Genomic and non-genomic alterations in melanoma contributing to disease progression and therapy resistance

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Remarks

Here I present a cumulative thesis consisting of two published original articles and one unpublished manuscript.

The work presented in this thesis was carried out between January 2016 and July 2019 under the supervision of the principal investigator Prof. Dr. rer. nat. Annette Paschen of the Dermatology Department of the University Hospital Essen (DE), Group ‘Molecular Tumor Immunology’.

I hereby declare that this thesis, submitted for the consideration of Dr. rer. nat award, has been composed by me.

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1. Abstract

Melanoma is an aggressive cancer that arises from transformed melanocytes of the skin and the ocular region, the uvea or conjunctiva. Two main therapies are currently applied to patients with cutaneous melanoma, small molecule inhibitors blocking dysregulated oncogenic pathways (in case of *BRAF*^{V600E} mutant melanoma), and antibody-based immunotherapy, boosting cytotoxic T cell responses against cancer cells. So far, none of these therapies has been approved for conjunctival melanoma. Given the rarity of this disease, it has been difficult in the past to thoroughly study the molecular mechanisms and genetic alterations driving this type of melanoma. By analyzing a large cohort of 67 tumor samples, we aimed to elucidate recurrent genetic alterations in conjunctival melanoma. Targeted next-generation sequencing was performed covering genes recurrently mutated in cutaneous and uveal melanoma. This led to the identification of *NF1* mutations in conjunctival melanoma, frequently detected in tumors lacking *BRAF* and *RAS* mutations. Based on these results, we proposed the classification of conjunctival melanoma into *BRAF*-mutated (25%), *RAS*-mutated (19%), *NF1*-mutated (33%) and triple-WT tumors, similar to cutaneous melanoma (paper Scholz et al., British J Cancer, 2018, in this thesis). Overall, our study provides insights into the mutational make-up of conjunctival melanoma and reveals the genetic similarities between cutaneous and conjunctival melanomas. Given the therapeutic success of inhibitor therapy and immunotherapy in skin melanoma, we propose both therapies can be considered as a valid option for patients suffering from conjunctival melanoma.

As indicated above, T cell-based immunotherapy of cutaneous melanoma has achieved great success over the past few years. But approximately half of the treated patients are intrinsically resistant or acquire resistance to therapy, indicating the urgent need to elucidate resistance mechanisms. The T cell-derived cytokine IFNγ plays a fundamental role in tumor cell killing under immunotherapy. Inactivation of the kinase JAK2, an IFNγ signaling pathway component, is known to contribute to immunotherapy resistance in tumor cells. In Horn, Leonardelli et al., we provide insight into the evolution of *JAK2* deficiency. The *JAK2* locus maps closely to the *CDKN2A* locus, encoding the tumor suppressor p16 on chromosome 9p. Allelic *CDKN2A* loss is an important step in melanoma development and caused by large deletions on chromosome 9p. Analyzing SNP array data of 46 melanoma cell lines and copy number data of the TCGA melanoma cohort (n= 367), we found *CDKN2A*-

associated *JAK2* losses in the majority of samples analyzed (> 60%). Thus, allelic *JAK2* loss occurs early in development of the disease, followed by inactivating gene mutations in single tumor clones finally establishing *JAK2* deficiency (paper Horn, Leonardelli et al., JNCI, 2018, in this thesis). Our results suggest that screening of tumor lesions for genetic *JAK2* alterations could be a valuable strategy to identify patients with an enhanced risk of developing immunotherapy resistance.

While the contribution of genetic alterations to IFNγ resistance is now well accepted, the role of non-genetic mechanisms is poorly defined. We postulated that non-genomic mechanisms could protect melanoma cells from IFNγ-induced apoptosis thereby interfering with the efficacy of immunotherapy. To study this, we established IFNγ-resistant variants from IFNγ-sensitive parental tumor cells. The sensitive and resistant tumor cell pairs were analyzed for transcriptional signatures associated with resistance to cytokine-induced apoptosis and phenotypic alterations were confirmed at the protein level. This led to the identification of an EMT (epithelial to mesenchymal transition)-like phenotypic switch in cells with acquired resistance to IFNγ going along with the downregulation of IFNγ signaling pathway genes (paper Leonardelli et al., unpublished, in this thesis). Interestingly, the EMT signature has previously been reported to be associated with unresponsiveness to both treatment with targeted inhibitors and immune-modulating antibodies, suggesting a broader relevance of our findings for melanoma therapy.

In summary, the different studies of this thesis provide further insight into the genetic and phenotypic heterogeneity of melanoma cells and its role in resistance to immunotherapy, which should guide therapeutic decisions to improve the clinical outcome of patients.

Zusammenfassung

Das Melanom ist ein aggressiver Tumor, der infolge einer malignen Transformation von Melanozyten der Haut und der okkularen Region, Uvea und Konjunktiva, entsteht. In der Therapie des kutanen Melanoms kommen hauptsächlich zwei Verfahren zur Anwendung: die Behandlung mit Inhibitoren, welche fehlregulierte onkogene Signalwege blockieren (im Falle des *BRAF*^{V600E} mutierten Melanoms) und die Antikörper-basierte Immuntherapie, welche die zytotoxische Aktivität Tumor-reaktiver T Zellen verstärkt. Im Falle des konjunktivalen Melanoms ist bislang keine dieser Therapien als Standardtherapie zugelassen. Aufgrund der Seltenheit des Melanom Subtyps war es bislang schwierig die zugrunde liegenden molekularen Mechanismen und genetischen Alterationen zu definieren. Diese Arbeit hatte zum Ziel, in einer Kohorte von 67 konjunktivalen Melanomproben wiederholt auftretende Mutationen zu identifizieren. Dazu wurde das Verfahren der zielgerichteten “Next-Generation“ Sequenzierung angewandt. Dies führte zur Identifizierung von *NF1* Mutationen, die häufig in *BRAF* und *RAS* Wildtyp Tumoren auftreten. Basierend auf unsere Ergebnissen kann das konjunktivale Melanom in *BRAF*-mutierte (25%), *RAS*-mutierte (19%), *NF1*-mutierte (33%) and 3-fach Wildtyp Tumore unterteilt werden vergleichbar zum kutanen Melanom (Manuskript Scholz et al., British J Cancer, 2018, in dieser Dissertation). Daher schlagen wir eine Behandlung dieses seltenen Melanom Subtyps mit zielgerichteten Inhibitoren und spezifischen Immuntherapien vor, entsprechend dem Vorgehen beim kutanen Melanom.

Wie vorangehend dargestellt, hat die T Zell-basierte Immuntherapie des kutanen Melanoms in den letzten Jahren große Erfolge erzielt. Allerdings ist etwa die Hälfte der behandelten Patienten von einer Therapieresistenz des Tumors betroffen, die bereits zu Beginn der Behandlung vorliegt oder in deren Verlauf erworben wird. Daraus ergibt sich die dringende Notwendigkeit Resistenzmechanismen aufzuklären. Das T Zellzytokin IFNγ übernimmt eine zentrale Rolle in der Eliminierung von Tumorzellen unter Immuntherapie. So wurde bereits gezeigt, dass die Inaktivierung der Kinase JAK2, einer Komponente des IFNγ Signalwegs, zur erworbenen Immuntherapieresistenz des Melanoms beiträgt. In Horn, Leonardelli et al., wurde die Entstehung der *JAK2* Defizienz untersucht. Auf Chromosom 9p befindet sich der *JAK2* Lokus in relativer Nähe zum *CDKN2A* Gen, welches für den Tumorsuppressor p16 kodiert. Der allelische *CDKN2A* Verlust ist ein wichtiger Schritt in der Entstehung des Melanoms und tritt infolge großer Deletionen auf Chromosom 9p auf. Die

Analyse von SNP Array Daten aus 46 Melanomzelllinien und der TCGA Melanom Kohorte (n = 367) zeigte, dass ein *CDKN2A*-assoziiierter *JAK2* Verlust in der Mehrheit der untersuchten Proben vorlag (> 60%). Folglich treten allelische *JAK2* Verluste früh im Verlauf der Erkrankung auf, während inaktivierende Genmutationen im Anschluss durch einzelne Tumorzellklone erworben werden, was schließlich zur *JAK2* Defizienz führt (Manuskript Horn, Leonardelli et al., JNCI 2018, in dieser Dissertation). Auf der Grundlage unserer Ergebnisse schlagen wir ein Screening von Tumorläsionen auf genetische Alterationen in *JAK2* vor, um Patienten mit einem erhöhten Immuntherapieresistenz Risiko zu identifizieren.

Während die Rolle von genomischen Alterationen in der IFNγ Resistenz akzeptiert ist, sind nicht-genetische Mechanismen bislang kaum untersucht. Wir postulierten, dass nicht-genetische Mechanismen in Melanomzellen einen Schutz gegenüber IFNγ-induzierter Apoptose vermitteln und somit die Wirksamkeit von Immuntherapien beeinträchtigen. Um dies zu untersuchen, wurden aus IFNγ-sensitiven parentalen Tumorzellen durch kontinuierliche Kultivierung in Gegenwart des Zytokins resistente Varianten etabliert. Die sensitiven und resistenten Tumorzellpaare wurden dann hinsichtlich potentieller Resistenz-vermittelnder transkriptioneller Signaturen untersucht und korrespondierende phänotypische Veränderungen der Tumorzellen auf Proteinebene bestätigt. Dies führte zur Identifizierung von EMT (epithelial-mesenchymal transition) Signaturen und entsprechenden phänotypischen Veränderungen in Zellen mit erworbener IFNγ Resistenz (Manuskript Leonardelli et al., unpubliziert, in dieser Dissertation). Ähnliche EMT Signaturen wurde bereits mit der Resistenz des Melanoms gegenüber zielgerichteten Inhibitortherapien und Antikörper-basierten Immuntherapien in Zusammenhang gebracht, was auf eine breitere Relevanz unseres Befundes hindeutet.

Zusammenfassend ergeben sich aus den verschiedenen Studien dieser Dissertation neue Erkenntnisse zur genetischen und phänotypischen Heterogenität des Melanoms und deren Rolle in der Immuntherapieresistenz, welche richtungsweisend für Therapieentscheidungen sein sollten, um das klinische Ansprechen der Patienten zu verbessern.

2. Introduction

2.1. Melanoma

Melanoma is an aggressive cancer and the deadliest malignancy of the skin (1–3). It arises from transformed melanocytes, which are located throughout the body; they can be found in the bottom layer of the skin epidermis, in the eye, both in the conjunctiva and in the uvea, in the vaginal epithelium, in the heart and in bones. Melanocytes are responsible for producing melanin, to protect from ultraviolet (UV)-induced damage (1–5). Melanocytes are originally derived from neural crest cells, which in turn originate from the neural tube. During development, neural crest cells form from the dorsal part of the neural tube by epithelial to mesenchymal transition (EMT). These cells have high migratory capacity and they can give rise to melanocytes, Schwann cells and peripheral neurons in the embryo by migration, proliferation, differentiation. Melanocyte precursors migrate along the body to fully differentiate into pigment-producing melanocytes. The high migratory capacity and the plasticity of melanocyte precursors and neural stem cells are one of the reasons that melanoma is also plastic, aggressive and highly metastatic (5).

At the molecular level, melanoblasts/glial bipotent progenitors arise from the neural crest cells by specific expression of *SOX10*. Melanoblasts are therefore further differentiated by turning on expression of the *MITF*, *DCT* and *KIT*. This population forms the pigment-producing melanocytes and another dedifferentiated population (*MITF* / *KIT*-negative but *DCT*-positive), the melanocyte stem cells (**Figure 2.1**). These stem cells have the aim of replacing melanocytes during the course of life (5).

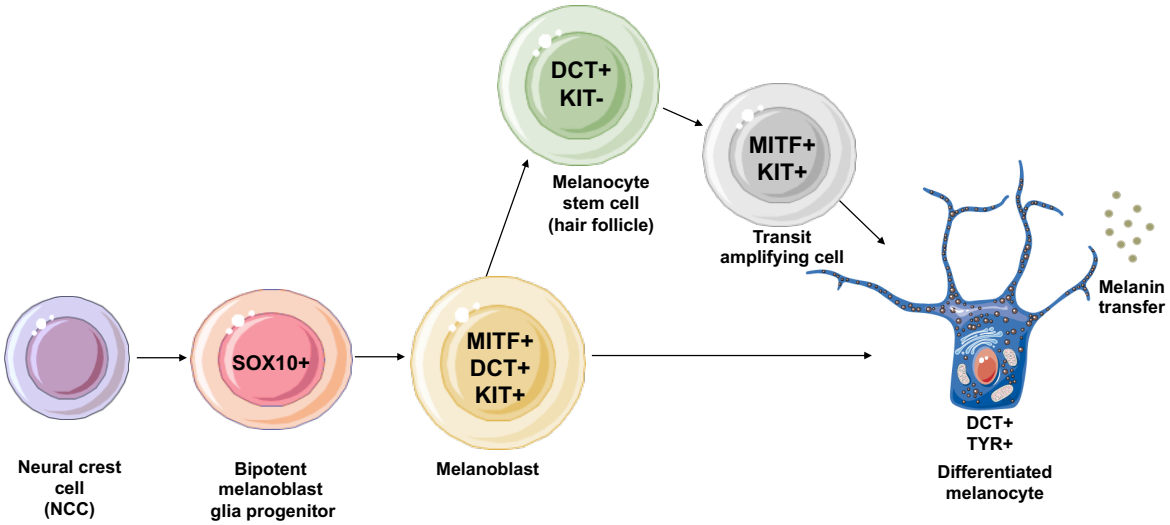

Figure 2.1 Differentiation marker expression throughout melanocyte development.

Expression of different differentiation marker throughout the development of melanocytes, starting from neural crest cell (NCCs) in mammals and image of a mouse embryo with a blue X-Gal staining of melanoblasts. Adapted from (5) and produced using Servier Medical Art (https://smart.servier.com/).

2.2. Cutaneous melanoma

Cutaneous melanoma accounts for almost 1% of all cancer-related death. It is the 7th most common cancer type in Europe, increasing every year since 1970, and the most frequent type of melanoma. There is an increasing need for preventative measures to cut the frequency of melanoma worldwide (6).

The incidence and mortality rates of this disease differ by country. In 2012, the incidence of cutaneous melanoma was calculated to be 0.2 per 100 000 person/years in Asia, and 7.7 per 100 000 person/years in the Americas. New Zealand and Australia, where the ozone depletion is higher, have the highest incidences, accounting for 35.8 per 100 000 person/years and 34.9 per 100 000 person/years, respectively (**Figure 2.2**) (7).

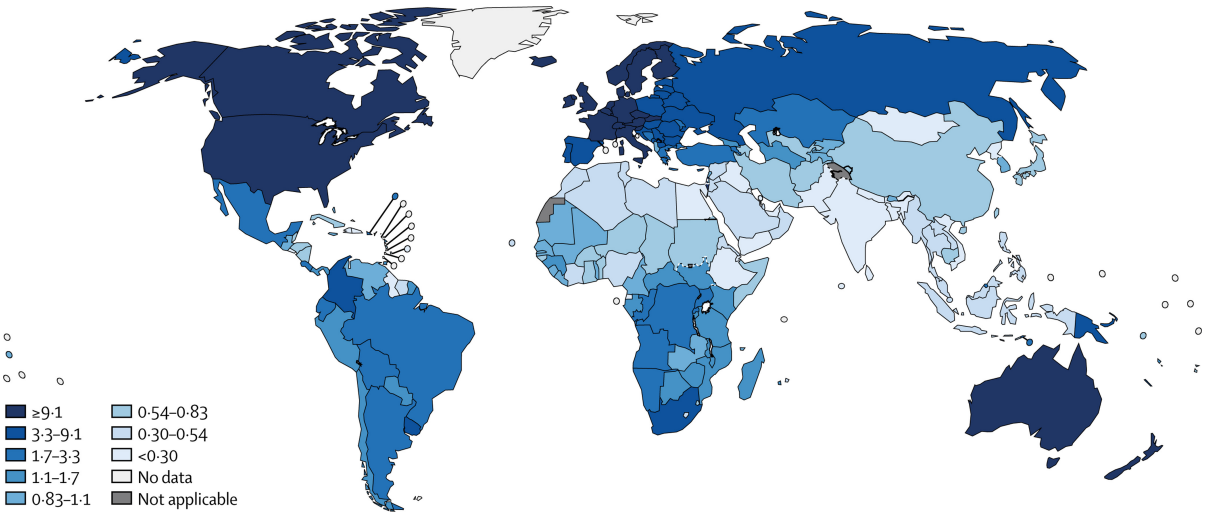

Figure 2.2 Incidence of cutaneous melanoma worldwide in 2012.

Incidences are age-standardized and representing both men and women together. Expressed as the number of cases per 100 000 person/years (7).

2.2.1. Risk factors of cutaneous melanoma

Cutaneous melanoma has both genetic and environmental risk factors. The most common risk factor is UV radiation exposure. Sun UV light exposure causes genomic aberrations in melanocytes. There are three types of UV rays: UV-A (315-400 nm wavelength), UV-B (280-315 nm wavelength) and UV-C (100-280 nm wavelength). UV-A is not absorbed in the ozone layer and penetrates in the skin where melanocytes are. They are mainly responsible for damaging the skin. Most of UV-B is absorbed in the ozone layer, depending on ozone layer status and weather conditions, but can also cause damage in the skin and tanned appearance. UV-C is prevented to enter the atmosphere by the ozone layer (8–10).

