DNA samples from 15 clinically characterized MCC patients to be analyzed by WES (publication 1, table 1). Of the 15 cases, 7 had MCPyV DNA integrated into the tumor genome, whereas the remainder 8 did not show viral antigens detectable by PCR and either by immunohistochemistry (IHQ). We called this group of 15 samples the "discovery cohort", as it was utilized to "discover" the somatic genomic alterations harbored by tumors. After sequencing, we performed a broad genomic analysis of these cases, searching for somatic mutations, but also extending then our study to explore those significantly altered signaling pathways and molecular mechanisms, by means of bioinformatics analysis of mutational data. Through this analysis of mutations and altered mechanisms, we identified a series of biomarkers, chosen on the basis of the mechanistic interpretation of the mutational data. Such biomarkers were then assessed in a larger cohort, called the "validation cohort", consisting of 48 MCC cases, amongst which the previous 15 were included (publication 1, supplementary table S1). A little more than a half of these cases (27/48) were MCPyV-positive and the rest (21/48) were MCPyV-negative. Analysis performed with this cohort allowed the identification of new disease mechanisms, whose implication in MCC had not been proposed before, in both MCPyV-positive and -negative cases. Furthermore, we utilized clinical and immunohistochemical data from the validation cohort to identify prognostic predictors amongst our set of biomarkers, which were significantly related with survival of patients.

5.2. INVERSE CORRELATION BETWEEN MERKEL CELL POLYOMAVIRUS INTEGRATION AND ULTRAVIOLET MUTATIONAL SIGNATURE IN MCC.

As aforesaid, we sequenced the whole exome of the 15 MCC cases which compound the discovery cohort and searched for the somatic single nucleotide variants (SSNVs) in each tumor. At this point, we already detected a huge difference between the number of mutations harbored by MCPyV-positive and by MCPyV-negative tumors; cases without viral antigens displayed extremely high mutational rates, with an average of 1939 SSNVs per tumor, whereas samples with viral antigens had much lower rates, with an average of only 73 SSNVs per case (publication 1, supplementary table S3). Restricting our analysis to solely those mutations that provoke an amino-acid change, the difference was maintained; an average of 595 nonsynonymous SSNVs per each MCPyV-negative case and an average of only 22 nonsynonymous SSNVs per MCPyV-positive case were found (publication 1, supplementary table S4 and figure 1B). Mutational rates shown by those samples without viral integration

were comparable to values reported in cutaneous melanoma tumors [Berger, M. F. et al., 2012; The_Cancer_Genome_Atlas, 2015].

We then sought the ultraviolet signature by counting the number of C → T transitions located specifically at dipyrimidine sites and the number of CC → TT tandem substitutions in each sample [Brash, D. E. et al., 1982; Rochette, P. J. et al., 2003; Runger, T. M., 2008; Brash, D. E., 2015]. Again, we found a clear difference between MCPyV-positive and -negative tumors; none of the MCPyV-positive ones showed the genomic signature attributed to UV radiation effects whereas all MCPyV-negative cases but one showed such signature. These data are represented in figure 1A of publication 1, where the difference between the two MCC etiologies is noticeable.

Interestingly, as 5 of the matched tumor/normal samples of our discovery cohort came from formalin-fixed paraffin-embedded (FFPE) samples whereas the remainder 10 came from freshly frozen (FF) tissues, we had the opportunity to ascertain whether the origin of samples affected the number or the type of mutations. Fixation artefacts have been reported to increase the number of transitions in FFPE samples [Williams, C. et al., 1999; Srinivasan, M. et al., 2002]. However, the distribution of C → T transitions at dipyrimidine sites among our samples did not correlate with their FFPE or FF origin. Actually, the sample with the highest number of C → T changes came from FF tissue and 2 out of 5 DNA samples coming from FFPE tissues showed relatively low mutational burdens, low number of C → T transitions and absence of CC → TT tandem substitutions.

Thus, tumors without integrated viral DNA accumulated most of the detected somatic mutations and had a UV mutational signature. Those cases showing viral antigens seemed to have a very different pattern of genomic alterations. These results were obtained as an original piece of information, but they appeared published by other groups during the preparation, submission and evaluation of our manuscript. In any case, our data are in accordance with those recently reported by independent publications [Harms, P. W. et al., 2015; Wong, S. Q. et al., 2015; Goh, G. et al., 2016].

5.3. A NUMBER OF GENES AND SIGNALING PATHWAYS ARE RECURRENTLY ALTERED IN MCC.

Focusing this time our attention in those SSNVs that cause an amino-acid change, we identified a number of genes which appeared recurrently mutated in tumors from different patients (publication 1, table S6). Some examples of the most recurrently mutated genes are:

- *FAT4* and *TTN*, which were mutated in 6 patients each. All patients but one had MCPyV-negative tumors;

- *TP53* and *RYR2*, each one mutated in 5 patients, again MCPyV-negatives with one exception;
- *RPTOR* and *RB1*, which appeared mutated in 4 patients each, all of which were MCPyV-negative cases.

Taking into account all recurrently mutated genes, we perceived that their mutations were clearly concentrated in MCPyV-negative cases. This was not very surprising given the fact that these cases harbored the majority of overall mutations. Some of recurrently mutated genes and the distribution of their mutations are represented in figure 1C of publication 1.

Next, to delve into the functional relevance of all found SSNVs, we performed an unbiased analysis of them by means of OncodriveFM, a tool designed to uncover driver genes or gene modules, as well as to elucidate pathways and biological processes significantly altered, from the whole ensemble of mutations of a set of samples [Gonzalez-Perez, A. et al., 2012]. Using this approach, we identified 9 genes with both p and q values below 0.5: *TP53*, *CDK5RAP1*, *FAT4*, *ADAM8*, *GLB1LD*, *CACNA1C*, *OGG1*, *HIVEP2* and *RB1* (publication 1, table S7). These genes could be hence considered as possible drivers of Merkel cell carcinoma. However, other 60 genes which did not meet the q value cutoff did have a significant p value, among which were *CIT*, *CLASP2*, *MLL3*, *RPS6KB1*, *HDAC2*, *RPTOR*, *ROS1*, *NFATC4* or *MAPK8IP3*, but to name a few (publication 1, table S7). These genes could be also playing a role in carcinogenesis, so consider them as possible targets for therapy could be therefore interesting. We are conscious that the restricted number of cases included in this study may have limited the statistical power of some of these results. Therefore, future studies with bigger cohorts could confirm the relevance of these genes in carcinogenesis, or even extent the number of potential driver genes.

In addition, we found through the analysis with OncondriveFM several significantly altered gene modules publication 1, table S8). Among them, there were a number of modules that coincided described as altered in melanoma, small cell lung carcinoma (SCLC), non-small cell lung carcinoma (NSCLC) and basal cell carcinoma, suggesting hence some similarities between molecular processes directing these cancer types and MCC. Interestingly, as explained above in this thesis, the histopathologic and clinic similarities between MCC and SCLC are noticeable [Pulitzer, M. P. et al., 2009; Wang, T. S. et al., 2011], as well as the shared genomic features (mutational index and UV signature) between some MCCs and most cutaneous melanomas [Harms, P. W. et al., 2015; The_Cancer_Genome_Atlas, 2015]. Furthermore, OncodriveFM returned a number of signaling pathways significantly altered in our samples, including PI3K-AKT-mTOR, P53 signaling and WNT signaling.