UV light exposure causes two main types of DNA damage: the production of free radicals, which can be mutagenic, and the promotions of changes between pyrimidines, though the absorption of UV energy directly by cytosines and thymines. Skin cancer arises when unrepaired or incorrectly repaired UV-induced DNA damage in the melanocytes disturbs important growth signaling pathways and cell cycle control mechanisms, causing aberrant proliferation and behavior (1,8,10,11).

People having fair skin types, light-colored eyes and hair, freckles have increased risk of skin cancers (1,8,10–12). Studies have also shown a higher risk of getting

melanoma in people who had sunburns in childhood and adolescence. Other risk factors include a high number of nevi and a family history of melanoma (1,3,6).

2.2.2. Mutational landscape of cutaneous melanoma

Melanocytes in the skin are characterized by a very slow rate of proliferation. As soon as they become neoplastic, they start dividing more frequently, accumulating mutations and chromosomal alterations. Cutaneous melanoma is one of the tumors with the highest mutational load (> 10 mutations per megabase) (**Figure 2.3**) and a high number of UV signature mutations (1,3,12,13).

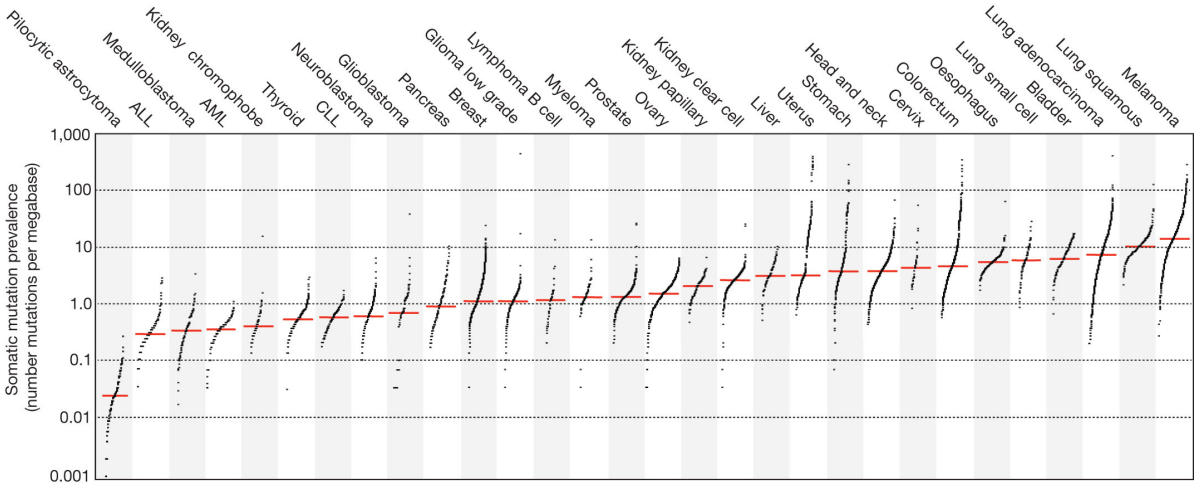

Figure 2.3 Somatic mutational load in cancers across human cancer types.

Somatic mutation prevalence, shown in number of mutations per megabase (vertical axis in log scale) in different cancer types (horizontal axis), ordered by median (red line) of somatic mutations. ALL =acute lymphoblastic leukaemia; AML =acute myeloid leukaemia; CLL = chronic lymphocytic leukaemia (14).

The malignant transformation from melanocyte to cutaneous melanoma follows a sequence of accumulating mutations. *BRAF*^{V600E} activating mutation in the mitogen-activated-protein-kinase (MAPK) pathway initiates aberrant proliferation resulting in nevus development and consequent cell cycle arrest. The MAPK pathway is central for cellular proliferation, and constitutive activation of the RAS/RAF/MEK/ERK pathway is critical for the development of cutaneous melanoma. This mutation is not sufficient to drive cutaneous melanoma formation (15). *BRAF* mutation is usually

followed by a mutation in the promoter region of the telomerase reverse transcriptase (*TERT*), the fundamental unit of the telomerase complex, and by alterations in *CDKN2A*, a cell-cycle-controlling gene. Further mutations are acquired in the chromatin remodeling gene *ARID1A, 1B or 2*, together with the appearance of extensive chromosomal aberrations during the transition to invasive cutaneous melanoma. At this stage, mutations and aberrations are also found in the *PTEN* or the tumor protein p53 (*TP53*). Altogether, these aberrations affect the activity of the MAPK pathway and the PI3K/AKT/mTOR pathway (1,3,8,9). The genetic makeup of cutaneous melanomas changes when cells go deeper into the skin and become invasive. At this stage, there is no further increase in the mutational burden, consistent with the fact that UV is a major contributor of DNA alterations in this disease (**Figure 2.4**).

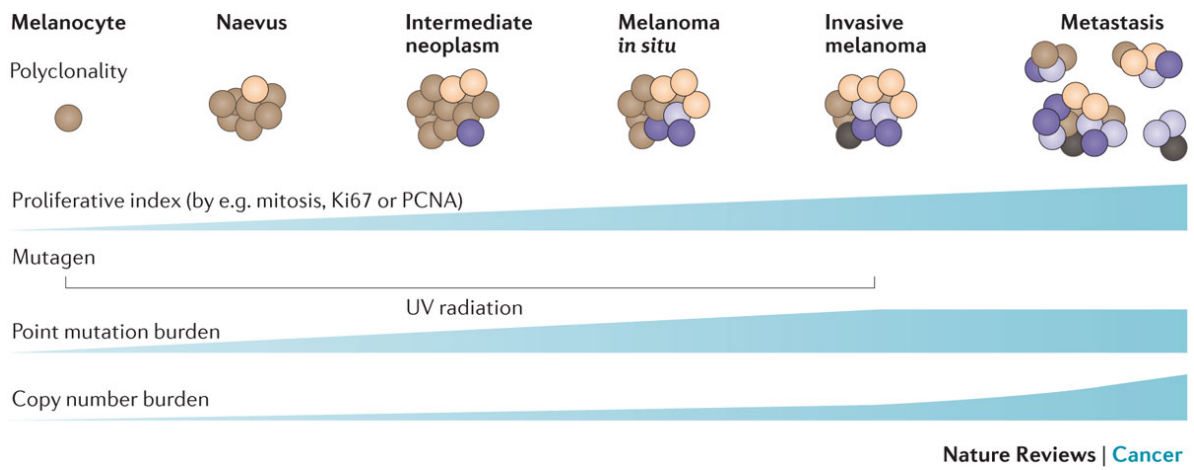

Figure 2.4 Melanoma development.

During malignant development of melanocytes into melanoma, cells become more proliferative, accumulate point mutations, mostly caused by UV radiations, and other genomic alterations as copy number alterations (9).

The Cancer Genome Atlas (TCGA) conducted an extensive analysis of more than 300 cutaneous melanoma patients and presented a new genetic classification of this disease, based on the most commonly occurring mutations (**Figure 2.5**) (13):

- Hot-spot *BRAF*-mutant tumors accounted for around 50% of all cutaneous melanomas, characterized by the presence of activating *BRAF* mutations of

codon 600 (V600E, V600K, and V600R). Hot-spot *BRAF* mutations anti-correlated with hot-spot *NRAS* mutations.

- *RAS* hot-spot activating mutations, the second most common in cutaneous melanomas, affecting all three *RAS* family members *NRAS*, *KRAS,* and *HRAS*. *NRAS* mutations were found to be the most common.

- *NF1* mutations, found in 14% of cutaneous melanomas. Mainly, they are predicted to be loss-of-function mutations. They are characterized by the highest mutation rate. *NF1* is a GTPase-activating protein that can downregulate MAPK activity. Therefore, loss of *NF1* function can constitutively activate this pathway. NF1 mutations correlated inversely with hot-spot *BRAF* mutations.

- Triple wild-type (WT) tumors accounted for around 10% of cutaneous melanomas, characterized by low-frequency non-hotspot mutations. Only one third of the samples were characterized by UV-signature mutations, higher copy-number alterations and structural rearrangements in genes as *KIT, CDK4, MDM2,* and *TERT*, as compared to the three other subtypes.

TERT-promoter mutations C228T and C250T are detected in 30-80% of cutaneous melanomas, and mutually exclusive between each other's (13,16).

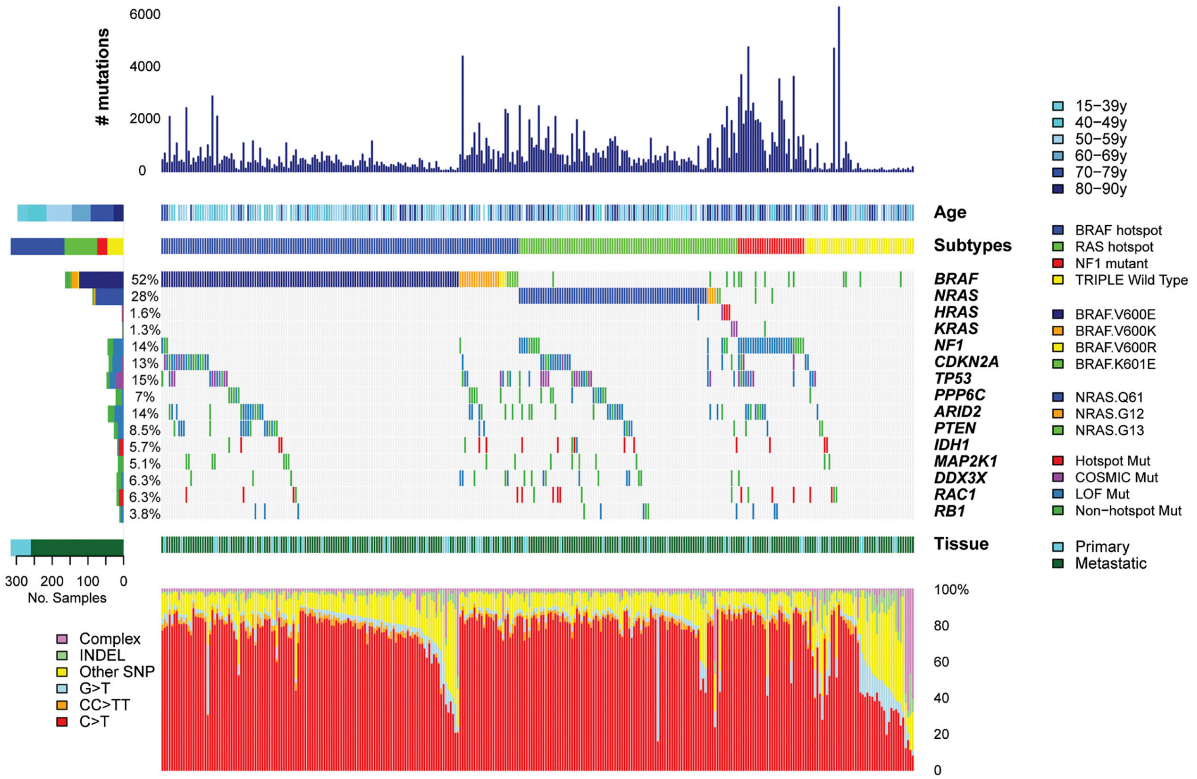

Figure 2.5 Landscape of cutaneous melanoma driver mutations.

Number of mutations, age at melanoma occurrence, and mutation subtype (BRAF, RAS, NF1, and Triple-WT) (top part). Individual mutations are color-coded, primary and metastatic samples are indicated (middle part). Mutation type is indicated per sample (bottom part) (13).

2.3. Melanoma of the eye

Melanoma of the eye is the second most common type of melanoma after cutaneous melanoma, very rare but often fatal. In the USA, incidence of ocular melanoma is 6 cases per million people (17). Similarly to cutaneous melanoma, the incidence of eye melanoma in Europe decreases from north to south, related to better protection of darker ocular pigmentation more usually found in the southern regions (18).

Eye melanoma most commonly occurs in the uvea (around 80%), which is the middle layer of the eye. The uvea is formed of three parts: the iris (the colored part of the eye), the choroid layer (where blood vessels are) and the ciliary body (controls the shape of the lens and produces the humor). Eye melanoma can also rarely occur in

the conjunctiva (5%), the outermost thin and transparent layer of the eye (**Figure 2.6**) (4,17,19,20).

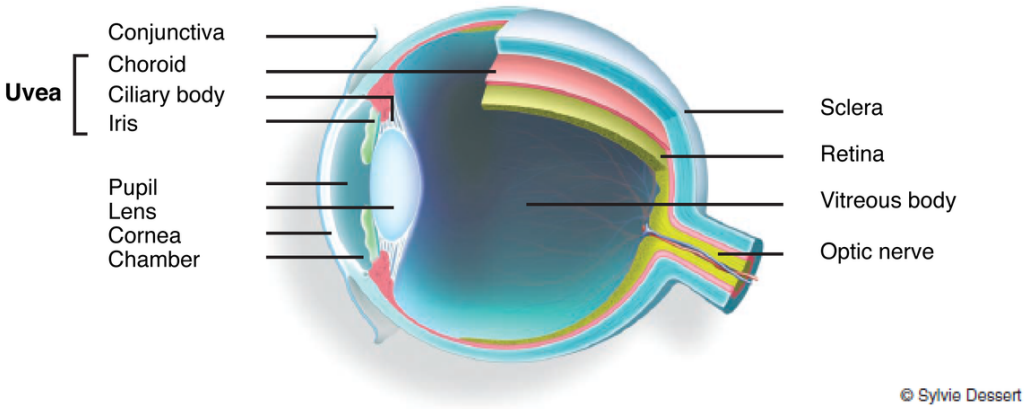

Figure 2.6 Anatomy of the eye.

The eye is composed of three layers. The sclera is the exterior of the eyeball and is formed by a white fibrous layer. Inside, the uvea can be found, which is rich of blood vessels and includes three structural components the iris, the ciliary body and the choroid. The conjunctiva is the in front part of the eye and the eyelid (https://www.umcure2020.org/en/uveal-melanoma/general-information/).

2.3.1. Uveal melanoma

Uveal melanoma is the most common intraocular tumor. The susceptibility factors for uveal melanoma include fair skin, light hair, and eye color. Uveal melanoma is more common in people who sunburn easily compared with those who tan well, related to the fact that there is less melanin in the skin and eye for protection against UV rays (21). Other risk factors include the presence of iris nevus, which could transform into uveal (and most likely iris) melanoma, even though this is a rare event (21).

Uveal melanoma is characterized by mutations in the G Protein Subunit Alpha Q (*GNAQ*) and G Protein Subunit Alpha 11 (*GNA11*), found in around 90% of cases. These receptors activate the PKC, MAPK and YAP signaling pathways, which control proliferation and differentiation (22–27). Mutations in *GNAQ* and *GNA11* are considered to be early events in the development of this disease (28). Other detected mutations include the eukaryotic translation initiation factor 1A (*EIF1AX*), splicing factor 3b subunit 1 (*SF3B1*), and BRCA1-associated protein 1 (*BAP1*) (29,30).

2.3.2. Conjunctival melanoma

Conjunctival melanoma (CoM) arises from the melanocytes in the basal cells of the conjunctival epithelium, with an incidence of 0.24 to 0.8 cases of CoM per million population in North Europe and the USA (31). The rarity of this tumor makes it difficult to make strong statements on the genetic basis of the disease, but there is growing evidence that CoM is genetically closer to cutaneous melanoma, rather than to uveal melanoma (32,33).

Similarly to cutaneous melanoma, previously reported mutations in CoM include *BRAF* mutations, found in 29% of tumors analyzed and *NRAS*, found in 18% of samples (32,33). *KIT* mutations have been detected in only 7.7% of cases (34). CoM is characterized by losses in the chromosome (chr.) 9p, gains in chr. 7 and amplifications in chr. 11, similarly to cutaneous melanoma (32,35).

Treatment options for patients with conjunctival melanoma are limited at the moment. Topical chemotherapy in the form of eye drops is used together with surgical excision, to limit the spread of the disease, but reoccurrence happens in over 50% of patients after surgery (31,36).

MAPK inhibitors (MAPKi) could be a therapeutic option for patients with CoM, given the reported cases of *BRAF* and *RAS* mutations. To provide better treatment options, a deeper understanding of the cellular and molecular mechanisms underlying CoM is required.

2.4. Heterogeneity and plasticity in melanoma

Heterogeneity is a hallmark of human cancers (37). At the cellular level, the development of melanoma follows an evolution-based process, with a gradual accumulation of genomic alterations, and the positive selection of certain cell lineages with survival advantages and the depletion of others. This process leads to tumor heterogeneity. Tumor heterogeneity is defined as the presence of genetic and phenotypic differences in subpopulations of cells within one tumor or different tumors in the same patient (intra-tumor) or between tumors of different patients (inter-tumor)

(38). Inter- and intra-tumor heterogeneity represents a challenge for the selection of specific biomarkers for clinical decisions and the design of successful therapies (39,40).

The progress of genome-wide next-generation sequencing (NGS) and single-cell sequencing technologies is helping to uncover the genomic heterogeneity of melanoma and other cancer entities. TCGA project expedited the understanding of the molecular basis of many malignancies. This resulted in the increased use of NGS in clinical settings and the high number of new drug discoveries towards altered genes or pathways. Furthermore, single-cell sequencing technologies can help understanding intra-tumor heterogeneity, as they ultimately enable the classification of a single tumor into different populations based on their genetic makeup. This could lead to an understanding of why therapies are failing (13,41,42).

Cutaneous melanoma not only is highly heterogeneous, characterized by many genomic alterations, but it is also highly plastic and capable of adapting quickly to changes in the microenvironment. Plasticity is a state in which cancer cells exist in reversible phenotypic conditions, expressing properties normally related to stem cells, appearing as undifferentiated, embryonic-like cells (43–46). Both heterogeneity and plasticity contribute to different therapy responses between melanoma patients. In response to drugs, proliferative melanoma cells can undergo a transcriptional and phenotypic switch and become more invasive and dedifferentiated. This is called “the phenotype switching model” (47–49).

The two phenotypic states described in the model, termed “invasive” and “proliferative”, defined also by high and low expression of the differentiation factor *MITF*, are interchangeable and reversible. High expression of *MITF* is fundamental for the proliferative state, also characterized by a high level of pigmentation-relates genes. On the other hand, the invasive cell state has low or no expression of *MITF* but shows EMT - like phenotype and expression of factors related to a more dedifferentiated phenotype (13,50–53).

In the past years, many groups aimed to better understand the heterogeneity and plasticity of melanoma. Recent studies reported EMT and wound healing-related gene signatures as predictors of unresponsiveness to MAPKi in melanoma patients (54,55). Single-cell RNA sequencing (RNA-seq) of MAPKi-treated melanoma cells allowed for the identification of four different transcriptional states in response to

drug-induced stress, one of which is characterized by the downregulation of MITF activity and consequent induction of de-differentiation towards a neural crest stem cell (NCSC) - like state (41).

Melanoma heterogeneity and plasticity are contributors to therapeutic failure, with an increased likelihood of resistance to future therapies.

2.4.1. MITF and the phenotype switching model

MITF is the master regulator of differentiation of neural crest cells into melanin-producing melanocytes and is important for the expression of pigmentation-related genes. *MITF* is amplified in 15-20% of metastatic melanoma and was previously linked to poor survival. Mutations affecting this gene are very rare (56,57).

MITF expression is regulated by several transcription factors, important in development and differentiations, including PAX3, SOX10, CREB and MITF itself (58,59). Melanocyte-stimulating hormone (αMSH) binds to melanocortin 1 receptor (MC1R), and cAMP production follows. CREB transcription factors, activated by cAMP, stimulate the expression of *MITF*. As both SOX10 and CREB are needed for the expression of neural-crest derived genes, the expression of *MITF* is very cell-type specific. Post-transcriptionally, MITF is phosphorylated by many kinases, as the MAPK, important for regulating MITF activity upon microenvironment changes.

MITF regulates the transcription of a variety of genes involved in cell cycle (as *CDK2*, *CDKN2A*), differentiation and pigmentation (Melan-A (*MLANA*), Tyrosinase (*TYR*)), metastasis (*MET*) and apoptosis (B-cell CLL／lymphoma 2 (*BCL2*) and hypoxia-inducible factor 1-alpha (*HIF1*α)) (**Figure 2.7**) (13,60–62).

MITF expression and activity have a fundamental role in melanocyte and melanoma behavior. Based on the MITF activity in melanoma cells, Carreira et al. described the “rheostat model” (62), where high activity of MITF characterizes differentiation, mid-level activity promotes proliferation, low-level activity stimulates an invasive, stem cell-like phenotype, and the total absence of MITF activity causes senescence or cell death (62). This concept was further developed by Hoek et al., who studied expression profiles of melanoma cells and identified two transcription signatures associated with different expression levels of *MITF*, corresponding with “proliferative”

and “invasive” cellular states (50,64). Accordingly, the proliferative state is characterized by high expression of *MITF* and differentiation genes as *MLANA* and *TYR*, while the invasive state is characterized by low levels of *MITF* and high expression of several receptor tyrosine kinases (*RTK*s) including *AXL*, epidermal growth factor receptor (*EGFR*) and EMT-associated transcription factors, promoting a more dedifferentiated state (42,64–67).

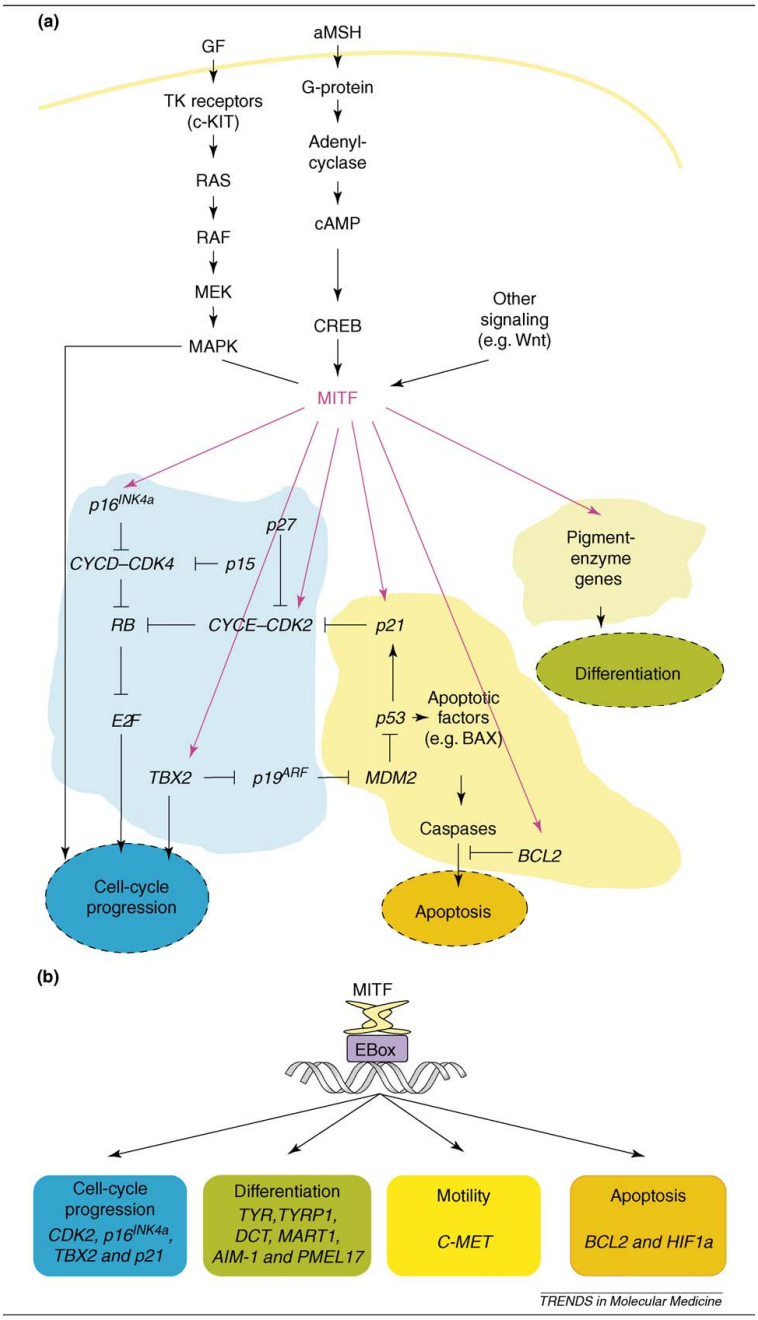

Figure 2.7 MITF regulation and targets.

Representation of the pathways targeting MITF and its targets. Mainly two signaling pathways are connected to MITF; growth factors (GFs), which activate MAPK pathway and the MAPK pathway which has a role in MITF regulation, and the aMSH, which binds to the MC1R expressed at the melanocyte surface. MITF regulates melanocyte differentiation, cell-cycle progression, survival and melanoma development (63).

2.4.2. Epithelial to mesenchymal transition

Although the switching of melanoma cellular phenotypes was only recently discovered, the trans-differentiation of epithelial cells into mesenchymal cells, known as EMT, is an essential process in development, wound healing, stem cell behavior, and cancer progression. The involvement of EMT in cancer biology is also studied as it confers metastatic properties and resistance against therapies to many cancers (49,68–72). The process starts with cells losing junctions and polarity. Then, cells undergo changes in the cytoskeleton and transcriptional programs to downregulate epithelial gene expression signatures and activate genes involved in the mesenchymal and invasive phenotype (73,74). During the transition, junctions between cells are degraded together with the epithelial cadherin (E-cadherin). Loss of E-cadherin is accompanied by a gain of mesenchymal neural cadherin (N-cadherin). This “cadherin switch” alters the adhesion properties of cells and facilitates their motility. N-cadherins is linked to the cytoskeleton through α-catenin and β-catenin and interacts with many RTKs (68,75). Transitioning cells downregulate epithelial integrins while overexpressing other integrins and proteases, such as metalloproteases (MMPs), which promote the extracellular matrix (ECM) degradation. These phenotypic changes are initiated and promoted by master regulators of the mesenchymal program SNAI, Twist Family BHLH Transcription Factor (TWIST) and zinc-finger E-box-binding (ZEB) transcription factors. These transcription factors contribute substantially to the establishment of the EMT transcription program and drive EMT progression (76–78).

The WNT signaling pathway is important for the regulation of EMT during neural crest formation, together with the Transforming Growth Factor Beta (TGFβ) family members. WNT and TGFβ signaling work together to control gene expression changes during EMT. TGFβ enables β-catenin to accumulate in the nucleus and

upregulate the WNT signaling, to further fuel the EMT transcriptional reprogramming (74,77).

2.5. Therapeutic options

Before the discovery and the development of MAPK inhibitors, patients with advanced melanoma were treated with dacarbazine, a chemotherapeutic drug. There is now evidence that melanoma is insensitive to cytotoxic chemotherapy since no survival benefit was measured (79–82). In the last decade, new strategies were developed to target oncogenic signaling pathway - like BRAF inhibitors, or to boost the tumor-specific cytotoxic T cell responses - like antibodies impairing inhibitory checkpoints as cytotoxic T-lymphocyte-protein 4 (CTLA-4) and programmed T cell death 1 (PD-1).

The development of these new therapies for melanoma patients was possible thanks to the characterization of the oncogenic signaling pathways and the identification of new targets of clinical impact with NGS and modern genomic platforms (83,84).

2.5.1. Targeted therapy

Targeted therapy uses small molecules directed at the most significant oncogenic signaling pathways and specifically recognizing a mutated protein. 70% of patients with cutaneous melanoma carry mutations in genes of key signaling pathways, as the MAPK pathway, associated with hyperproliferation or malignant phenotype (83,85,86).

The discovery by Davies et al., in 2002, that a substantial subset of cutaneous melanomas are driven by an oncogenic, gain-of-function mutation in *BRAF* position V600E in the MAPK pathway was a major incentive for research in oncogene-directed targeted melanoma therapies and has led to the identification of selective inhibitors for $BRAF^{V600E}$ (15).

The MAPK pathway regulates many important cellular processes such as proliferation, differentiation, and apoptosis. In normal conditions, binding of mitogens or hormones to surface receptor tyrosinase kinases triggers the activation of

oncogenic RAS and the consequent formation of the MAPK complex with downstream RAF, MEK1/2, ERK, and other scaffolding proteins. This is the beginning of the MAPK cascade. When RAF proteins are activated, ERK is able to regulate directly or indirectly the expression of genes important for cell proliferation and survival. Dysregulation of the MAPK pathways is very common in many other cancers, causing tumor cells to continuously proliferate without the need of external mitogenic signals (**Figure 2.8**) (87,88).

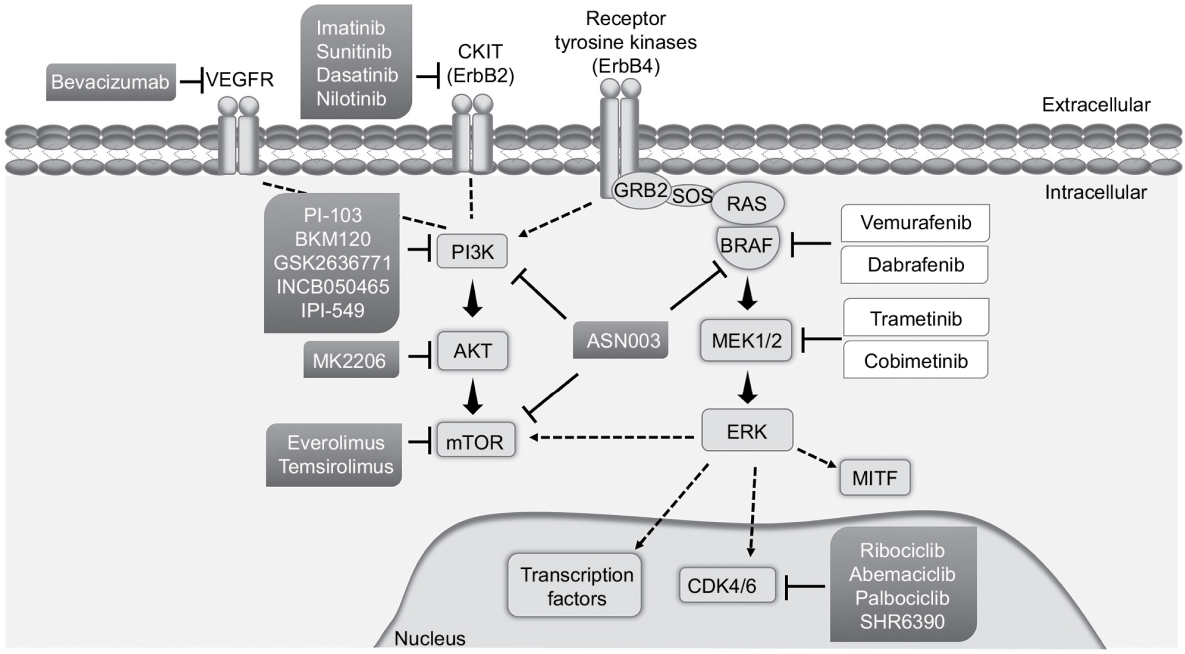

Figure 2.8 MAPK pathway in melanoma and targeted therapies.

Mitogens are able to activate the RTKs on the cell surface, which activate the RAS/RAF/MEK/ERK pathway on one hand and PI3K on the other hand. RAS/RAF/MEK/ERK pathway activates MITF, CDK4/6 and other transcription factors. Targeted therapies for cutaneous melanoma against mutations in this pathway are indicated (in white, approved by the FDA; in grey, in clinical trials). The PI3K/AKT/mTOR cascade, together with the MAPK pathway, promotes proliferation, cell survival, and metastasis (86).

Vemurafenib was developed as a highly specific $BRAF^{V600E}$ inhibitor (85,89). The BRIM clinical trial, in which they compared vemurafenib with dacarbazine in cutaneous melanoma patients carrying *$BRAF^{V600E}$* mutations, showed an 84% overall survival (OS) rate compared to 64% OS rate in the dacarbazine group after six

months. Patients treated with vemurafenib had a median longer progression-free survival (PFS) of 5.3 vs 1.6 months. The response rate was also higher for vemurafenib-treated patients (48% vs 5%) (89).

Clinical trial data indicate that almost all the vemurafenib-treated patients with *BRAF*^{V600E} melanomas respond to treatment. However, most patients develop resistance to vemurafenib, indicating that tumor cells are highly dependent on the MAPK pathway and are able to reactivate it for survival. Recently, other inhibitors of the MAPK pathway, like MEK inhibitors (MEKi), have been used in combination with BRAF inhibitors (BRAFi) treatment (90–92). Long et al. published recently a clinical trial on patients with unresectable stage IIIC or stage IV melanoma with a *BRAF*^{V600E} or *BRAF*^{V600K} mutation. They patients received a combination treatment with dabrafenib plus trametinib (n = 211) or dabrafenib monotherapy (n = 212). 3-year PFS was 22% with combi-treatment compared to 12% of monotherapy. 3-year OS was shown to be 44% and 32% respectively. This data showed a long-term durable survival for patients with *BRAF*^{V600E} tumors under combination therapy (≥3 years) (93).

2.5.2. Immunotherapy

The idea of using the immune system to fight against cancer is not new, but this concept is currently changing the landscape of cancer treatment. The concept behind currently applied immunotherapies is based on the recognition of cancer cells, presenting tumor-specific molecules (antigens) on their surface, by cytotoxic CD8+ T cells.

2.5.2.1. Immune recognition and escape

Melanoma and many other cancers are characterized by the accumulation of mutations which can 1) activate cancer cell signaling and lead to cancer formation and metastasis, or 2) be passenger mutations that do not contribute to the malignant phenotype but show high immunogenicity. These tumor-specific mutations result in the production of neoantigens, defined as antigens that are absent from the normal

human genome and derived from tumor-specific genetic alterations. Neoantigens can generate a potent cytotoxic anti-tumor T cell responses (14,94).

Upon antigen recognition, CD8+ T cells secrete cytotoxic granules, containing perforins and granzymes, to kill their direct tumor target cells. Moreover, activated T cells release cytokines such as tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ), inducing apoptosis in surrounding cells (54,95–101).

The binding of IFNγ to its receptor IFNGR1/2 on tumor cells causes the activation of the JAK1 and JAK2 receptor-associated kinases, which in turn phosphorylate STAT1. Phosphorylated STAT1 forms homodimers which directly activate the transcription of primary response genes, including *IRF1*. IRF1 is a transcriptional activator that in turn activates the expression of secondary response genes causing cell cycle arrest and cell death (**Figure 2.9**) (102–104).