5.4. COMMON AND DIVERGENT DISEASE MECHANISMS BETWEEN MCPyV-POSITIVE AND MCPyV-NEGATIVE MCC TUMORS.

According to results yielded from the analysis of mutations performed with OncodriveFM [Gonzalez-Perez, A. et al., 2012] we had found in our cohort a number of significantly mutated genes, which were in turn encompassed in several significantly altered pathways or biological processes. It is important to note that most of these processes had not been associated with MCC before. The most significantly altered mechanisms (publication 1, supplementary table S8 and figure 1C) were:

- focal adhesion and interactions with nearby cells and with intracellular matrix,
- transcriptional regulation,
- metabolism,
- calcium signaling,
- WNT signaling,
- signaling by cAMP,
- signaling by RAS and MAPKs,
- RTKs,
- PI3K/mTOR pathway.

Considering these data, we selected a number of transcription factors which could be considered as endpoints, or surrogated markers, of these altered functions, and which could be used as readouts of their activity. These markers could be assessed by IHQ, more affordable than WES and, in addition, easily applicable to the routine characterization of tumors. This allowed us to expand our study to 48 clinically characterized cases (the "validation cohort") (publication 1, table S1), within which the 15 previously sequenced cases were included. Thus, we analyzed in the validation cohort the accumulation in the nucleus of c-MYC, P-STAT3, P-CREB, LEF1 and NFAT, as indicators of the activity of significantly altered mechanisms (two examples of immunohistochemical analyses are in publication 1, figure 2). We also included p53 and RB1, because they were two significantly altered genes of our cohort, besides being known drivers of MCC [Zager, J. S. et al., 2011]. We added p63 to be assessed, in spite of not being one of our significantly altered genes, because it had been described as relevant in MCC [Llombart, B. et al., 2005]. Finally, we assessed the polyomaviral DNA integration by analyzing the expression of its LT antigen.

IHQ results (publication 1, table 2) showed that most biomarkers had a heterogeneous pattern of expression across MCPyV-positive and -negative tumors: they were detected positive in approximately a half of cases of each group. By contrast, other biomarkers were specifically detected in MCPyV-negative tumors. Almost exclusive of MCPyV-negative cases were MYC (accumulated in the nucleus of only one MCPyV-positive sample) and LEF-1 (also in one MCPyV-positive sample). However, not all cases of MCPyV-negative group

were positive for these biomarkers, but they were detected in approximately a half of them, indicating thus an existing heterogeneity amongst those MCC tumors that not express viral antigens.

Interestingly, only 2 out of 27 MCPyV-positive samples (7 %) showed RB loss, whereas 11 out of 21 MCPyV-negative cases (52 %) did present RB loss. Again, this reflected higher homogeneity among MCPyV-positive cases, while those MCPyV-negatives appeared to be more heterogeneous. But also, and what is more important, this could suggest the presence of some mechanism in tumors expressing viral antigens able to avoid or counteract RB protein effects. This hypothetical mechanism is perhaps replaced by RB loss in those cases without integrated viral genome. In fact, as described in section 1.3.2. of this thesis, a RB binging domain are conserved in large T and 57 kT MCPyV antigens [Shuda, M. et al., 2009; Stakaityte, G. et al., 2014]. In addition, interaction with RB protein has been proposed as critical for the observed growth-promoting effects of LT viral antigen [Houben, R. et al., 2012]. Furthermore, truncating RB1 mutations have been found specifically in tumor samples from cases with polyomavirus undetectable by sensitive PCR [Cimino, P. J. et al., 2014]. Therefore, it seems feasible that those tumors which express viral antigens do not need to loss RB, whereas those tumors which not express these antigens can have mutations that mimic their effects. Thus, despite the differences detected by IHQ between MCPyV-positive and MCPyV-negative groups in the expression of RB1, this marker could be part of a shared mechanism of disease.

On the other hand, most markers were heterogeneously expressed across MCPyV-negative and also across MCPyV-positive cases. For example, p63 was identified as mutated in 48 % of MCPyV-positives and in 67 % of MCPyV-negatives. Likewise, NFAT, P-CREB, P-STAT and p53 were positively stained in comparable proportions between both groups of samples.

Hence, we could infer from our immunohistochemical analysis of 48 MCCs that molecular processes that at the end increase the levels of p63, NFAT, P-CREB, P-STAT and p53 in the nucleous, such as cAMP signaling, calcium signaling or JAK-STAT pathway, are commonly deregulated in MCPyV-positive and -negative cases, perhaps being mechanisms of disease shared between both etiologies of MCC. By contrast, processes which lead to MYC and LEF1 expression, such as MAPK or PI3K signaling, are specifically overactivated in those cases which are not expressing viral proteins. A schematic representation of these shared and private mechanisms is in publication 1, figure 4.

5.5. CORRELATION BETWEEN SIGNIFICANTLY MUTATED GENES AND BIOMARKERS EXPRESSION.

Interestingly, there were amongst our sequencing data a number of mutations with a previous COSMIC-ID (publication 1, supplementary table S3), fact which allowed us to examine whether the expression of the analyzed biomarkers in MCC samples was in accordance with the expected effect of such mutations. In this regard, we had detected in patient 5 two inactivating mutations in *TP53*, which provoke the amino-acid changes R280K (COSM129830) and H47Y (COSM129851), respectively, as well as a truncating *RB1* mutation, which provoke the amino-acid change W195* (COSM214151). In agreement with this, immunohistochemical data revealed an accumulation of p53 protein in the nucleous, but a negative RB protein staining (publication 1, table 2). In the same line, a correlation between mutations and IHQ results was found related with *ERBB4* gene. This gene was mutated in two patients; patient 1, with two missense mutations, and patient 6, with one mutation described in cosmic (COSM160827). Both patients, 1 and 6, had a positive immunohistochemical staining for cMYC and P-STAT3 (publication 1, table 2), factors whose expression can be induced by several RTKs, including ERBB4. In the same vein, patient 1 had mutations in *ADCY10* (COSM899133) gene –which codify an adenylate cyclase– and in *GRM3* (COSM229505) gene –which produce a GPCR–. Adenylate cyclases and GPCRs are involved in cAMP/CREB activation and, accordingly, P-CREB staining was positive in patient 1 (publication 1, table 2).

In other cases, in the absence of a previous description of found mutations, their effects could be inferred from the activity of the signaling pathway associated with the mutated gene. As an example of this, *HIVEP2* gene (also known as MYC Intron Binding Protein 1, MIBP1), which has been identified as a significantly mutated gene in this work (publication 1, supplementary table S7), appeared mutated in patients 1, 3, 6 and 7 (publication 1, supplementary table S3). Interestingly, immunohistochemical data revealed a positive cMYC expression in patients 1, 3 and 6, suggesting thus a correlation between *HIVEP2* mutations (presumably inactivating) and cMYC expression. Indeed, it has been reported that this gene can inhibit the transcription of *cMYC* [Iwashita, Y. et al., 2012] and that the expression of *HIVEP2* and *cMYC* genes is inversely correlated in human cells [Zajac-Kaye, M. et al., 2000].

These described cases can serve as examples that show how the expression of specific biomarkers can reflect the effect of specific mutations and, therefore, the analysis of the expression IHQ biomarkers could be really useful to infer what mechanisms have resulted deregulated by the presence of certain mutations in the tumor.