Many are the mechanisms that allow melanoma cells to escape T cell recognition. Immune escape mainly depends on defective antigen presentation in melanoma cells or the development of an immunosuppressive microenvironment.
High-throughput sequencing studies in melanoma suggest that high tumor mutational load and neoantigen load are associated with clinical benefit and increased overall survival (105). Nevertheless, the acquisition of new mutations can result in the loss of tumor-specific antigens, which help melanoma cells escape immune surveillance (14,106–109).

T cell responses are regulated by a balance between co-stimulatory and inhibitory signals, the latter received via inhibitory receptors, so-called immune checkpoints. Under physiological conditions, inhibitory immune checkpoints are important for preventing autoimmunity and T cell overactivation during an acute infection. But tumor cells can express ligands of inhibitory checkpoint receptors thereby dampening T cell activity. The two most intensively studied inhibitory immune-checkpoint receptors are cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed T cell death 1 (PD-1). Antibodies blocking these two inhibitory immune checkpoints release T cells from inhibitory signaling in order to enhance endogenous anti-tumor activity (110).

CD8+ T cells are the main producers of IFNγ following tumor antigen recognition. Activation of the IFNγ signaling pathway can induce cell cycle arrest and apoptosis in melanoma cells (102,111). IFNγ is also able to upregulate antigen presentation

enhancing the immunogenicity of tumor cells and making them more vulnerable to T cell recognition and killing (111,112). Loss-of-function mutations in *JAK1/2* can result in resistance to T-cell mediated killing and immune checkpoint blockade (ICB) in patients with metastatic cutaneous melanoma (113,114). On the other hand, IFNγ can contribute to tumor progression as it induces the expression of PD-1 ligands on tumor cells (115–117).

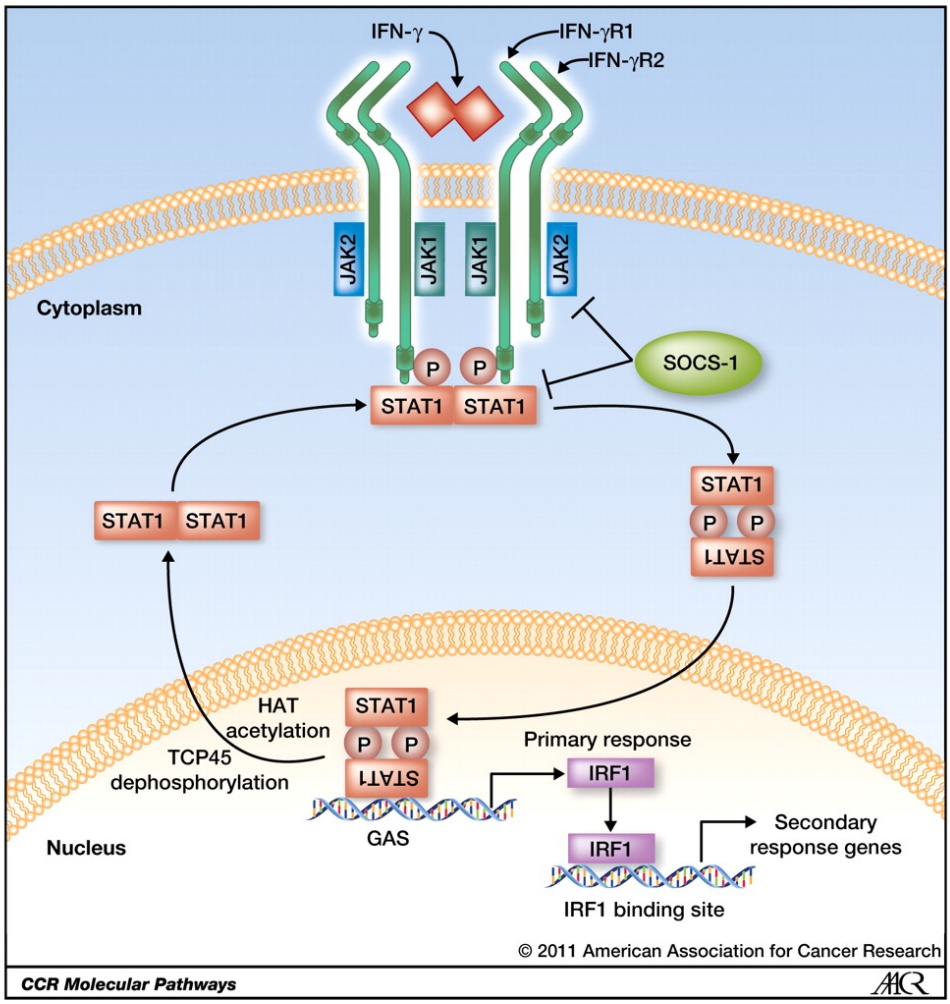

Figure 2.9 The IFNγ signaling pathway.

When IFNγ binds to the IFNgR1/2, dimerization of receptors leads to activation of JAK 1 / 2. The phosphorylates JAK proteins bind to STAT1. STAT1 then forms homodimers which translocate into the nucleus to regulate primary response genes (as IRF1). STAT1 homodimers bind to a specific regulatory region on the DNA called GAS. IRF1 is then able to bind the DNA itself and activate the secondary response genes (104).

Cutaneous melanoma is known to be a very plastic tumor; previously it was shown to be able to switch from a proliferative, or epithelial-like, to more invasive, or mesenchymal-like, state, similarly to the classical EMT (50,65). Expression levels of differentiation antigens as MITF and Melan-A is significantly lower in the invasive and mesenchymal-like state. Dedifferentiated melanoma cells are recognized less by CD8+ T cells.

EMT-like phenotype switching has been shown already to play a role in immune escape under anti-Melan-A adoptive T cell therapy (ACT) (118–122). Proinflammatory cytokines, like TNFα, released during therapy and therapy-induced tumor-tissue injury, are critical for the induction of a dedifferentiated melanoma cell phenotype (122–125). Thus, genomic and non-genomic mechanisms are involved in melanoma immune escape.

2.5.2.2. Types of immunotherapies

In the past thirty years, T-cell activating cytokines as interleuking-2 and interferon-alpha were the only type of immunotherapy available. These treatments showed a low clinical success rate and high toxicity for the patients (126,127). The real breakthrough in melanoma therapy was the discovery of immune checkpoint inhibitors. Immune checkpoints are regulators of the immune system and are essential for self-tolerance mechanisms. CTLA-4 and PD-1 are inhibitory receptors of T cell activity and their task is to prevent exceeding immune responses leading to long-lasting inflammation and autoimmunity. The discovery and development of antibodies specific to CTLA-4 and PD-1 revolutionized cancer treatment and patients' survival (128). When bound to their ligands, CTLA-4 receptors inhibit T cell activation during T cell priming, which is the initial phase of T cell activation happening in the lymph nodes. PD-1 inhibitory receptors have the function to block activated T cells in the peripheral tissue upon binding to its ligands on tumor cells, PD-L1 or on the APCs, PD-L2. CTLA-4 and PD-1 checkpoint blockers are therefore able to relief T cells from inhibitory signals and lead to enhanced T cell activation (**Figure 2.10**) (129,130).

Since 2011, seven different agents targeting CTLA-4 (i.e. ipilimumab), PD-1 (i.e. nivolumab, pembrolizumab, and cemiplimab) and PD-L1 (i.e., atezolizumab, avelumab, and durvalumab) have been approved by the US Food and Drug

Administration (FDA), for a variety of different cancers including advanced melanoma, non-small-cell lung carcinoma (NSCLC), head and neck and cutaneous squamous cell carcinoma, renal cell carcinoma (RCC) and others. (131).

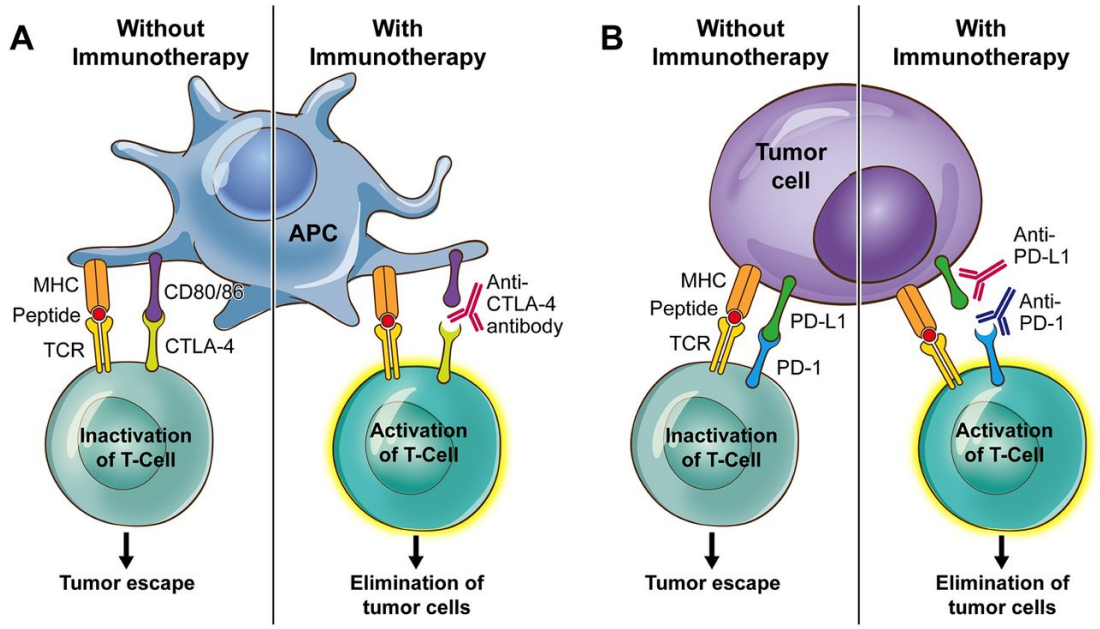

Figure 2.10 Immune checkpoint blockade effects.

Anti-CTLA-4 (A) and anti-PD-1/anti-PD-L1 (B) antibodies bind to the corresponding inhibitory receptor on T-cells and melanoma cells. T-cells are activated by the binding of the T-cell receptor to the antigen/MHC complex. CD80/86 are the ligand to CTLA-4 checkpoint, and their binding downregulates T-cell activation. PD-1 has two ligands: PD-L1 and PD-L2. PD-L1 is expressed by cancer, PD-L2 is expressed by APCs. Both CTLA-4 and PD-1/PD-L1 blockade by monoclonal antibodies can lead to an antitumor immune reaction (132).

Ipilimumab (anti-CTLA-4), nivolumab (anti-PD-1) and pembrolizumab (anti-PD-1) were the first tested ICB in malignant cutaneous melanoma. In 2015, Schadendorf et al. analyzed multiple studies and to estimate with more precision how ipilimumab was affecting long-term survival in patients with advanced melanoma. Ipilimumab showed good responses in melanoma patients, with a median OS of 11.4 months and a plateau in the OS curve around the third year. At this point, survival rates were reported to be around 20% to 26% (133). Pembrolizumab showed superiority in clinical responses compared to ipilimumab; after 6 months of treatment, patients free of disease progression was measured to be 51.1% with PD-1 inhibition compared

with 39.3% with CTLA-4 inhibition; OS was 72.2% compared with 50.4% at 12 months, and 59.3% compared with 28.6% at 24 months. The combination of CTLA-4 plus PD-1 blockade showed an OS rate of 73.1% at 12 months, showing superiority compared with a single-agent PD-1 inhibitor (128).

Another promising type of immunotherapy is ACT. It consists of the administration of ex vivo expanded tumor specific T cells. In brief, tumor-infiltrating lymphocytes (TILs) are isolated from the tumors, activated and expanded ex vivo (**Figure 2.11**). ACT induces durable response rates against melanoma (from 49 - 72 % in three sequential trials), and 22 % of patients show a complete remission (134,135). ACT is a patient-specific immunotherapy, therefore very expensive. The clinical benefit of ACT has already been associated with high mutation burden and putative neoantigens, correlating with clinical benefit (95,136).

Despite good response rates seen in clinics, many patients still do not respond to immunotherapy or progress soon due to acquired resistance. Currently, there is no effective way for clinicians to know which patients will respond or will become resistant. Resistance to immunotherapy happens mainly due to alterations in the antigen presentation machinery (e.g. *B2M*), genetic alterations in the IFNγ signaling, impaired immune infiltration or T cell exhaustion (137). Currently, ongoing clinical trials are testing more immune checkpoint blockers to be combined with each other or with targeted therapies, in order to achieve a better OS for melanoma patients (138).

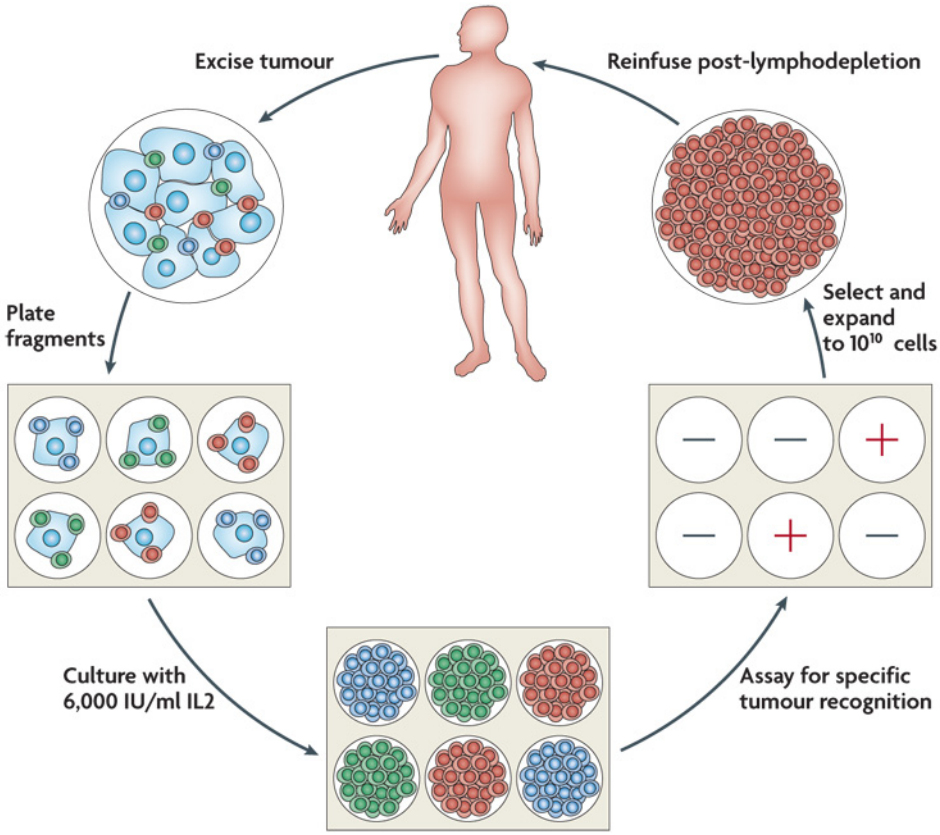

Figure 2.11 Adoptive T-cell therapy workflow.

Different tumor cultures are established from the excised mass. Each one is tested for specific tumor recognition. Only cultures with high anti-tumor reactivity are chosen and are expanded. Reinfusion into the patient after lymphodepleting chemotherapy. IL2, interleukin-2.

2.5.2.3. Immune checkpoint blocking antibodies

In 1996 James Allison and colleagues were able to identify the crucial role of CTLA-4 and demonstrate that blocking antibodies against CTLA-4 could help to treat tumors in immune-competent animal models. More than 15 years later, in 2010, ipilimumab was approved by the FDA for treatment of melanoma patients (84). In 2010, Hodi et al. carried out a clinical trial with the goal of registering an improvement in OS among patients with metastatic melanoma. They performed a phase 3 study, studying ipilimumab (anti-CTLA-4) administered with or without a glycoprotein 100 (gp100) peptide vaccine and compared with gp100 mono treatment in 676 previously treated patients. Monotherapy with gp100 cancer vaccine has been shown to induce immune responses but with limited effects (139,140). Median OS was shown to be 10.0

months for patients receiving ipilimumab plus gp100, versus 6.4 months for patients receiving gp100 alone. There was no difference in OS between patients receiving ipilimumab mono treatment or in combination with gp100. This study showed the superiority in OS improvement of ipilimumab compared with gp100 alone (84).

A second checkpoint receptor, PD-1, and its ligand PD-L1, was characterized between 1992 and 2000 (141,142). Antibodies against PD-1 can block this inhibitory mechanism and show durable responses (143). Robert et al. tested the differences in OS between PD-1 immune checkpoint inhibitor nivolumab and the chemotherapeutic agent dacarbazine. This clinical trial involved 418 previously untreated metastatic melanoma patients with *BRAF* WT tumors. After one year, the OS rate was shown to be 72.9% for patients treated with nivolumab versus 42.1% in the dacarbazine group. The median PFS was 5.1 months in the andi-PD-1-treated group and only 2.2 months for the chemotherapy-receiving patients. Nivolumab was shown to have a significant betters and PFS compared with dacarbazine (144). In another study, pembrolizumab, a second anti-PD-1 immune checkpoint antibody approved for clinical use, achieved 26% of objective responses in ipilimumab-resistant patients (145).

Given the plasticity of cancers and their ability to develop resistance mechanisms to monotherapy, combination therapy of anti-CTLA-4 and anti-PD-1/PD-L1 was approved in 2015 for advanced-stage melanoma, after reaching improved clinical response. Larkin et al. carried out a double-blind, phase 3 study, where they compared nivolumab (anti-PD-1) alone or nivolumab plus ipilimumab (anti-CTLA4) with ipilimumab alone in 945 previously untreated patients with unresectable stage III or IV melanoma. Treatment was assigned in a 1:1:1 ratio. Median PFS was shown to be 11.5 months for patients in the with nivolumab plus ipilimumab group versus 2.9 months with ipilimumab monotherapy and 6.9 months with nivolumab monotherapy. Therefore, combination therapy with nivolumab with ipilimumab or nivolumab alone reached better PFS compared to ipilimumab alone (146).

2.6. Resistance to therapies

Despite the great success of targeted therapy and immunotherapy for the treatment of cutaneous melanoma, the majority of the people still develop resistance or are

innately resistant. Resistance to therapy can be either intrinsic, therefore patients do not respond to therapy from the beginning, or acquired when patients respond initially but then progress (147).

Acquired resistance to MAPKi occurs via acquisition of genetic alterations aiming to reactivate the MAPK pathway. The plasticity of melanoma transcriptomes also plays an important role in drug adaptation (55).

ICB has been shown to produce durable responses, but around 60% of patients show primary resistance to PD-1 inhibition and ca. 30% of responders develop secondary resistance (148).

A better understanding of resistance mechanisms is necessary to develop biomarkers for prediction of efficacy and respond to both targeted therapy and ICB.

2.6.1. Resistance to targeted therapy

Although MAPKi treatment has shown great benefit for patients with metastatic melanoma, patients eventually become resistant to the treatment and progress. This happens by acquisition of mutations or other alterations restoring the activity of the MAPK pathway in tumor cells (149,150).

The main mechanisms leading to MAPKi resistance involve molecular MAPK pathway reactivation by either *BRAF* splice variants or *BRAF* amplification, further mutations in the MAPK pathway components *NRAS* and *MEK1/2* (151,152). The PI3K/AKT/mTOR pathway is the second most commonly re-activated pathway during the development of MAPKi resistance in melanoma, via deletion or gene mutation of *PTEN* or via activation of RTKs (153,154).

While drug resistance is often initiated by acquisition of genetic alterations, evidence is emerging that non-genomic alterations can cause drug-tolerant states leading to minimal residual disease (41,42,155). Melanoma cells with low expression levels of *MITF* and high levels of *AXL*, characteristics of a mesenchymal-like or “invasive” gene expression signature, are resistant to MAPKi (49,155,156). The phenotype switching from a proliferative to an invasive cell state was recently proposed as a mechanism of MAPKi resistance (49) and confirmed recently by Rambow et al. (41). In their recent work, Rambow et al. demonstrated that residual disease from

melanoma lesions carrying *BRAF* mutations after MAPKi treatment shows four distinct drug-tolerant states, exhibiting heterogeneity. One of these, characterized by a transcriptional program matching the neural crest stem cell markers and low *MITF* expression, increased upon drug exposure, indicating that undifferentiated cells were driving drug tolerance in their model (41).

2.6.2. Resistance to immunotherapy and IFNγ

CD8+ T cells are able to specifically recognize tumor-specific antigens and eliminate cancer cells. Still, tumors continue to grow, implying that they can develop mechanisms to avoid recognition. It was previously demonstrated that loss of antigen presentation machinery, as B2M or MHC molecules, has a role in acquired resistance to different immunotherapies (157,158). In recent years, inactivating mutations and aberrations in the IFNγ pathway in tumor cells have emerged as key in the development of resistance to ICB therapy (54,97,98,114,159,160).

Mutations are not the only mechanisms used by cancer cells to avoid immune-recognition. The production of pro-inflammatory cytokines by activated CD8+ T cells results in phenotypic changes in the cancer cells. Landsberg et al. previously demonstrated in an *in vivo* model of ACT therapy against the gp100 differentiation antigen, that tumors only responded transiently to therapy. During the ACT protocol, tumor-infiltrating cells released TNFα and melanoma cells adapted to the stimulus by downregulating gp100 and switching into a more dedifferentiated phenotype (122). Previously, it was demonstrated that an active immune response (i.e. release of TNFα, interleukin-6 (IL-6) and TGFβ) can induce reprogramming through an EMT-like differentiation (118,161). Accordingly, the Innate PD-1 Resistance Signature (IPRES), generated by Hugo et al., shows upregulation of genes involved in immunosuppression, angiogenesis, extracellular matrix remodeling, TGFβ signaling, and EMT (55).

In the last years, the search for biomarkers for anti-CTLA-4 and anti-PD-1/PD-L1 response was intense. Mutational load and neoantigen load are used for predicting clinical response to immune checkpoint inhibitors, still, some patients with high mutational load still do not respond (162). The status of the IFNγ pathway or the antigen presentation machinery may also be an important biomarker for ICB response, as inactivation of these pathways was shown to contribute to resistance to

anti-PD-1 therapy (113,114). High copy number loss, especially chr.10 and *PTEN* loss, were previously associated with poorer response to ICB. Analysis of copy number status might be an informative prognostic marker (163).

2.7. Next generation sequencing technologies and their impact in cancer research

The development of high-throughput technologies such as NGS has allowed the study of thousands of genes from a bulk tumor to a single-cell level. Whole exome sequencing (WES) and RNA-seq are tools often used to study the genomic and non-genomic landscape of a tumor. WES is applied to study the coding regions of DNA, therefore it is widely used for mutation detections (13,87,164,165). It has increasingly been used to determine tumor mutational burden and predict neoantigens in melanoma tumors, given the high mutation rate of melanoma (13). TCGA and the international cancer genome consortium (ICGC) have analyzed more than 50 000 cancer genomes, revealing that most driver genes with frequent mutations in cancer have been already discovered (87,166).

The exploration of cancer somatic mutation in different cancer types has shown the power of NGS technologies; their implementation into research and clinics not only has changed the basic knowledge of cancer biology, but it has also opened the door to many different clinical applications, as targeted treatments and prognosis prediction (167).

RNA-seq reveals to be fundamental in cancer research, for the exploration of transcriptomes of tumor cells. RNA-seq is employed for studying the alterations in gene signatures in groups of samples or gene expression changes in treated patients, to find biomarkers or signature to predict response and stratify patients before treatment (41,77,122,168–171). RNA-seq is exploited to quantify the differentially regulated transcripts under different conditions. Many studies in the past have shown its application for understanding transcriptional changes in the cells or patients upon treatment or during the development of resistance to a specific drug (169–171).

High-throughput technologies help to better understand of the heterogeneity of melanoma and other tumor entities and they allow the exploration of the genomic and non-genomic evolution processes that cancer undergoes in the context of disease progression and therapy resistance (172).

As NGS is becoming more often used in many clinics, the development of predictive biomarkers will help to stratify the patients for response to ICB, avoiding both useless costs and inadequate therapy for unresponsive patients.

2.7.1. The Cancer Genome Atlas

Bioinformatic approaches recently have become essential support for cancer research in general. Cancer is a complex disease that involves dynamic changes in the genome. Each cancer type has a set of unique aberrations, spanning from mutations, copy number variants, abnormal gene expression, and altered methylation profiles, and all of this complicates the understanding of the disease and possible treatments. The willingness of better understanding of the genetic changes of cancer and the development of genome-wide sequencing technologies have let to the birth of the TCGA in 2005 (166,173). This project had the aim to accelerate the understanding of the genetics of different cancers using the new technical and bioinformatic tools, to find new therapies, diagnostic tools and preventive strategies (166,173).

TCGA project and all the analysis platforms provided for users to explore the genomic profiling of large cancer patient cohorts have become valuable and essential resources for nowadays cancer research. TCGA researches have worked on the genome sequencing of large cohort of patients from more than 30 different cancer types, with the major goal of providing information to study in depth one cancer type or be able to do comprehensive pan-cancer analysis, characterizing the molecular changes that happen within cancer cells and map their molecular alterations (87,166,173,174). This approach has helped the understanding of tumorigenesis and improving treatment standards.

2.8. Aims of the study

The past few years were marked by the improvement of melanoma therapy based on small-molecule inhibitors, targeting the aberrantly active MAPK pathway in tumor cells, and immunotherapy, reactivating cytotoxic tumor-specific T cells and enhancing T cell responses against cancer cells. Both therapies are very successful in treatment of cutaneous melanoma, but innate and acquire resistance remains a major problem. Moreover, no standard treatment is currently approved for conjunctival melanoma which still remains poorly characterized due to the rarity of this disease subtype.

Overall, melanoma therapy is challenged by the genetic heterogeneity and phenotypic plasticity of the tumor cells. To further elucidate this heterogeneity and determine it role in resistance to therapy, the following aims of the study were defined:

- To determine the mutational landscape of conjunctival melanoma in order to understand its development and guide therapeutic decisions
- To define genomic and non-genomic tumor resistance mechanisms involved in resistance to immunotherapy, focusing on resistance to the T cell effector cytokine IFNγ.

3. Publications

3.1. *NF1* mutations in conjunctival melanoma

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Own contributions:

- Conception: 0% - Study design.
- Experimental work: 10% - Sample selection and histopathology, DNA isolation, Targeted sequencing.
- Data analysis: 20% - Sequence analysis.
- Statistical analysis: 0% - Associations of mutation status with clinical and pathological parameters with IBM SPSS Statistics software.
- Writing the manuscript: 15% - Visualisation of the results, literature research, conceptual design, writing all chapters, preparation of supplemental material
- Revising the manuscript: 15% - Revision of the reviewed manuscript, upload of manuscript, corresponding author

Essen 9.7.2019 Place, Date	Klaus Griewank
_____ Place, Date	_____ Annette Paschen
_____ Place, Date	_____ Sonia Leonardelli

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ARTICLE

Genetics and Genomics

NF1 mutations in conjunctival melanoma

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BACKGROUND: Conjunctival melanoma is a potentially deadly eye tumour. Despite effective local therapies, tumour recurrence and metastasis remain frequent. The genetics of conjunctival melanomas remain incompletely understood.
METHODS: A large cohort of 63 conjunctival melanomas was screened for gene mutations known to be important in other melanoma subtypes by targeted next-generation sequencing. Mutation status was correlated with patient prognosis.
RESULTS: Frequent mutations in genes activating the MAP kinase pathway were identified. *NF1* mutations were most frequent ($n = 21$, 33%). Recurrent activating mutations were also identified in *BRAF* ($n = 16$, 25%) and *RAS* genes ($n = 12$, 19%; 11 *NRAS* and 1 *KRAS*).
CONCLUSIONS: Similar to cutaneous melanomas, conjunctival melanomas can be grouped genetically into four groups: *BRAF*-mutated, *RAS*-mutated, *NF1*-mutated and triple wild-type melanomas. This genetic classification may be useful for assessment of therapeutic options for patients with metastatic conjunctival melanoma

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INTRODUCTION

Conjunctival melanoma accounts for 5–10% of all ocular melanomas with a 10-year local recurrence rate of 38–69% and disease-related mortality of 13–38%.[1–6] A better understanding of the genetics of conjunctival melanoma may help identify improved therapeutic options for patients with advanced disease.

In recent years, major melanoma subtypes have been genetically characterised. Cutaneous melanomas frequently harbour activating mutations in *BRAF* (~50%)[7] or *NRAS* (~20%), as well as mutations in *NF1*.[8–11] *BRAF*, *NRAS* and *NF1* mutations lead to activation of the mitogen-activated protein (MAP) kinase pathway.[9, 12, 13] Based on these findings, a genetic classification of cutaneous melanomas has been proposed distinguishing four genetic groups: *BRAF*-mutated, *RAS*-mutated, *NF1*-mutated or triple wild type.[11]

Uveal melanomas exhibit a different mutation profile, and harbour mutations in *GNAQ*,[14] *GNA11*,[15] *CYSLTR2*,[16] *PLCB4*,[17] *EIF1AX*,[18] *SF3B1*[19] and *BAP1*,[20] which are rarely found in other melanomas.[15, 21–23]

Conjunctival melanomas have not been characterised genetically as well as other melanoma subtypes. $BRAF^{V600E}$ and *NRAS* mutations are present in 14–50%[24–27] and 18%,[28] respectively, of conjunctival melanomas. *TERT* promoter mutations were identified in 32–41% of conjunctival melanomas.[29, 30] One study reported a *KIT* mutation in 1/14 (7%) tumours.[31] Copy number analysis identified alterations reminiscent of cutaneous and mucosal melanomas, including *CDKN2A* and *PTEN* losses.[28] These data suggest that conjunctival melanomas are genetically similar to cutaneous melanomas, but aside from *BRAF*, *NRAS* and *TERT* promoter mutations, recurrent mutations in other genes have not been identified.

There are two main therapeutic avenues for metastatic melanoma. Firstly, targeted small inhibitors dampening pathologically activated cell-intrinsic signalling mechanisms, with the most effective to date being a combination of BRAF and MEK inhibitors in *BRAF*-mutated melanoma.[32] Secondly, immunotherapies applying anti-CTLA-4 and anti-PD-1 antibodies have shown impressive response rates in cutaneous and mucosal melanoma.[33–35] Both approaches may be clinically useful in advanced conjunctival melanoma.[36]

Our study aimed to further elucidate genetic events in conjunctival melanoma by analysing a large tumour cohort with a targeted next-generation sequencing assay covering genes that are recurrently mutated in cutaneous and uveal melanoma.

MATERIALS AND METHODS

Sample selection and histopathology

Sixty-seven conjunctival melanoma samples were obtained from the tissue archives of the Departments of Ophthalmology,

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Table 1. Correlation between mutation status and clinical features in conjunctival melanomas

		Total		$BRAF^{WT}$		$BRAF^{V600E}$		*P*-value ($p < 0.05$)	RAS^{WT}		RAS^{MUT}		*P*-value ($p < 0.05$)	$NF1^{WT}$		$NF1^{MUT}$		*P*-value ($p < 0.05$)
		%	(*n*)	%	(*n*)	%	(*n*)		%	(*n*)	%	(*n*)		%	(*n*)	%	(*n*)	
Total			63	74.6	47	25.4	16		81.0	51	19.0	12		66.7	42	33.3	21	
Sex	Female	50.8	32	38.1	24	12.7	8	0.94	44.4	28	6.3	4	0.18	36.5	23	14.3	9	0.37
	Male	49.2	31	36.5	23	12.7	8		36.5	23	12.7	8		30.2	19	19	12	
Eye	Right	55.6	35	39.7	25	15.9	10	0.63	42.9	27	12.7	8	0.59	34.9	22	20.6	13	0.52
	Left	41.3	26	31.7	20	9.5	6		34.9	22	6.3	4		28.6	18	12.7	8	
	N/A	3.2	2	3.2	2	0	0		3.2	2	0	0		3.2	2	0	0	
TNM	1	55.6	35	41.3	26	14.3	9	0.77	44.4	28	11.1	7	0.44	38.1	24	17.5	11	0.56
	2	23.8	15	17.5	11	6.3	43		22.2	14	1.6	1		14.3	9	9.5	6	
	3	15.9	10	11.1	7	4.8	0		11.1	7	4.8	3		9.5	6	6.3	4	
	N/A	4.8	3	4.8	3	0			3.2	2	1.6	1		4.8	3	0	0	
Tumour origin	PAM	52.4	33	41.3	26	11.1	7	0.1	44.4	28	7.9	5	0.35	37.5	24	14.3	9	0.43
	Naevus	17.5	11	7.9	5	9.5	6		15.9	10	1.6	1		11.1	7	6.3	4	
	De novo	22.2	14	19	12	3.2	2		15.9	10	4.8	4		11.1	7	11.1	7	
	N/A	7.9	5	6.3	4	1.6	1		4.8	3	3.2	2		6.3	4	1.6	1	
Relapses	No	46	29	36.5	23	9.5	6	0.26	33.3	21	12.7	8	0.22	27	17	19	12	0.46
	Yes	47.6	30	31.7	20	15.9	10		42.9	27	4.8	3		34.9	22	12.7	8	
	N/A	6.3	4	6.3	4	0	0		4.8	3	1.6	1		4.8	3	1.6	1	
Metastasis	No	68.3	43	49.2	31	19.0	12	0.32	54.0	34	14.3	9	0.34	42.9	27	25.4	16	0.55
	Yes	22.2	14	15.9	10	6.3	4		20.6	13	1.6	1		17.5	11	4.8	3	
	N/A	9.5	6	9.5	6	0	0		6.3	4	3.2	2		6.3	4	3.2	2	
Exenteration	No	76.2	48	57.1	36	19.0	12	0.36	60.3	38	15.9	10	0.64	47.6	30	28.6	18	0.44
	Yes	17.5	11	11.1	7	6.3	4		15.9	10	1.6	1		14.3	9	3.2	2	
	N/A	6.3	4	6.3	4	0	0		4.8	3	1.6	1		4.8	3	1.6	1	
Age at diagnose	Median 67.4 years, Range 40.1–88.8 years																	

Clinical and pathological stage is according to TNM 7th edition AJCC 2010 for conjunctival melanoma *N/A* not assessable, *PAM* primary acquired melanosis

Dermatology and Pathology of the University Hospital Essen, and the Department of Ophthalmology, University Hospital Tübingen, Germany. The study was approved by the local ethics committee of the University of Duisburg-Essen.

DNA isolation
Formalin-fixed, paraffin-embedded tumour tissues were sectioned, deparaffinised and manually microdissected as previously described.[37] Genomic DNA was isolated using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany).

Targeted sequencing
A custom amplicon-based sequencing panel covering 29 genes known to be mutated in melanoma was used (genes listed in Supplemental Table 1), as previously described.[37] Mean coverage of 2094 reads, with a minimum coverage of 30 reads in >80% of the target loci, was achieved. Four samples were excluded from analysis due to low coverage.