5.6. P-CREB AS AN INDEPENDENT PROGNOSTIC MARKER IN MCC.

We addressed whether some of our selected biomarkers would be related with the clinical outcome of patients. To this end, we analyzed all death events caused by MCC in our validation cohort. Through this analysis we found two tendencies that did not meet statistical significance (publication 1, supplementary figure S1), but also two significant correlations (publication 1, figure 3).

Thus, we found that those patients whose tumors expressed MCPyV antigens tended towards a longer survival. This result is in accordance with observations reported by different groups, which point at MCPyV infection as a marker of better prognostic [Inoue, T. et al., 2000; Paulson, K. G. et al., 2011; Touze, A. et al., 2011; Higaki-Mori, H. et al., 2012; Sihto, H. et al., 2012]. Likewise, we also observed that those cases with RB loss tended towards worse outcomes. However, these two results did not meet statistical signification (publication 1, supplementary figure S1).

On the other hand, we observed that cases whose tumors had accumulation of p53 protein (so presumably mutated p53 protein) did not show any clear tendency towards increased or reduced survival. In other cancer types, such as non-small cell lung cancer (NSCLC), alterations in this protein have been linked to worse prognosis [Massoni Neto, L. M. et al., 2007; Molina-Vila, M. A. et al., 2014; Said, R. et al., 2014], but such relation has not been demonstrated in MCC so far. Similarly, p63 and NFAT markers did not show any clear tendency (publication 1, supplementary figure S1).

By contrast, we found a significant correlation between P-CREB and P-STAT expression and survival (publication 1, figure 3); those patients whose tumors were positive for P-CREB staining lived less than those patients with P-CREB-negative tumors ($p=0.011$). Likewise, patients whose tumors were positive for P-STAT staining showed a significant reduction in survival, compared to those whose tumors had undetectable P-STAT ($p=0.024$).

In light of these significant results, as were conscious of more than one variant could be concurrently affecting survival, we performed a multivariate data analysis, including in it P-CREB, P-STAT, sex, age, disease stage and MCPyV status (publication 1, figure 3). In a first univariate analysis, both P-CREB and P-STAT were strongly associated with higher mortality, with hazards of 3.89 and 3.37, respectively. However, after including the rest of factors in a multivariate analysis, the P-CREB hazard was even higher, but the hazard of P-STAT diminished considerably, indicating that this marker was not totally independent of all included variants.

Consequently, two biomarkers that are heterogeneously expressed in both MCPyV-positive and MPCyV-negative MCCs, being part of two commonly deregulated disease

mechanisms, were significant prognostic markers. Nevertheless, only P-CREB has been shown to be a strong predictor, independent of sex, age, stage, MCPyV and P-STAT. These findings could have an interesting application to the field of immunotherapy, as will be broader commented below. This opens the possibility of envisaging future studies aimed to explore the potential of this marker as predictor of the response of patients to some therapies, like those with immune checkpoint blockade agents.

5.7. CURRENT STATUS OF MELANOMA THERAPIES.

Melanoma is nowadays a disease positioned at the forefront of the development of systemic therapeutics with both molecular targeted therapies and immune checkpoint inhibitors as cornerstones of treatment. The awareness of the great complexity and heterogeneity that characterize melanoma tumors, coupled with the significant disparities observed in the response of patients to treatments, has given rise to a gradual change in therapies, which have been, and still are progressively evolving from a generalist point of view to a more personalized approach.

Numerous treatment regimens have been developed for patients with metastatic disease over the past several years, each with its strengths and weaknesses. The survival of patients has increased due to these available therapies, but a substantial portion of these patients do not respond to the existing treatments and just a minority show a long-term, durable remission [Hodi, F. S. et al., 2010; Topalian, S. L. et al., 2014; Robert, C. et al., 2015]. Such is the case that metastatic melanoma remains a fatal disease with a high rate of mortality, especially amongst those patients who do not respond to immunotherapy and do not carry activating mutations in *BRAF* gene, fact which makes them no appropriates to receive targeted therapies in the manner as they are being applied now [Hodi, F. S. et al., 2010; Topalian, S. L. et al., 2012; Robert, C. et al., 2015].

Current targeted therapies in melanoma are virtually limited to the use of inhibitors of the BRAF mutated protein, either alone or in combination with MEK inhibitors, in those cases harboring activating mutations in *BRAF*. After the rapid initial response detected in most patients to this treatment, unfortunately, drug resistance is developed after a short time, followed by a clinical relapse [Flaherty, K. T. et al., 2010; Sosman, J. A. et al., 2012; Robert, C. et al., 2015]. In addition, about 15 % of patients do not achieve tumor regression at all [Flaherty, K. T. et al., 2010; Sosman, J. A. et al., 2012; Robert, C. et al., 2015]. Another troubling circumstance is that no targeted therapy is being used in those patients without activating mutations in *BRAF*, who represent approximately a half of all melanoma cases [Davies, H. et al., 2002; The_Cancer_Genome_Atlas, 2015].

Immunotherapy produces more sustained responses than targeted therapies (reviewed by [Sharma, P. et al., 2015]) and, currently, it is often the first line treatment in melanoma. However, only a minority of patients derives benefit from this kind of treatment, while from 60 % to 70 % of them do not show any response [Hamid, O. et al., 2013; Delyon, J. et al., 2015; Sharma, P. et al., 2015].

Consequently, further efforts directed to enlarge the scope of therapies could bring benefits for many patients with advanced disease. In this regard, we wondered if a molecular characterization of individual melanoma cases could uncover unknown mechanisms of disease and facilitate, thus, an extensive understanding of this cancer. Hopefully, this could lead us to change the current conception of targeted therapies and manage a more rational use of them, yielding an improved efficacy.

As aforesaid, current targeted therapies for melanoma patients are limited to the inhibition of MAPK signaling pathway. However, it has been described that MAPK signaling is not enough for melanocytic transformation, being needed other signaling pathways (sometimes called cooperatives), like PI3K [Mishra, P. J. et al., 2010]. Indeed, high MAPK signaling induces cytotoxicity and senescence in melanocytes; the expression of the $BRAF^{V600E}$ protein in preclinical models has been associated with a phenomenon known as "oncogene-induced senescence (OIS)" [Michaloglou, C. et al., 2005], which results in a cell-cycle arrest, by involving the activation of both RB and p53 pathways. OIS brings about the stopping of the growth of some benign tumors, including melanocytic nevi harboring activating *BRAF* mutations, and avoids their progression to cancer [Michaloglou, C. et al., 2005; Chandeck, C. et al., 2010]. Therefore, some additional alteration must be to overcome OIS, to counteract the cytotoxic effects of high MAPK signaling and, finally, induce the malignant transformation of melanocytes.

In this regard, a number of clinical trials have been done with PI3K pathway inhibitors in advanced melanoma patients, amongst other cancer types, for example, those identified as NCT01616199, NCT01390818 or NCT01820364 (https://clinicaltrials.gov/), in which PI3K and mTOR inhibitors were used in combination with BRAF or MEK inhibitors. The starting with the use of such inhibitors in the clinic was based on the apparent importance of this signaling pathway in overcoming OIS, prompting the appearance of cancer and, later, of metastases [Cheung, M. et al., 2008; Dankort, D. et al., 2009; Vredeveld, L. C. et al., 2012]. This kind of therapies are offering a promising alternative for melanoma treatment and, depending on their toxicity profiles and antitumor activities, they could be approved to treat this and other forms of cancer. However, melanoma cases included in these trials were those with *BRAF* activating mutations, staying out those cases with wild-type *BRAF*. Therefore, further efforts in the development of targeted therapies are necessaries. In this respect, an impending clinical study (NCT01960829) is now recruiting participants with metastatic

melanoma, no previously treated, in whom test the efficacy of an mTOR inhibitor. The inclusion criteria of this trial contain the necessity of showing alterations in the mTOR protein, but disregard the *BRAF* mutational status. Possibly, studies like this can make a difference in the use of targeted therapies.