Sequence analysis
CLC Cancer Research Workbench from QIAGEN® was used for sequence analysis, as previously reported.[37] Mutations were considered if coverage of the mutation site was ≥30 reads, ≥10 reads reported the mutated variant and the frequency of mutated reads was ≥10%.

Associations of mutation status with clinical and pathological parameters
Associations of mutation status with available clinico-pathological parameters (listed in Table 1) were explored. Analyses were performed with IBM SPSS Statistics software (version 20.0; International Business Machines Corp., Armonk NY, USA). A *p*-value of <0.05 was considered statistically significant.

RESULTS

Tumours and patients
Conjunctival melanomas occurred equally in male and female with a median age of 67 years (range 40–89 years). Of the samples for which information was available, 52% (33/63) originated from primary acquired melanosis (PAM), 18% (11/63) from naevi and 22% (14/63) arose de novo. Clinical stage at initial presentation was stage 1, stage 2 and stage 3 in 56% (35/63), 24% (15/63) and 16% (10/63) of patients, respectively (American Joint Committee on Cancer staging system for conjunctival melanoma, 7th edition, 2010). Adjuvant treatment was received by 87% (55/63) of patients (21 ruthenium, 17 proton, 6 percutaneous radiotherapy, 7 cryotherapy and 3 mitomycin C). Tumours recurred in 47% (30/63) and metastasised in 22% (14/63) of cases. Additional information is listed in Table 1.

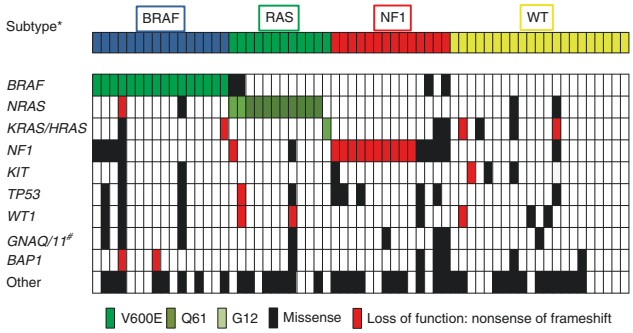

Fig. 1 Mutations in conjunctival melanoma. Distribution of mutations identified by amplicon panel next-generation sequencing. Green: mutations known or assumed to be activating; Red: nonsense or frameshift loss-of-function mutations; Black: missense mutation with unknown functional consequences. Mutations listed as "Other" include mutations detected in *CDK4*, *FLT4*, *PIK3CA*, *PIK3R1*, *FBXW7*, *MITF*, *MAP2K1*, *MAP2K2*, *ARID1A*, *ARID2*, *SF3B1*, *CTNNB1*, *PTEN*, *CDKN2A*, *SMARCA4A*, *EZH2*, *IDH1* and the protein-coding area of *TERT* (the promoter region of *TERT* was not covered by the amplicon-based sequencing panel used in this study). *Subtype according to TCGA genomic classification of cutaneous melanoma. #None of the *GNAQ* or *GNA11* mutations identified were the known activating Q209 or R183 mutations recurrently identified in uveal melanomas (details in Supplemental Table 2)

Identified mutations

Activating $BRAF^{V600E}$ (c.1799A>T) mutations were detected in 16/63 (25%) tumours. Additionally, 4 *BRAF* mutations with unknown functional consequences were identified (Supplemental Table 2).

Activating *RAS* mutations (11 *NRAS* and 1 *KRAS* mutation) were identified in 12/63 (19%) tumours (Table 2). We also detected 4 *NRAS*, 3 *KRAS* and 5 *HRAS* mutations with unknown functional consequences (Supplemental Table 2).

NF1 mutations were identified in 21/63 (33%) tumours. Clearly inactivating *NF1* mutations were observed in 10 tumours. *NF1* mutations co-occurred with *BRAF* and *RAS* gene mutations in some tumours, but also frequently occurred alone. All identified mutations are shown in Fig. 1 and listed in Table 2, and shown in Supplemental Figs. 1 and 2.

Additionally, mutations in various genes frequently mutated in cutaneous melanoma were detected. The majority of these mutations were of unknown functional consequences (Supplemental Table 2). While a few *GNAQ* and *GNA11* mutations were identified (Fig. 1, Supplemental Table 2), they presumably represent functionally non-relevant bystander mutations, as none of the identified mutations were the activating R183 or Q209 mutations known to occur in uveal melanomas.[14, 15, 21]

Statistical analysis

There were no statistically significant associations between clinicopathological parameters with *BRAF*, *RAS* and *NF1* mutation status (Table 1).

DISCUSSION

To our knowledge, the present study represents the most detailed analysis of gene mutations in conjunctival melanoma to date.

Activating *BRAF* mutations were detected in 25% of samples, lying within the range of previous studies reporting 14–50%.[24–27, 38] This variation may be due to sample bias or technical differences. In view of the recent development of effective BRAF and MEK inhibitors, the presence of *BRAF* V600 mutations in conjunctival melanomas is of considerable therapeutic relevance.[39]

In addition to known activating *NRAS* mutations in 18% (11/63) of tumours, we identified an activating *KRAS* G12A mutation. Being the first report on these mutations in conjunctival melanoma, this finding is reminiscent of cutaneous melanoma, in which *KRAS* mutations are rare but occur in a mutually exclusive fashion with *NRAS* mutations.[11] In the proposed TCGA (The Cancer Genome Atlas) genomic classification of cutaneous melanoma, mutations in all three *RAS* genes are grouped together as *RAS*-mutated melanomas.

Our study is the first to identify *NF1* as a frequently mutated oncogene (33%) in conjunctival melanoma. *NF1* has recently been recognised as the third most commonly mutated gene (after *BRAF* and *RAS*) in cutaneous melanoma, activating the MAP kinase pathway.[11] In our conjunctival melanoma cohort, *NF1* mutations were also present in samples harbouring activating *RAS* or *BRAF* mutations (Fig. 1). This is similar to the situation in cutaneous melanoma where the co-occurrence of *NF1* with *BRAF*, *RAS* and other mutations is well recognised.[11, 12, 40]

NF1 mutations are particularly frequent in melanoma subtypes rarely harbouring *BRAF* and *NRAS* mutations,[8, 12, 41] including melanomas associated with high sun exposure.[8, 12] Ultraviolet exposure is a known pathogenic factor in conjunctival melanoma and could explain the high number of *NF1* mutations detected. *NF1* mutations have been associated with high tumour mutational load and affected patients have been reported to benefit from anti-PD-1 therapy in cutaneous melanoma.[42] This suggests that *NF1* mutation status has potential as a biomarker for immunotherapy in conjunctival melanoma.

In summary, our study identifies a range of mutations in conjunctival melanoma. The distribution of activating mutations, with *RAS* gene mutations occurring not only in *NRAS* but also *KRAS*, and *NF1* mutations being frequent in tumours lacking *BRAF* or *RAS* mutations, suggests that the proposed genetic classification of cutaneous melanomas into *BRAF*-mutated, *RAS*-mutated, *NF1*-mutated or triple-wild-type tumours is also applicable to conjunctival melanoma.

Table 2. MAP kinase pathway activating mutations in conjunctival melanoma

Gene		Mutation type	Tumours harbouring mutation	
			N	%
BRAF		All mutations	16	25
		V600E	16	25
RAS		All mutations	12	19
	NRAS	Q61R	5	8
		Q61K	2	3
		Q61H	1	2
		Q61L	1	2
		G13D	1	2
		G12N, G12C	1	2
	KRAS	G12A	1	2
NF1		All mutations	21	33
		T60del	1	2
		R262C	1	2
		C42Y, G2397R, S2587L	1	2
		S2751N, L552P, G2392E	1	2
		D176E	2	3
		L847P, P866S, V1762I	1	2
		C1899Y	1	2
		M1180I, S52F; T60I	1	2
		A2715V; A2208T	1	2
		G2397R, R2517fs	1	2
		I1824fs	1	2
		L1892[a]	1	2
		N1451L	1	2
		Q1815[a]	1	2
		Q756fs	1	2
		R1362[a]	1	2
		R440[a], Q2239[a]; S1497F; V1393A	1	2
		S168L	1	2
		S1786[a], L1102[a]; Q1815fs	1	2
		Y1678fs	1	2
Wild type			14	22
Total			63	

MAP mitogen-activated protein, *fs* frameshift mutations [a]Nonsense mutations

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AUTHOR CONTRIBUTIONS

Literature search: S.L.S., K.G.G., B.S., H.W., R.M.; study design: K.G.G., K.P.S., D.S.; data collection: S.L.S., I.C., D.S., R.M., I.M., H.R., S.T., S.L., E.H., C.F., K.G.G., H.W., A.P., D.S.; data analysis: S.L.S., K.G.G., R.M., T.S., H.W., A.S., A.P.; data interpretation: S.L.S., K.G.G., A.P., A.S., H.W.; manuscript writing: all authors.

ADDITIONAL INFORMATION

Supplementary information is available for this paper at https://doi.org/10.1038/s41416-018-0046-5.

Competing interests: Dirk Schadendorf is on the advisory board or has received honararia from Roche, Genetech, Novartis, Amgen, GSK, Boehringer Ingelheim and Merck. The other authors declare no competing interests.

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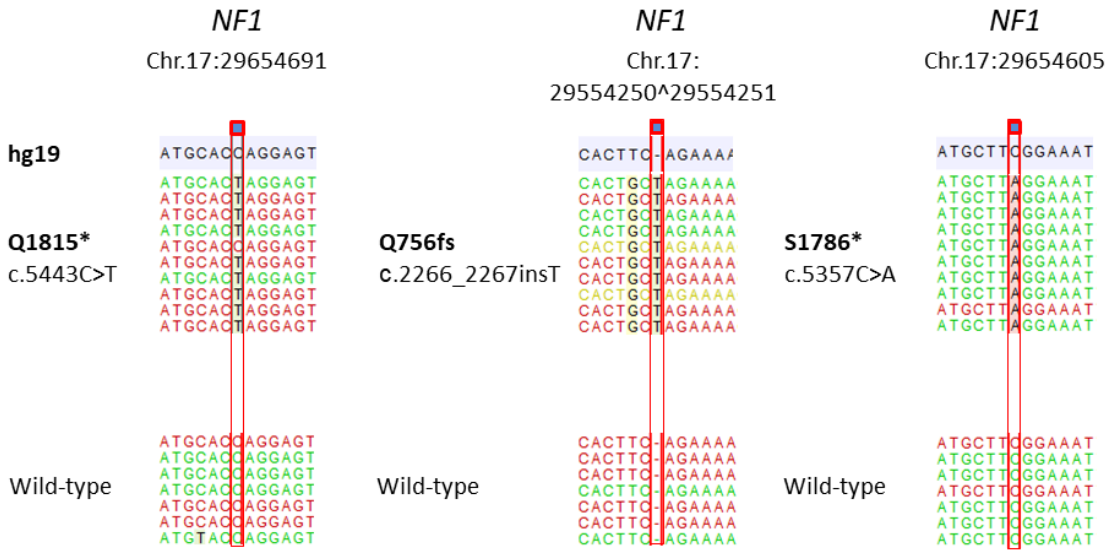
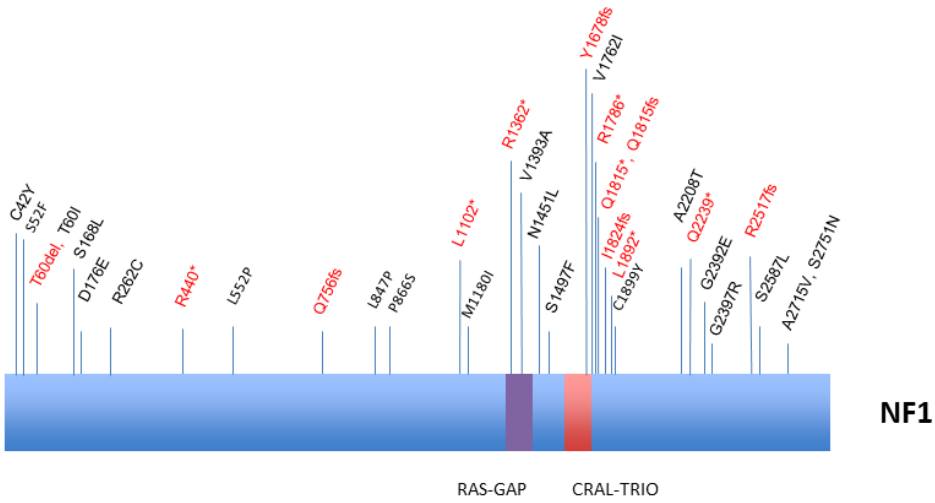
Supplemental Figure 1. Inactivating *NF1* mutations



Supplemental Figure 2: Distribution of identified *NF1* mutations



SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1: Examples of inactivating *NF1* mutations

Shown are the results of 3 samples in which inactivating mutations were detected by amplicon-based next-generation sequencing. Demonstrated on top are the sequenced results where a nucleotide alteration was determined. A wild-type result from another sample is shown underneath for comparison.

Supplemental Figure 2. Distribution of identified *NF1* mutations

Demonstrated a scheme of the NF1 protein with the mutations identified at different locations in the protein. Functionally inactivating mutations leading to a premature stop-codon (non-sense) or frameshift are demonstrated in red.

SUPPLEMENTAL TABLES

Supplemental Table 1: Genes covered in the applied sequencing panel

Supplemental Table 2: List of all mutations for each tumour sample

Supplemental Table 1

Nr.	Gene	Primary melanoma type	Customary mutation type	Target bases	Bases covered	Primer pairs
1	BRAF	cutaneous	activating	2860	2456	40
2	NRAS	cutaneous	activating	650	650	10
3	KIT	cutaneous	activating	3354	3264	51
4	HRAS	cutaneous	activating	780	667	11
5	KRAS	cutaneous	activating	787	787	13
6	CDKN2A	cutaneous	tumour suppressor	1184	713	14
7	PTEN	cutaneous	tumour suppressor	1392	1248	22
8	CDK4	cutaneous		1052	1052	19
9	TP53	cutaneous	tumour suppressor	1503	1396	26
10	RAC1	cutaneous		776	721	14
11	NF1	cutaneous	tumour suppressor	9900	9167	143
12	PIK3CA	cutaneous		3607	3313	50
13	MAP2K2	cutaneous		1423	1240	24
14	PIK3R1	cutaneous		2637	2627	42
15	MITF	cutaneous		2066	2066	35
16	TERT*	cutaneous		3719	2371	39
17	ARID2	cutaneous	tumour suppressor	5928	5830	82
18	ARID1A	cutaneous	tumour suppressor	7258	6132	81
19	SMARCA4	cutaneous	tumour suppressor	5761	5040	88
20	MAP2K1	cutaneous		1436	1436	26
21	CTNNB1	cutaneous		2626	2626	40
22	EZH2	cutaneous		2680	2680	46
23	IDH1	cutaneous		1405	1394	22
24	FBXW7	cutaneous		2898	2808	43
25	WT1	cutaneous		1784	1282	24
26	GNAQ	uveal	activating	1220	1064	17
27	GNA11	uveal	activating	1220	944	14
28	BAP1	uveal	tumour suppressor	2599	2380	39
29	SF3B1	uveal		4455	4412	72

* the protein-coding area (but not the promoter region) of the *TERT* gene was covered by the panel.

Supplemental Table 2 : is available for this paper at 10.1038/s41416-018-0046-5

3.2. Tumor *CDKN2A*-associated *JAK2* loss and susceptibility to immunotherapy resistance

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- Conception: 25% - Study design.
- Experimental work: 75% - Cell culture, Western Blots, FACS staining, quantitative PCR, targeted sequencing, SNP array
- Data analysis: 50% - SNP array analysis, TCGA data analysis
- Statistical analysis: 50% - analysis of TCGA data
- Writing the manuscript: 25% - Visualization of the results, literature research, conceptual design, writing all chapters

_____ Place, Date _____Annette Paschen

_____ Place, Date _____Sonia Leonardelli

JNCI 17-0885R1

Brief Communication

Tumor *CDKN2A*-associated *JAK2* loss and susceptibility to immunotherapy resistance

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Abstract

Poor clinical responses to checkpoint blockade with anti-CTLA-4 and anti-PD-1 antibodies in melanoma have recently been associated with acquired IFNγ resistance that protects tumor cells from the antiproliferative and pro-apoptotic cytokine activity. IFNγ-resistant melanoma cells very often lack functional expression of the IFNγ signaling pathway gene JAK2 due to gene deletions or inactivating gene mutations. Analyzing melanoma cell lines (n = 46, applying next-generation targeted sequencing and single nucleotide polymorphism arrays) as well as available genomic data sets from The Cancer Genome Atlas (TCGA) tumor tissue samples (cutaneous melanoma n = 367, lung squamous cell carcinoma n = 501, bladder urothelial carcinoma n = 408, breast invasive carcinoma n = 768, colorectal adenocarcinoma n = 257), we demonstrate that the frequent chromosomal losses of the tumor suppressor *CDKN2A* in melanoma and other tumor entities enhance the susceptibility to IFNγ resistance by concomitant deletion of the *JAK2* gene (odds ratio = 223.17, 95% confidence interval = 66.91 to 1487.38, two-sided P = 7.6 $x10^{-46}$). Tumors with *JAK2* mutations or homozygous *JAK2* deletions demonstrate allelic losses covering both *CDKN2A* and *JAK2*. This suggests that patients with tumor chromosomal *CDKN2A* losses are susceptible to developing immunotherapy resistance and should be screened for JAK2 deficiency prior to and under immune checkpoint blocking therapy.

In recent years, therapies with immune checkpoint blocking antibodies have revolutionized the treatment of many different cancer entities, demonstrating impressive clinical response rates (1). However, resistance to therapy is a problem, and considerable effort is underway to understand the mechanisms involved. Recently, resistance to anti-PD-1 and anti-CTLA-4 antibody therapies has been associated with tumors acquiring genetic resistance to IFNγ, thereby losing sensitivity to the anti- proliferative and pro-apoptotic cytokine activity (2–5). IFNγ-resistant melanoma cells frequently lack functional expression of the IFNγ pathway component JAK2 that is caused by inactivating the *JAK2* gene, located at chromosome 9p24.1 (Chr.9p24.1), either by mutation of one and deletion of the other allele or by homozygous gene deletions (3–5). In our study, we identify that chromosomal loss of a tumor suppressor increases the susceptibility to acquired IFNγ resistance.

CDKN2A is one of the most frequently lost tumor suppressor genes in human cancers. Two alternative *CDKN2A* reading frames encode the tumor suppressors $p14^{ARF}$ and $p16^{INK4A}$, both critically involved in cell cycle regulation. *CDKN2A* deficiency, developing early in disease, is observed in 50% to 80% of melanomas and frequently associated with larger chromosomal alterations affecting the Chr.9p21.3 gene locus (6–8).

The relative proximity of *CDKN2A* and *JAK2* on Chr.9p led us to ask whether there is an association of allelic losses of both genes. To answer this, we first studied genomic alterations and protein expression as described in detail in the Supplementary Methods (available online) on specimens from melanoma patients obtained after written informed consent and local ethics approval.

IFNγ-resistant Ma-Mel-54a cells established from a cutaneous metastasis of patient Ma-Mel-54 showed mutational inactivation of *JAK2* (p.Q959P) and *CDKN2A* (p.R80*, nonfunctional truncated protein), whereas IFNγ-sensitive Ma-Mel-86a cells from patient Ma-Mel-86 demonstrated wild-type sequences of the corresponding genes (defined by targeted sequencing and analyses of available exome data (F**igure 1A; Supplementary Figure 1, A–C**) (3,9). Accordingly, Ma-Mel-54a as well as Ma-Mel-54b cells, obtained from a consecutive metastasis of patient Ma-Mel-54, lacked JAK2 and p16 expression, as demonstrated by immunoblot analyses and immunohistochemical staining (**Figure 1, B and C**). JAK2-deficient Colo857 melanoma cells, previously described as IFNγ signaling defective (10), were also

negative for p16 (**Figure 1D; Supplementary Figure 1, B and C**), suggesting an association of genetic alterations affecting *JAK2* and *CDKN2A*.

Screening single nucleotide polymorphism (SNP) array data from five melanoma cell lines established from metastatic lesions of five patients (NCBI GEO database: accession No. GSE60218 for Ma-Mel-48a and Ma-Mel-100a, GSE80736 for Ma- Mel-86a, GSE96884 for Ma-Mel-54a and Ma-Mel-61a) for allelic *JAK2* and *CDKN2A* losses (applying the Chromosome Analysis Suite program, Affymetrix, Santa Clara, CA) demonstrated large deletions on Chr.9p encompassing *CDKN2A* and *JAK2* in all cases (**Figure 1E**).

Analysis of an independent SNP microarray data set (GEO accession number GSE17534) using Affymetrix genotyping console software (Affymetrix, Santa Clara, CA) confirmed the high frequency of *CDKN2A*-associated *JAK2* losses (75.6%, 31 of 41) in melanoma cell lines (**Figure 1F**). This suggests that *CDKN2A*-associated *JAK2* deletions occur early in the course of disease and that a subsequent mutation or deletion in the remaining *JAK2* allele leads to IFNγ resistance.

To screen for association of *CDKN2A* and *JAK2* deletions in tumor tissues, we assessed The Cancer Genome Atlas (TCGA) data sets, applying cBioPortal for Cancer Genomics (14). Considering that losses of *CDKN2A* are frequent not only in melanoma but in a range of different malignancies, including many approved for treatment with anti-PD-1 such as lung squamous cell carcinoma and bladder carcinoma (11), we studied five different malignancies (skin cutaneous melanoma n = 367, lung squamous cell carcinoma n = 501, bladder urothelial carcinoma n = 408, breast invasive carcinoma n = 768, colorectal adenocarcinoma n = 257) for deletions in *CDKN2A* and *JAK2*. Compared with samples demonstrating deletions in only one or none of these genes, all analyzed cancer entities showed a statistically significant increase in the proportion of samples carrying losses of both *JAK2* and *CDKN2A* (**Figure 2A**), considering P values of less than .05 (for melanoma: odds ratio [OR] = 223.17, 95% confidence interval = 66.91 to 1487.38, P = 7.6x10-46, calculated in R v3.1.1 using the function “oddsratio” applying two-sided chi- square test) (**Figure 2B**). This was also observed in cancers such as colorectal adenocarcinoma, where *CDKN2A* deletions are less frequent. Visualization of the lost chromosome segments in TCGA tissue samples (skin cutaneous melanoma n = 481, lung squamous cell carcinoma n = 626, bladder urothelial carcinoma n = 436, breast invasive carcinoma n = 1247, colorectal adeno- carcinoma n = 551) using the UCSC Xena Browser

(http://xena. ucsc.edu/) confirmed that larger deletions on Chr.9p result in concurrent allele losses of *CDKN2A* and *JAK2* (**Figure 2C; Supplementary Figure 2, A and B**). Notably, the majority of samples harboring mutations and homozygous deletions in *JAK2* showed concurrent *CDKN2A* and *JAK2* allelic losses in melanoma (75.0%, 9 of 12), lung squamous cell carcinoma (90.5%, 19 of 21), and bladder urothelial carcinoma (80.0%, 16 of 20) (**Figure 2A**).

These data demonstrate that *CDKN2A*-associated loss of *JAK2* is a very frequent event in the indicated tumor entities. Our study has some limitations as the results have been obtained by retrospective data analysis. Prospective studies analyzing larger numbers of tumor samples and samples obtained sequentially during the course of therapy will be valuable to confirm our findings.

Our data suggest that chromosomal losses of the tumor suppressor *CDKN2A* by associated deletion of *JAK2* increase the susceptibility of tumors to become resistant to IFNγ and immunotherapy. These data demonstrate, on an individual gene level, the recently published concept of chromosomal alterations predisposing to immunotherapy resistance (12,13). Based on our findings, one may need to consider whether tumors harboring *CDKN2A* losses require more detailed genetic screening prior to or during immunotherapy, or should potentially be prioritized for targeted (i.e., BRAF inhibitor) therapies. Our findings also suggest that assessing the proximity of therapy resistance genes and frequently lost tumor suppressors may identify additional genetic associations of therapeutic relevance.

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Notes

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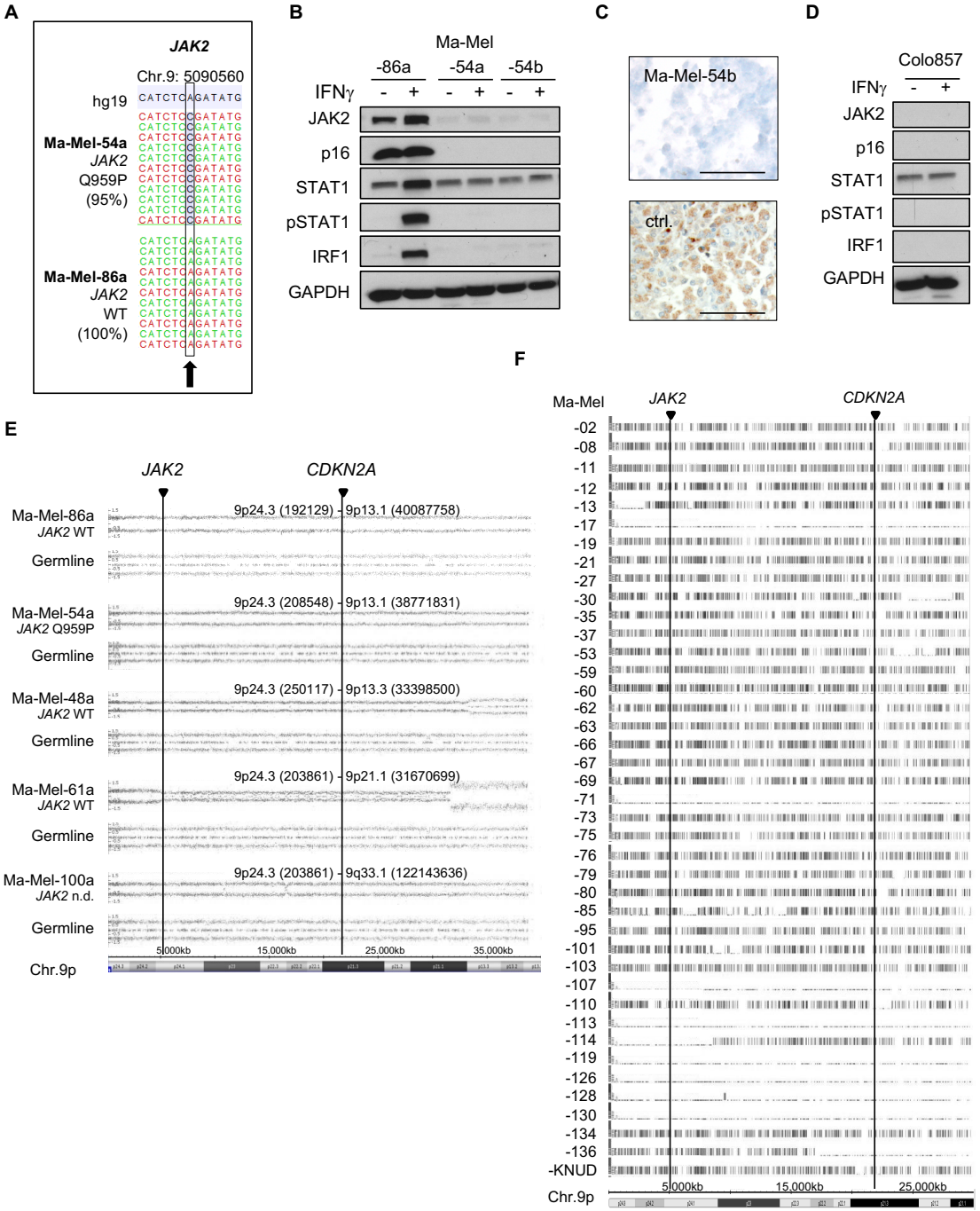
Figure Legend

Figure 1. Screening for allelic *CDKN2A* and *JAK2* losses. A) Targeted sequencing of DNA from melanoma cells Ma-Mel-54a and Ma-Mel-86a. Plots of aligned sequencing reads in the *JAK2* gene. Wild-type sequence in Ma-Mel-86a shown on the bottom; arrow highlights *JAK2* c.2876A>C, p.Q959P mutation site in Ma-Mel-54 cells. %, frequency of mutation or wild-type sequence in sequencing reads. B) Lysates from Ma-Mel-86a and Ma-Mel-54a cells treated with IFNγ (500 U/mL; 24 hours) or left untreated, analyzed by immunoblot for ex- pression of p16 and IFNγ signaling pathway components (JAK2, STAT1, pSTAT1, IRF1); GAPDH, loading control. Representative data from one of three independent experiments. C) Sections from cryopreserved Ma-Mel-54b tissue stained for p16 in comparison with paraffin tissue from a positive control metastasis by immunohistochemistry. Bars represent 50 mM. D) Lysates from Colo857 cells treated with IFNγ (500 U/mL; 24 hours) or left untreated, analyzed by immunoblot for expression of p16 and IFNγ signaling pathway components (JAK2, STAT1, pSTAT1, IRF1); GAPDH, loading control. Representative data from one of three independent experiments. E) Single nucleotide polymorphism (SNP) results given as allelic distribution of Chr.9p shown for DNA obtained from Ma-Mel-86a, Ma-Mel-54a, Ma-Mel-48a, Ma-Mel-61a, Ma-Mel-100a, and autologous peripheral blood cells as normal control (germline). Vertical lines indicate the locations of *JAK2* at Chr.9p24.1 and *CDKN2A* at Chr.9p21.3. Numbers indicate the regions deleted on Chr.9p. F) SNP array data from the indicated 41 melanoma cell lines and paired corresponding germline DNA were assessed for allelic losses on Chr.9p. Vertical gray lines indicate allelic losses. WT = wild-type.

Figure 2. Deletions of *CDKN2A* and *JAK2* in various cancer types. A) Visualization of *CDKN2A* and *JAK2* copy number data of The Cancer Genome Atlas (TCGA) tumor samples, obtained from https://www.cbioportal.org (14). Horizontal bars indicate the total number of samples with available copy number data for skin cutaneous melanoma (SKCM), lung squamous cell carcinoma, bladder carcinoma, breast carcinoma, and colorectal adenocarcinoma. Blue and green colors depict samples with losses in either *CDKN2A* or *JAK2*. Samples showing concomitant heterozygous deletions in *CDKN2A* and *JAK2* are shown in light red. Dark red indicates homozygous deletions of both genes as well as hom*CDKN2A*/het*JAK2* and het*CDKN2A*/hom*JAK2* loss. Gray color indicates samples with no losses in either gene. TCGA tissue samples with *JAK2* homozygous deletions (*JAK2* hom del; boxed) or single nucleotide variants (*JAK2* SNV) are listed and allocated to the different groups. B) Odds ratios, 95% confidence intervals, and P values were calculated to define association of losses in both genes over one gene lost or no loss at all (not differentiating between heterozygous and homozygous losses). Odds ratios were calculated in R v3.1.1 using two-sided chi-square tests to calculate the P values. C) Alterations in copy number of Chr.9p segments in TCGA melanoma (SKCM) samples were visualized using the UCSC Xena Browser (http://xena.ucsc.edu/). Thresholds for segment losses (blue) and gains (red) were calculated automatically. BLCA = bladder carcinoma; BRCA = breast carcinoma; CI = confidence interval; COAD = colorectal adenocarcinoma; LUSC = lung squamous cell carcinoma; OR = odds ratio; SKCM = skin cutaneous melanoma; SNV = single nucleotide variant.

Figure 1


A
JAK2
Chr.9: 5090560
hg19
CATCTCAGATATG
Ma-Mel-54a
JAK2
Q959P
(95%)
Ma-Mel-86a
JAK2
WT
(100%)
B
Ma-Mel
-86a
-54a
-54b
IFNγ
- + - + - +
JAK2
p16
STAT1
pSTAT1
IRF1
GAPDH
C
Ma-Mel-54b
ctrl.
D
Colo857
IFNγ
- +
JAK2
p16
STAT1
pSTAT1
IRF1
GAPDH
E
JAK2
CDKN2A
Ma-Mel-86a
JAK2 WT
9p24.3 (192129) - 9p13.1 (40087758)
Germline
Ma-Mel-54a
JAK2 Q959P
9p24.3 (208548) - 9p13.1 (38771831)
Germline
Ma-Mel-48a
JAK2 WT
9p24.3 (250117) - 9p13.3 (33398500)
Germline
Ma-Mel-61a
JAK2 WT
9p24.3 (203861) - 9p21.1 (31670699)
Germline
Ma-Mel-100a
JAK2 n.d.
9p24.3 (203861) - 9q33.1 (122143636)
Germline
5,000kb
15,000kb
25,000kb
35,000kb
Chr.9p
F
Ma-Mel
JAK2
CDKN2A
-02
-08
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-KNUD
5,000kb
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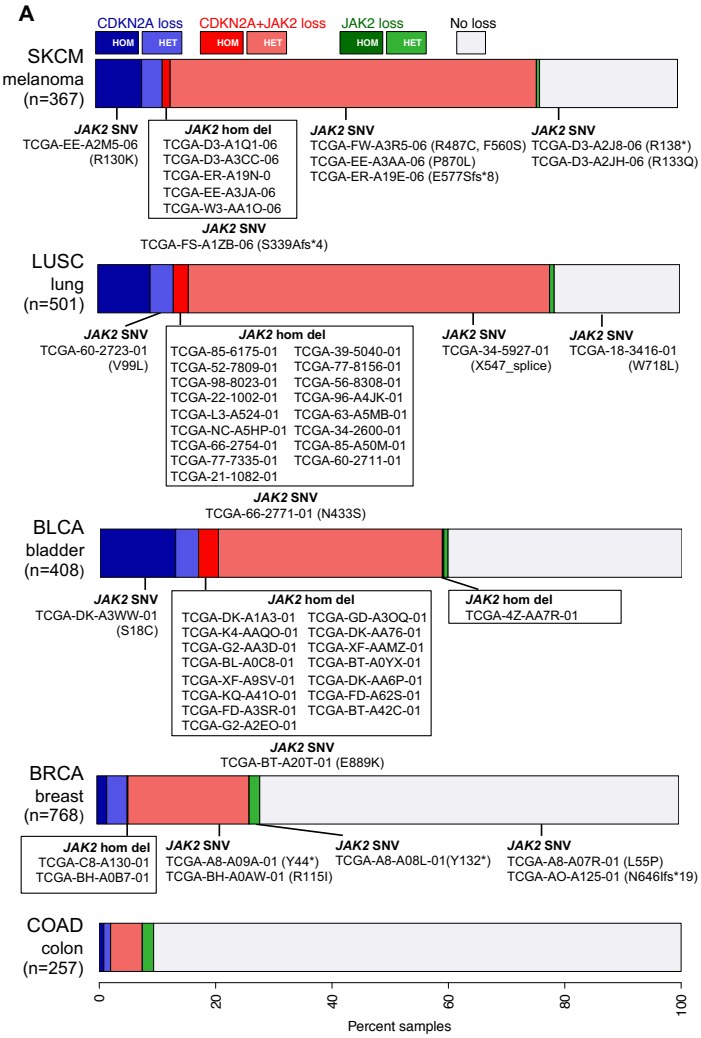
25,000kb
Chr.9p