Data from Next Generation Sequencing (NGS) are showing that more mutated genes than were initially expected are participating in tumorigenesis of different cancer types, including melanoma [Stransky, N. et al., 2011; Berger, M. F. et al., 2012; Stark, M. S. et al., 2012]. Actually, a single melanoma lesion can harbor up to 1,500 somatic mutations only in exons [Berger, M. F. et al., 2012]. Such a heterogeneity and complexity of melanoma tumors imply a critical challenge for medicine. Molecular analysis of tumors at diagnosis may allow us to find private disease mechanisms that can be used to guide a rational selection of treatments in a personalized way.

Taking into account all these data, it seems that, alongside to MAPK signaling, other pathways are involved in the complex process of tumorigenesis. Related with that can be the fact that both clinical and pre-clinical studies have shown that combination strategies may be advantageous and can overcome the shortcomings of individual monotherapy approaches [Aziz, S. A. et al., 2010; Gopal, Y. N. et al., 2010; Shi, H. et al., 2011; Reuben, A. et al., 2015; Robert, C. et al., 2015]. Our data reinforce these findings, showing that combinatorial approaches, targeting two or three altered pathways at the same time, are often more efficient at braking cell proliferation *ex vivo* and tumor growth *in vivo*.

Resistances to actual treatments are a major hindrance to improve current data of survival of patients with advanced melanoma. A dynamic process of competition between clones and subclones within the tumor could address the appearance of multifactorial resistances [Greaves, M. et al., 2012; Shi, H. et al., 2014]. Data returned from the study of this process involve a wide range of mechanisms, some of them consisting of alternative ways to activate MAPK signaling [Emery, C. M. et al., 2009; Nazarian, R. et al., 2010; Villanueva, J. et al., 2010; Poulikakos, P. I. et al., 2011] whereas some others are independent of this pathway, such as PI3K/AKT pathway activation [Gopal, Y. N. et al., 2010; Villanueva, J. et al., 2010; Xing, F. et al., 2012] or MEK-independent effects mediated by COT [Johannessen, C. M. et al., 2010]. In this context, it acquires sense the possibility that the repression of MAPK signaling in cells addicted to this pathway thwarts their proliferation but facilitates the propagation of other clonal or subclonal cell populations, changing the network of pro-proliferative mechanisms of the whole tumor, which can be able to continue growing in the presence of MAPK inhibitors. From this perspective, provided evidences of frequent cross-resistances to BRAF and MEK inhibitors are barely surprising (reviewed by [Grazia, G. et al., 2014]). As multiple mechanisms seem to be involved in the process of resistance to therapy, once again, combinatorial regimens of treatment are likely to be more effective, either being

used simultaneously or consecutively. In this regard, patients treated with AKT inhibitors after progressing to the treatment with BRAF inhibitors responded to this therapy during a period of time (clinical trial identified as NCT01519427). However, no prior molecular study was performed with tumors of these patients, so it is possible that not all of them had the same level of activation (or tumor dependence) of PI3K/AKT pathway. On the other hand, as aforesaid, no trials have been done with patients in progression whose tumors lack activating mutations in *BRAF* oncogene.

Compiling this information, we hypothesized that, as part of an intricate network of transforming mechanisms in melanoma, this disease can use multiple, and probably case-specific, oncogenic mechanisms simultaneously. Some of these mechanisms can be, for example, PI3K, MET, GNAQ, Rho and JAK/STAT [Sanz-Moreno, V. et al., 2011; Straussman, R. et al., 2012; Turajlic, S. et al., 2014; Sadok, A. et al., 2015], which, alongside with MAPK signaling, participate as mechanistic drivers of this disease and promote resistance to therapy deriving in cancer progression. Therefore, we thought that the molecular characterization of tumor lesions was probably the key to achieving a rational selection of the appropriate treatment for each case. This could give us the opportunity to develop genetically defined therapeutic strategies, which may offer novel possibilities for treatment that would complement currently used therapies.

In this work, we selected 217 genes to be studied, basing this selection on three major criteria: a) previous evidences of their relevance in melanoma, b) availability of an associated pharmacological inhibitor with potential clinical use, c) their involvement in intracellular signaling or chromatin architecture. As many analyzed genes were targets of available inhibitors, results obtained from our genomic analysis could be directly translated into *ex vivo* and even *in vivo* assessment of functional and biological effects of relevant treatments.

5.8. MULTIPLE MECANISMS ARE ALTERED IN CUTANEOUS MELANOMA TUMORS.

We first compared *in silico* our selection of genes with previously published sequencing data from a total of 158 melanoma patients, originating from the sequencing of 133 exomes [Hodis, E. et al., 2012; Nikolaev, S. I. et al., 2012; Stark, M. S. et al., 2012] and 25 genomes [Berger, M. F. et al., 2012] from cutaneous melanoma cases, most of them metastatic. We found an average of 12.9 mutated genes (of our selection) per patient, among which, 3.8 genes per patient had a direct or indirect inhibitor available for being used in the clinical practice (publication 2, figure 1A and supplementary table SI). Likewise, we analyzed sequencing data from 11 cell lines, 10 of which were commercial and the remaining one was stablished in our laboratory from a biopsy of metastatic melanoma. In this case, we found

3.82 mutated genes and 2.73 mutated druggable genes per line (publication 2, figure 1A and supplementary table SI). Compiling all these data, we found an average of 3.73 mutated genes per case which can be rationally related with specific inhibitors currently (or to be soon) in clinical use.

These mutated genes belonged to different sets or pathways, which resulted in this way altered with different frequencies (publication 2, figure 1B and supplementary table SI). Thus, the two pathways which appeared more frequently deregulated by found mutations were PLC, as 25 % of druggable mutated genes were part of this pathway, and MAPK, affected by 23 % of mutations. Consequently, calcium signaling could be possibly playing, along with MAPK signaling, an important role in either carcinogenesis or resistance to therapies, as studies from which data came had included in the same work cases at diagnosis, cases in different moments of treatment and cases in relapse [Berger, M. F. et al., 2012; Hodis, E. et al., 2012; Nikolaev, S. I. et al., 2012; Stark, M. S. et al., 2012]. Receptor tyrosine kinases (RTKs) and G-protein-coupled receptors (GPCRs) represented, respectively, 18 % and 9 % of druggable mutated genes. Both types of membrane receptors trigger a number of signaling pathways related with cellular proliferation, survival, differentiation and migration [Ullrich, A. et al., 1990; Blume-Jensen, P. et al., 2001; Gschwind, A. et al., 2003; Zhu, H. et al., 2015]. Apart from MAPK, PLC, RTKs and GPCRs, several pathways, such as PI3K, JAK–STAT and YAP-mediated signaling, appeared also deregulated, although in lower proportions of tumors. Altogether, these findings reflect the great heterogeneity recurrently reported in melanoma, but also, they suggest the possibility of utilizing 3 or 4 inhibitors in each patient, affecting specifically deregulated mechanisms, either individually, sequentially or simultaneously.

Bearing in mind these results, we started to collect melanoma cases with intent to identify mutations harbored by melanoma tumors through targeted deep sequencing. In contrast with previous studies performed by different groups [Berger, M. F. et al., 2012; Hodis, E. et al., 2012; Nikolaev, S. I. et al., 2012; Stark, M. S. et al., 2012], we stablished strict clinical criteria for the selection of patients in order to avoid mixing cases with different types or subtypes of melanoma, or in a different phase of disease evolution or treatment. We decided to include in this project cases of advanced cutaneous melanoma at diagnosis. Cases considered as “advanced” were those with worst prognosis, namely, those with metastatic disease or with a Breslow Index of 4 mm or greater. Cases considered as “at diagnosis” were those untreated, apart from surgical resection and some adjuvant therapy, like Interferon γ (IFNγ). Following these criteria, we collected tumor and normal samples from 18 patients (publication 2, supplementary table SII). Results obtained from the targeted ultrasequencing, and later validated, showed mutations in a variable number of genes (publication 2, table 1), being the average 3.17 genes per case. Some of these genes were repeated in different tumor samples, such as *RAF*, *FAT* or *PLC*, but taking together all the mutated genes of every tumor, we realized that each case had a unique mutational profile, with a differentiating combination

of mutated genes and, what is more, with a distinct allele frequency in each case. This information could be taken as a reflection of the clonal composition of each tumor. Some of our samples displayed no mutations in the selected genes, others showed mutations in genes that lack an associated inhibitor, so we could not envision a targeted therapy for these cases. For example, 3 tumors harbored mutations in *FAT* genes (2 of them, in *FAT4* and the remaining one, in *FAT2*), but no inhibitor are available to block the activity of FAT protein or pathway. However, 7 tumors (39 %) harbored mutations in genes that could be associated with one or more inhibitors, with direct or indirect effect over gene function, such as *RAS*, *RAF*, *FLT* or *PLC*. For example, one case of our cohort –melanoma 17– had mutations in 4 genes; *BRAF*, *FAT4*, *MAPK7* and *DCC*. Two of these altered genes –*BRAF* and *MAPK7* (also called *ERK5* and *BMK1,* respectively)– had associated inhibitors, such as Vemurafenib and XMD8-92, respectively, which could be used to treat this patient. Therefore, we used this patient as a model in which test our approach with *ex vivo* and *in vivo* experiments.

At this point we realized that we had 5 patients in our cohort who could be treated with inhibitors independent of MAPK signaling. In 3 of them, alterations in MAPK pathway were also presents, offering thus the possibility of combining the two approaches. Altogether, these data hint at the idea of designing personalized strategies for therapy, guided by specific mutational profiles, independently of the *BRAF* mutational status.

Keeping in mind the number of pathways, receptors and factors found altered in melanoma tumors, together with the number of cross-talk recurrently described between different pathways in many cellular types [Grammer, T. C. et al., 1997; Carracedo, A. et al., 2008; Carracedo, A. et al., 2008; Guo, X. et al., 2009; Mirzoeva, O. K. et al., 2009; Jiang, C. C. et al., 2011; Labouba, I. et al., 2015], including melanocytes, we could perceive the molecular processes that govern the biology of cells as a big network of complex interactions. Unbalancing this network could lead, amongst other outcomes, to an uncontrolled cellular proliferation.

5.9. TARGETED THERAPIES THAT DO NOT INHIBIT MAPK SIGNALING EXERT ANTI-PROLIFERATIVE EFFECTS IN CUTANEOUS MELANOMA.

As mentioned already, current targeted therapies in melanoma are practically limited to the use of BRAF and MEK inhibitors. Furthermore, they are being applied with a limited extent, focusing on those cases harboring activating *BRAF* mutations. We wanted to study the possibility of control cellular proliferation and tumor growth from a different perspective, exploiting other mechanisms of disease which could be targeted by inhibitors, together or not with MAPK signaling pathway.

Ex vivo effects of targeted therapies selected according to specific mutational signatures:

We firstly used 4 different commercial cell lines of cutaneous melanoma to test the effectiveness of targeted therapies selected based on the mutations found in the selected set of genes. For each cell line, we chose 2 or 3 mutated genes that could be associated with an inhibitor to test the effect of such inhibitors over cellular proliferation. During the development of this project, we have primarily been employing those drugs which were being used in patients, even if it was in clinical trials and even if it was for treating other diseases, in order to reach the possibility of translating our results to the clinic as directly and fast as possible. As well, during the development of this project, we have avoided treating cells with too high doses of drugs in order to prevent possible unspecific cytotoxic effects, especially when more than one inhibitor was used simultaneously. Thus, we selected and used inhibitors for A375, SKMEL2, SKMEL28 and MEWO cell lines. Most of chosen drugs blocked, specifically, altered molecules, but a few of them exerted an indirect effect over the altered pathway, inhibiting some point downstream the mutated protein. This happened, for example, with the use of Everolimus in SKMEL28 cells harboring a mutation in *PTEN*, with the use of HGFR inhibitors when detected mutations in *HGF*, and with the use of MEK inhibitors in cases with *RAS* mutations, such as SKMEL2.

We realized that a number of these inhibitors did not reduce the activation of ERK1/2, so they could be considered independent of MAPK pathway. This occurred with Everolimus (mTOR inhibitor) and Vargatef (FGFR inhibitor), used on A375 cells (publication 2, figure 2); with Dacomitinib (ERBB inhibitor) and XMD8-92 (ERK5 inhibitor), used on SKMEL2 cells (publication 2, supplementary figure S2); with Everolimus and Ruxolitinib (JAK inhibitor), used on SKMEL28 cells (publication 2, supplementary figure S3); and with the three inhibitors used on MEWO cells, Vargatef, Dacomitinib and Crizotinib –an HGFR inhibitor– (data not shown). All these inhibitors did have an effect on reducing cell proliferation, being stronger the effect of combining of two of them at their respective IC_{50} dose versus monotherapy. In addition, in the five cell lines it was observed that the greater number of inhibitors used together, the stronger effect yielded on cellular growth, regardless of whether this combination include or not the inhibition of MAPK-dependent mechanisms. As an example, MEWO cells were treated with tree different drugs, none of which affect the quantity of P-ERK1/2 detected by western blot (data not shown). Individually, these drugs were effective on reducing cellular proliferation in a dose-dependent manner. Combinations by twos returned enhanced effects versus monotherapies and, moreover, cells barely grew during 48 hours in presence of the three drugs together (publication 2, supplementary figure S5). Similar effects were perceived when treating A375, SKMEL2 and SKMEL28 cells, in whose cases the inhibition of three targets included MAPK-dependent and MAPK-independent mechanisms (publication 2, figure 2 and supplementary figures S2-S5).

These data leaded us towards two main appreciations: firstly, combinatorial targeted approaches seem to manifest more powerful anti-proliferative effects on melanoma cells than targeted monotherapies, as had been proposed by some other groups [Grazia, G. et al., 2014; Kwong, L. N. et al., 2014] secondly, targeted therapies which do not show any effect in inhibiting ERK1/2 activation do have an effect on braking cellular proliferation.

In order to deepen the awareness of the effects of our treatments in the biology of cells, we assessed their impact over DNA synthesis, using A375 cells as a model (publication 2, figure 2). We found that $BRAF^{V600E}$ inhibitor was very effective in minimizing DNA synthesis, but mTOR and FGFR inhibitors, both independent of MAPK activity, also exerted an effect, especially when both were used together. This experiment leaded us to reaffirm our previous conclusions: first, inhibitors which seem not to reduce ERK1/2 activation do inhibit cellular processes necessaries for cellular division, such as DNA synthesis; second, the treatment with those inhibitors do not seems to produce hard cytotoxic effects, as cells were still alive after 48 hours of treatment although synthesizing less DNA.

In the light of these results obtained with commercial cell lines, we then addressed whether the same approach would yield similar results MELANOMA17 cell line, which had been established in our laboratory from the tumor lesion of one of the patients included in this study (patient 17). Of the four mutated genes found in this tumor and, later, in this cell line (publication 2, figure 5B), two genes (*BRAF* and *MAPK7*) could be associated with inhibitors (Vemurafenib and XMD8-92, respectively). Therefore, we treated cells with these two drugs, which showed, individually, dose-dependent effects on reducing cellular growth. Interestingly, one of these drugs was independent of MAPK activity and did not affect the activation of ERK1/2 (publication 2, figure 5C), confirming our previous observations about the possibility of braking cellular growth without inhibiting MAPK activity. In addition, when cells were grown in presence of the two inhibitors (at their respective IC_{50} doses) simultaneously, their anti-proliferative effects were more powerful. Consequently, we reached again the best outcomes in reducing tumor cells growth by means of combinatorial targeted strategies, in this case affecting MAPK-dependent and -independent mechanisms.

In vivo effects of targeted therapies selected according to specific mutational signatures:

After these experiments with cellular cultures, we wanted to test our method *in vivo*. Therefore, A375 cells were injected in Balb/c Nu/Nu mice, originating tumors in xenograft (cell line-derived xenografts, CDX). Basing on the therapy designed for treating this cell line in culture, we divided mice with tumors into 4 different groups of treatment: 1. Control group, which receive only the vehicle; 2. Inhibited MAPK pathway group, which was given Vemurafenib (BRAF inhibitor); 3. Non-inhibited MAPK pathway group, which was given

Everolimus (mTOR inhibitor) plus Vargatef (FGFR inhibitor); 4. Triple therapy group, which receive the three drugs. During the treatment, we appreciated that tumors of those mice included in the control group grew faster than tumors of mice belonging to the treated groups (publication 2, figure 4). Indeed, after 13 days of treatment, mice given the BRAF inhibitor had tumors of a similar size to those given the mTOR inhibitor plus the FGFR inhibitor, but of a smaller size than mice in the control group. However, the smallest tumors were in those mice treated with the triple therapy.

Further than growth of tumors, we assessed the mitotic rate in tumors from the four groups, finding a lower number of mitosis in treated mice than in control mice (publication 2, figure 4). In those tumors treated with MAPK-independent inhibitors the number of mitosis was even lower than in those treated with Vemurafenib. In any case, tumors belonging to triple therapy group had a very low number of mitosis, much minor than tumors of each other group. As well, we assessed KI67 expression, finding lower levels in tumors from mice included in the triple therapy group. In addition, we confirmed our previous *ex vivo* results (obtained in A375 cells) regarding to P-ERK1/2 quantification, verifying that, this time *in vivo*, the combination of Everolimus and Vargatef did not significantly affect the amount of P-ERK detected by immunohistochemistry (IHQ).

These results showed that we had been able to reduce the growth of tumors and the number of mitosis by inhibiting MAPK pathway, but also by inhibiting other two mechanisms of disease, without affecting ERK1/2 activation. Furthermore, and in agreement with our previous observations *ex vivo*, the strongest effect in braking tumor growth were obtained with the triple therapy, which is, administering the higher number of drugs.

In order to go beyond in our pre-clinical studies, we generated patient-derived xenografts (PDX) by implanting pieces of a biopsied melanoma tumor in Nod Scid Gamma (NSG) mice. We chose for this experiment the tumor from patient 17, the same that was used in our previous *ex vivo* experiments. Again, mice were divided into 4 groups, each of which received a different treatment, accordingly with mutations found in this tumor. Thus, the first group was given the vehicle; the second group, Vemurafenib (BRAF inhibitor); the third group, XMD8-92 (ERK5 inhibitor); and the last group, a combination of both inhibitors. Tumor size was daily measured in order to monitor its evolution (which is graphically represented in figure 5G of publication 2). Since the 7^{th} day of treatment, tumors in the control group started to grow very fast whereas tumors in the 3 treated groups remained controlled. After 13 days of treatment, differences in both volume and weight of tumors between treated and non-treated mice were noticeable; showing that the 3 types of therapy had been effective (publication 2, figure 5H). Mice given the combinatorial therapy had tumors smaller than those treated with only one of the two drugs, albeit the statistical significance cutoff was not met in this case. This lack of statistical significance could be

attributed to the high efficacy of both types of monotherapy over the tumor growth, fact which makes the values of volume and growth of tumors in the two monotherapy groups too small to perceive great differences between them and the combinatory group.

As the ERK5 inhibitor had shown not to alter the amount of P-ERK1/2 in cells, we checked it in tumors collected from mice by IHC (publication 2, figure 5I). We found no significant differences between tumors treated with XMD8-92 and non-treated ones, but a reduced P-ERK1/2 staining in tumors belonging to the 2 groups which had received Vemurafenib. Thus, data from this experiment indicate again that we have the possibility of control the tumor growth by using drugs which not affect MAPK activity.

Our data lead us to envisage the possibility of designing targeted therapies, of course, for those cases with activating *BRAF* mutations, where the possibility of combining MAPK inhibitors with drugs that target other mechanisms of disease could be considered, but also for the remaining 50 % of melanomas. Conclusions extracted from our outcomes go on line with some current efforts directed to open the perspectives of targeted therapies, like the aforementioned clinical trial that will test the effects of an mTOR inhibitor in metastatic melanoma patients whose tumors show alterations in this pathway, regardless their *BRAF* mutational status (https://clinicaltrials.gov, NCT01960829).

5.10. TARGETED COMBINATORIAL THERAPIES ARE MORE EFFECTIVE WHEN USED WITHIN AN APPROPRIATE MUTATIONAL BACKGROUND IN CUTANEOUS MELANOMA CELLS.

Once proven the efficacy of the treatments designed according to the mutations found in cells and tumors, we wanted to know whether anti-proliferative effects of such treatments were dependent of the mutational profile of target cells. To assess this, we used the treatment designed for a certain cell line over others and compared the effects detected in each.

Thus, therapy designed for A375 was used to treat this cell line along with other five, including that established from the tumor of patient 17 (publication 2, figure 3). We used the IC_{50} dose of each inhibitor, individually and in different combinations, equally as done in previous experiments with A375 cells. $BRAF^{V600E}$ inhibitor was considerably more effective in A375 cells than in others. However, it did also show anti-proliferative effects over HT144, MALME and MELANOMA17 cells, all of which had the V600E mutation in the *BRAF* gene. We noticed that, in spite of this drug slowed down the proliferation of four cell lines, the level of efficacy was different in each one of them. These perceived variations in the effect of Vemurafenib over different cell lines with the same mutation can be easily explained considering the IC_{50} dose of each cell line. As we used the conditions calculated for A375, all

cell lines were growth in presence of Vemurafenib in a concentration of 0.07 μM, quantity similar to the IC_{50} of MALME cells, which were in fact more affected by the inhibitor than the other two, with higher values of IC_{50}. It is possible that the different mutational signatures and deregulated mechanisms present in each particular case could explain the different IC_{50} dosages calculated for Vemurafenib amongst cell lines. Consequently, it is possible that in patients with $BRAF^{V600E}$ tumors, this genetic heterogeneity could also play a role in the detected differences in clinical responses to BRAF inhibitors.

As occurred with Vemurafenib, we have recurrently observed that the same inhibitor had dissimilar IC_{50} doses across different cell lines, even if all of them had mutated the same gene, whose product would be the target of the inhibitor. Many factors can contribute to this circumstance, such as, for example, potential differences in the permeability of the plasmatic membrane for a particular drug, or the potential capacity of target cells for expelling the drug out [No_authors_listed, 2000]. But additionally, it might be also possible that the whole ensemble of mutations (or alterations) harbored by a cell determine the final biological effects of a particular drug, which inhibit one or a few of specific pathways of the whole altered network.

Beyond these effects of inhibitors individually used over different cell lines, we observed that combinations of these drugs by twos were mostly more effective in A375 cells than in the others, although the three possible combinations had substantial anti-proliferative effects in unless one cell line, apart from A375. However, the clearest difference between A375 and the rest of cell lines was observed when they all were grown in the presence of the three inhibitors which compose the triple therapy of A375. In this case, the proliferation rate of this cell line was reduced to 4 % versus the control whereas the rates of the other five lines ranged from 9 % to 43 % in relation to control.

Conversely, we tested the therapy designed for MEWO, SKMEL2, and MELANOMA17 cells over A375 (publication 2, figure 5E and supplementary figure S4). Again, the individual use of each drug exerted some effects, occasionally even stronger, over A375 than over the cell line for which the treatment had been intended. In this regard, especially noticeable was the powerful effect of Selumetinib in braking A375 cells growth, although it was expectable, taking into account the high levels of MAPK signaling in this cell line, fact which could mean a high dependence on MAPK signaling. However, consistently with our previous observations, combinatorial treatments, particularly those joining three different drugs, were significantly more effective in any cell line for which they had been intended.

In the same way, we did a series of experiments using the treatment designed for MELANOMA17, MEWO and SKMEL28 cells over several other lines (publication 2, figure

5E, supplementary figure S5C, and data not shown, respectively), reaching alike results than formerly.

Altogether, these experiments highlight the importance of the mutational background of cells in driving its response to a particular drug or group of drugs. This fact could explain, at least in part, the considerable differences observed across cancer patients in their evolution under a given treatment. The possibility that the whole ensemble of alterations in a tumor cell influences the degree of efficacy of a treatment sharpen the necessity of considering the treatment for cancer, and specifically for melanoma, in a more personalized way.

5.11. APPLICATION PROSPECTS OF MOLECULAR CHARACTERIZATION OF MCC AND MELANOMA.

The two projects that compound this thesis have been based on the use of sequencing techniques applied to the molecular characterization of tumors. The work conducted with Merkel cell carcinoma can serve as an example of the utilization of these techniques to achieve a broad analysis of molecular alterations driving cancer. By contrast, the work performed with cutaneous melanoma is an instance of a more defined molecular analysis. In both projects we have complemented sequencing results with functional and mechanistic studies, in one case basing on bioinformatics and statistical analysis and, in the other case, by means of *ex vivo* and *in vivo* experiments. Achieved results have allowed us to reach a better knowledge of the molecular basis of these two cancer types and, perhaps, they have even contributed tools which could be useful in a near future in diagnosis, prognosis or therapy.

In the case of Merkel cell carcinoma, we have found a set of pathways and biological processes frequently altered in our cohort, most of which had not been linked with MCC before. Some of these deregulated mechanisms could serve to guide therapy, in the same way as they are being already used in other cancer types. In this regard, *ex vivo* experiments with MCC cell lines could be really useful to test the effect of several inhibitors. Some examples of them could be:

- Tacrolimus (FK-506) to block signaling by calcium, which have been found significantly altered in our cohort.
- Dacomitinib (PF299804, PF299), Vargatef (BIBF 1120) and Regorafenib to inhibit several RTKs, including ERBB4, FLT4 and KDR, which are mutated in a number of our samples.
- BKM 120 −a PI3K and P-AKT inhibitor− or Everolimus −an mTOR inhibitor−, which could be used in those cases with alterations in PI3K-AKT-mTOR pathway.

As far as we know, these therapies have not been proposed or tested in MCC patients or cells, representing thus an interesting and novel choice that deserves to be explored.

Given the fact that the molecular characterization of all tumors is not always possible in the routine clinical practice, we have identified a number of biomarkers which can be easily assessed by IHQ. They could be useful in the near future to obtain a clue of molecular mechanisms probably altered in each MCC case in an affordable and fast manner.

In addition, through this work we have confirmed the two MCC etiologies recently proposed by independent studies [Goh, G. et al., 2015; Harms, P. W. et al., 2015; Wong, S. Q. et al., 2015]. As the etiology of a cancer could influence factors such as its aggressiveness or its response to treatments, taking into account this fact at the time of diagnosis may perhaps be interesting.

Probably, the most relevant finding achieved through this project is the discovery of P-CREB as an independent prognostic factor. Next step would be the validation of this finding in an independent cohort of MCC patients, so future works in this respect should be expected. In the event that the prognostic capacity of this marker is confirmed, its implementation in the regular characterization of MCC tumors could be recommendable.

Finally, there is now a real necessity of finding biomarkers that can serve as predictors of the response to immunotherapy. According with last reported data in this respect, the response of MCC patients to the treatment with anti-PD1 antibodies is similar between MCPyV-positive and –negative cases [Nghiem, P. T. et al., 2016]. Therefore, the study of markers subrogated to the activity of those disease mechanisms that have been identified in this work as common to the two MCC etiologies could be a great opportunity to characterize MCC cases and, perhaps, predict which of them could become more benefited by immunotherapy.

Regarding to cutaneous melanoma project, we have contributed an innovative perception of molecular mechanisms that could be driving this disease. While many efforts have been done for designing drugs capable of blocking MAPK signaling, making of this pathway the main objective of targeted therapies in melanoma [Chapman, P. B. et al., 2011; Larkin, J. et al., 2014; Robert, C. et al., 2015], a wide range of pathways whose inhibition could have an effect on breaking the growth of tumors or cells remains unveiled.

It is well known that a high proportion of melanoma tumors harbors alterations affecting MAPK pathway [The_Cancer_Genome_Atlas, 2015]. In addition, the activation of ERK1/2 over the levels detected in “normal” or “healthy” melanocytes has been reported in melanoma lesions at diagnosis [Jorgensen, K. et al., 2003; Zhuang, L. et al., 2005] and, moreover, in the time of relapse [Paraiso, K. H. et al., 2010; Lito, P. et al., 2012]. However,

the efficacy of current therapies in increasing the survival of patients is still limited, indicating that some mechanism must be developed by tumor cells to bypass the effect of inhibitors. Our data hint at the importance of different signaling mechanisms in prompt the melanocytic transformation, perhaps in cooperation with MAPK signaling. This can offer a broad range of therapeutic options to be used along with MAPK inhibitors, as schematically represented in figure 15.

On the other hand, our results provide a chance for trying a targeted therapy in so many cases which lack activating BRAF mutations, and thus, are not receiving targeted therapies now [Davies, H. et al., 2002; The_Cancer_Genome_Atlas, 2015] (figure 15). A possible future application of our outcomes could be a clinical trial with these patients, utilizing the approach that we have tested *ex vivo* and *in vivo*.

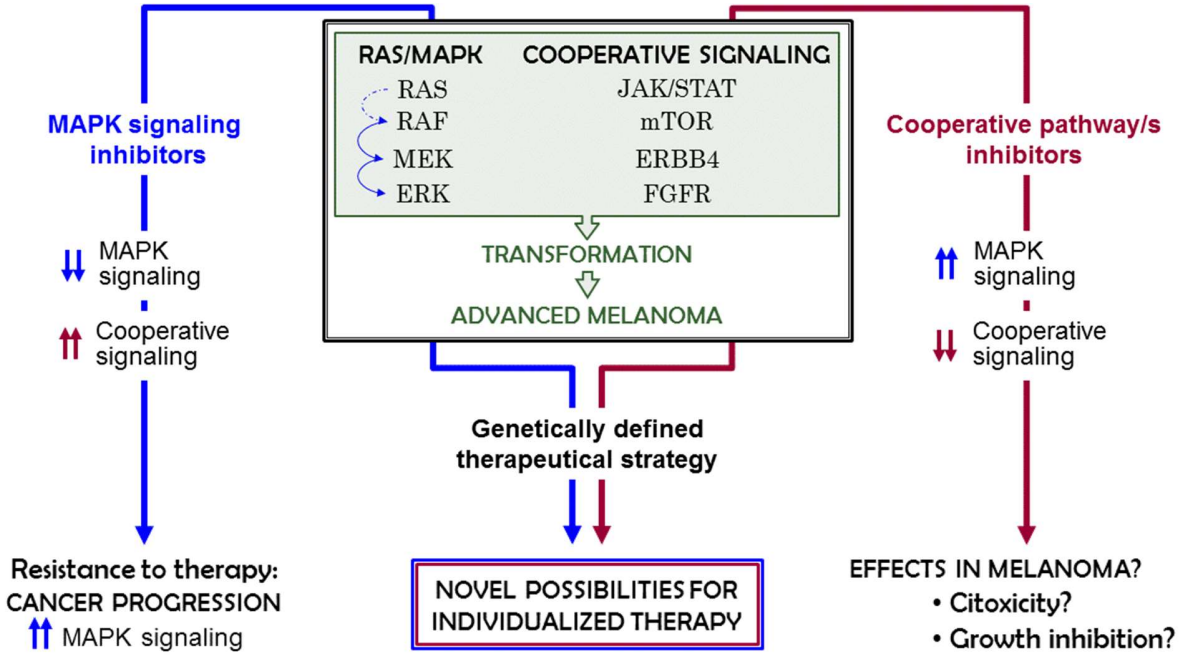

Figure 15. Schematic representation of the novel possibilities for guiding therapy in melanoma using combinatorial approaches guided by sequencing data. Current targeted therapy of advanced melanoma involves targeting aberrant MAPK signaling (blue arrows), guided by the detection of activating mutations in *BRAF*. This produces rapid but transient effects that end in the development of multiple mechanisms of resistance that have been shown to reactivate ERK1/2. Using the targeted approach proposed in this study, we can detect alterations in other potentially oncogenic signaling pathways that may contribute to promote melanocytic transformation and disease progression. Targeting these pathways without affecting MAPK (rosy arrows) may also inhibit melanoma growth, either directly (per se) or indirectly (due to cytotoxic effects of aberrant MAPK signaling). Thus, we have the possibility of designing novel therapeutic strategies (genetically defined) for individually characterized advanced melanoma cases.

Thus, the awareness of genomic alterations and aberrant signaling networks found in our cohort of melanoma tumors, as well as in other independent cohorts (through an *in silico*

analysis), could expand the range of possibilities for the design of targeted therapies in the future, either along with, or independently of, the inhibition of MAPK pathway activity, as represented in figure 15.

As mentioned before, this work with cutaneous melanoma has been focused on cases at diagnosis, before starting a treatment (apart from surgical resection and possible adjuvant therapy). Our next challenge would be to conduct a similar study with cases in progression after treatment, which means, cases that have become resistant to received therapy. Such analysis could lead us to untangle the mechanisms responsible of resistances to both immunotherapy and targeted therapies.

Considering the potential applicability of this work to the routine clinical practice, this approach would require several limitations to be overcome, which should entail:

1. Establishing efficient protocols to collect, manipulate and characterize tumor lesions representative of the different steps of the disease.
2. Managing the toxicity due to drug combinations.
3. Dealing with tumor heterogeneity and with interactions with the immune system, which could be responsible for the eventual resistance acquired after combinatory treatments.

Thus, although more work is needed to increase our understanding of the molecular mechanisms driving specific cases of Merkel cell carcinoma and advanced cutaneous melanoma, the work presented in this thesis offers novel possibilities and tools to develop approaches for diagnosis and treatment of these two types of malignant skin cancer.

6. Conclusions

6.1. CONCLUSIONS FROM ARTICLE 1: Shared oncogenic pathways implicated in both virus-positive and UV-induced Merkel Cell Carcinomas.

1. It has been designed an original approach in this field consisting in a combination of mutational and immunohistochemistry analysis that enabled the molecular characterization of MCC cases.
2. Genomic features of MCC tumors point at the existence of two main MCC subtypes:
   a) MCPyV-negative, with high mutational load and UV-induced genomic signatures.
   b) MCPyV-positive, with low mutational load.
3. Several biological processes and signaling pathways, not linked before with MCC, have been found significantly altered in our cohort.
4. Despite important genomic differences, MCPyV-positive and MCPyV-negative MCC tumors seem to develop similar mechanisms of disease: RB, p53, p63, NFAT, P-STAT and P-CREB.
5. P-CREB and P-STAT expression significantly correlate with poor prognosis in a univariate analysis.
6. In a multivariate analysis of our data, P-CREB expression has been identified as an independent prognostic marker for MCC patients (independent of age, sex, disease stage, P-STAT and MCPyV status).

6.2. CONCLUSIONS FROM ARTICLE 2: Individualized strategies to target specific mechanisms of disease in malignant melanoma patients displaying unique mutational signatures.

1. A targeted mutational analysis platform has been developed to study melanoma cases in a time compatible with the clinical practice (approximately 15 days).
2. Each tumor lesion has shown a unique mutational profile and an average of 3-4 mutated genes per case with potential to be directly or indirectly associated with a specific inhibitor.
3. Therapies that combine targeting MAPK-dependent and MAPK-independent mechanisms have been more effective than monotherapies, both *ex vivo* and *in vivo*.
4. Using this approach, it could be possible to suggest targeted therapies for advanced melanoma cases independently of *BRAF* mutational status.
5. Targeted therapies have been more effective *ex vivo* when used over appropriate mutational backgrounds.

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8. Annexes

8. ANNEXES

Following information can be found in the enclosed CD, placed on the back cover of this thesis:

- Thesis in digital format (pdf file).
- Publication 1:
  - Article (pdf file).
  - Supplementary material (pdf file).
- Publication 2:
  - Article (pdf file).
  - Supplementary material (pdf file).