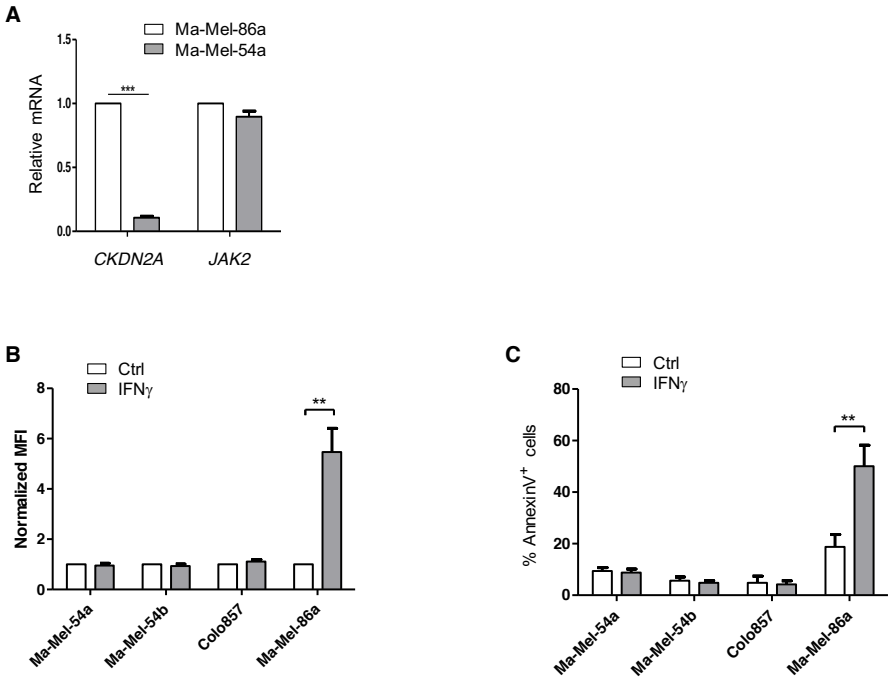
Figure 2



B

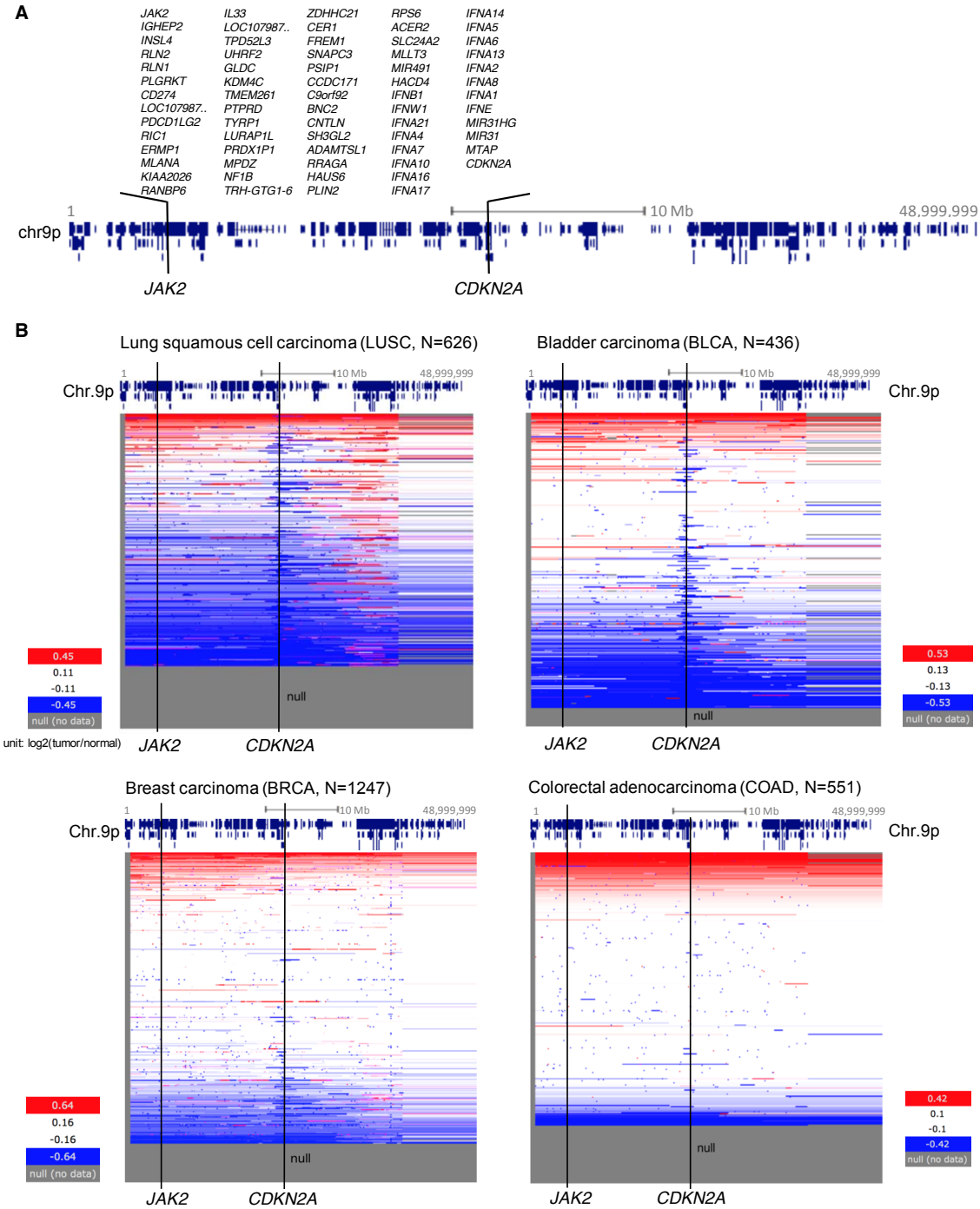
Cancer	OR	CI-lower	CI-upper	p
SKCM	223.17	66.91	1487.38	7.6e-46
LUSC	128.13	51.39	439.24	4.2e-55
BLCA	96.79	38.95	331.10	1.6e-43
BRCA	154.94	84.82	303.19	1.5e-112
COAD	117.35	32.79	527.29	1.7e-30

Supplementary Figure 1



Supplementary Figure 1. Comparison of IFNγ-sensitive and IFNγ-resistant melanoma cell lines for responses to cytokine treatment. **A)** Expression of *CDKN2A* and *JAK2* mRNA in IFNγ-resistant melanoma cells Ma-Mel-54a relative to IFNγ-sensitive Ma-Mel-86a cells defined by quantitative real-time PCR. Normalization of specific mRNA to endogenous GAPDH mRNA levels, relative expression calculated by the $2^{-\Delta\Delta CT}$ method. Mean values (+SD) from n = 3 independent experiments. Statistically significant differences defined by paired Student's t-tests, ***$P < 0.001$. **B)** Surface expression of PD-L1 on Ma-Mel-54a, Ma-Mel-54b, Colo857 and Ma-Mel-86a cells after IFNγ treatment (500 U/ml) for 3 days (n=3 replicates). Data are shown as mean fluorescence intensity of IFNγ-treated tumor cells normalized to control cells. Mean values (+SD) from 3 independent experiments. Statistically significant differences defined by paired Student's t-tests, **$P = 0.0021$. **C)** AnnexinV/PI staining of Ma-Mel-54a, Ma-Mel-54b, Colo857 and Ma-Mel-86a cells after 3 days of treatment with IFNγ (500 U/ml). Percentage of AnnexinV$^+$ cells is depicted as mean+SD (n=3). Statistically significant differences defined by paired Student's t-tests, **$P = 0.0039$.

Supplementary Figure 2



Supplementary Figure 2. Chromosome 9p copy number alterations in different cancers. **A**) List of genes located on Chr.9p covering the region between *JAK2* and *CDKN2A*. **B**) Alterations in copy number of Chr.9p segments in TCGA melanoma (SKCM) samples were visualized using the UCSC Xena Browser (http://xena.ucsc.edu/). Thresholds for segment losses (blue) and gains (red) were calculated automatically.

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Brief Communication, Supplementary Methods

Cell lines

Melanoma cell lines Ma-Mel-86a, Ma-Mel-54a and Ma-Mel-54b were established from tumor metastases after written informed consent given by the patients. Colo857 melanoma cells were purchased from American Type Culture Collection (ATCC, Manassas, USA). All cell lines were cultured in RPMI 1640 (Thermo Fisher Scientific, Waltham, USA) supplemented with fetal calf serum (10%) (Biochrom, Cambridge, UK) and penicillin/streptomycin (Biochrom, Cambridge, UK). Cells were treated for 24 h to 72 h with 500 U/ml IFNγ (Boehringer Ingelheim, Ingelheim am Rhein, DE).

AnnexinV/PI staining

Floating and adherent cells were harvested and resuspended in binding buffer, together with AnnexinV-APC (BD Biosciences, Franklin Lakes, USA) and propidium iodide (BD Biosciences, Franklin Lakes, USA). Cells were incubated for 15 min at room temperature. Apoptosis was measured on a Gallios flow cytometer (Beckmann Coulter, Brea, USA) and data was analyzed applying the Kaluza software (Beckmann Coulter, Brea, USA).

Immunohistochemistry

Expression of p16 in tumor tissue sections was detected using the CINtec p16 (clone E6H4) histology kit in combination with the OptiVIEW DAB IHC detection kit (both Roche Diagnostics, Rotkreuz, Switzerland).

Flow cytometry

Melanoma cells treated with IFNγ for 3 d or left untreated were analyzed for PD-L1 surface expression as follows: Cells were collected, resuspended in buffer (PBS + FCS (10%)) and anti-PD-L1-PE (29E.2A3, 1:20, Biolegend, San Diego, USA). Unstained cells were used to define background fluorescence. After fixation with paraformaldehyde (4%), cells were analyzed on a Gallios flow cytometer (Beckmann Coulter, Brea, USA) using the Kaluza software (Beckmann Coulter, Brea, USA) for data analysis. PD-L1 expression levels, given as mean fluorescence intensity (MFI), were normalized to background MFI.

Western blot analysis

Proteins were isolated from tumor cells treated with IFNγ or left untreated for 24 h. Isolated proteins were separated by SDS–polyacrylamide gel electrophoresis, blotted on nitrocellulose membranes and probed with the following primary antibodies: anti-CDKN2A/P16INK4A (clone EPR1473, 1:1000, Abcam, Cambridge, UK), anti-JAK2 (D2E12, 1:1000, Cell Signaling, Danvers, USA), anti-STAT1 (clone M-22, 1:1000, Santa Cruz, Dallas, USA), anti-pSTAT1 (clone 58D6, 1:1000, Cell Signaling, Danvers, USA), anti-IRF1 (clone H-205, 1:1000, Santa Cruz, Dallas, USA), anti-GAPDH (14C10, 1:5000, Cell Signaling, Danvers, USA). Membranes were incubated after washing with the rabbit secondary antibody linked to horseradish peroxidase (1:5000, Cell Signaling, Danvers, USA). Antibody signal was visualized with the enhanced chemiluminescence (ECL) system Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific, Waltham, USA).

Quantitative PCR

RNeasy plus Mini Kit (Qiagen, Hilden, DE) was used to isolate total mRNA from tumor cells, according to the manufacturer's instructions. Reverse transcription of RNA into cDNA was obtained with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Life Technologies, Grand Island, USA). Real-time PCR was carried out using TaqMan Gene Expression assays for *JAK2* (Hs01078136), *CDKN2A* (Hs00923894) and *GAPDH* (Hs02758991) (Thermo Fisher Scientific, Waltham, USA) and the StepOnePlus Real-Time PCR system (Applied Biosystems Life Technologies, Grand Island, USA). Relative RNA expression was calculated by the $2^{-\Delta\Delta}$Ct method after normalizing specific mRNA levels to the amount of endogenous *GAPDH* mRNA.

Targeted Sequencing

Tumor cells genomic DNA was isolated using the QIAamp DNA Mini Kit (Qiagen, Hilden, DE) according to the manufacturer's instructions. A custom amplicon-based sequencing panel covering 12 genes of the interferon pathway (1) was designed and prepared applying the GeneRead Library Prep Kit (Qiagen, Hilden, DE) according to the manufacturer's instructions. Individual samples were barcoded using a kit from New England Biosciences (Ipswich, USA) and 24 samples sequenced in parallel on an Illumina MiSeq Next Generation Sequencer (Illumina, San Diego, USA). Sequencing analysis was performed applying the

CLC Cancer Research Workbench from QIAGEN®. After trimming the primer sequences, the sequence reads were aligned to the human genome assembly 19 (hg19). Analysis for both insertions/deletions and single nucleotide variations followed. Single nucleotide polymorphisms (SNP) were filtered out by cross-referencing the dbSNP v.138 database, the 1000 genomes database and individual cases manually. Mutations affecting the coding region of the gene were considered if predicted to result in non-synonymous amino acid changes, overall coverage of the mutation site was ≥ 30 reads, > 5 reads reported the mutation variant and the frequency of mutated to non-mutated reads was ≥ 10%.

SNP array analysis

SNP arrays on DNA from tumor cell lines were carried out using the CytoScan HD Array (Affymetrix, Santa Clara, USA). Hybridization was done according to the manufacturer´s protocol and data analysis performed applying the Chromosome Analysis Suite program (Affymetrix, Santa Clara, USA).

Analyses of TCGA datasets

To analyze and visualize genomic data from The Cancer Genome Atlas (TCGA) the cBioPortal for Cancer Genomics (http://cbioportal.org) web interface was used (2). Association of *CDKN2A* and *JAK2* deletions was analyzed in the following available dataset: skin cutaneous melanoma (SCKM, provisional, 367 samples with information on copy number aberrations (CAN)), lung squamous cell carcinoma (LUSC, provisional, 501 samples with CNA), bladder urothelial carcinoma (BLCA, provisional, 408 samples with CNA), breast invasive carcinoma (BRCA, provisional, 768 sample with CNA) and colorectal adenocarcinoma (COAD, 257 samples with CNA). Mutations and putative copy number alterations (determined using GISTIC 2.0) for the query genes were downloaded from the cBioPortal website download section in tab-delimited format. Called CNA included the following values: -2 = homozygous deletion; -1 = hemizygous deletion; 0 = neutral / no change; 1 = gain; 2 = high level amplification.

CNA on chromosome 9p were visualized using the UCSC Xena Browser (http://xena.ucsc.edu/) in TCGA datasets of skin cutaneous melanoma (SKCM, 481 samples), lung squamous cell carcinoma (LUSC, 626 samples), bladder urothelial carcinoma (BLCA, 436 samples), breast invasive carcinoma (BRCA, 1247 samples) and colorectal

adenocarcinoma (COAD, 551 samples). Thresholds for segment losses (blue) and gains (red) were given in log2 (tumor/normal) and were calculated automatically by the Xena Browser.

Statistical analyses

Odds ratios [OR], 95% confidence interval [CI] and *P* value for concomitant losses in both genes over only one gene lost or no loss at all were calculated in R v3.1.1 using the function 'oddsratio' applying two-sided chi-square tests. Data obtained by quantitative PCR and flow cytometry are shown as mean (+SD) of three independent experiments. Statistical significance was calculated by two-sided paired Student's *t* test applying the GraphPad Prism 5 Software. Results were considered statistically significant with *P* values of less than 0.05.

References

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2. Gao J, Aksoy BA, Dogrusoz U, *et al*. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Sci Signal* 2013;6(269):pl1

4. Unpublished data

4.1. Acquired IFNγ resistance of melanoma cells linked to EMT-like transcriptional reprogramming

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Own contributions:

- Conception: 50% - Study design.
- Experimental work: 90% - Cell culture, selection of IFNγ-sensitive patient-derived cell lines, induction of IFNγ resistance in all cell lines apart from one (Ma-Mel-86c), Western Blots, FACS staining, xCELLigence experiments, Cristal Violet staining, isolation of the RNA from all the samples
- Data analysis: 100% - RNA Sequencing analysis by using R statistical software, applying DESeq2 and GSEA
- Statistical analysis: 100% - analysis of differentially expressed genes by R statistical software
- Writing the manuscript: 50% - Visualization of the results, literature research, conceptual design, writing chapters.

_____ Place, Date _____Annette Paschen

_____ Place, Date _____Sonia Leonardelli

Associating acquired IFNγ resistance of melanoma cells to EMT-like transcriptional reprogramming

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Abstract

A better understanding of mechanisms underlying resistance to T cell-based immunotherapy in melanoma is urgently needed in order to circumvent resistance and improve clinical outcomes. Interferon-gamma (IFNγ), released by activated tumor-reactive T cells, has an important role in cancer cell killing and determines the efficacy of immunotherapy. Genetic defects in distinct components of the IFNγ signaling pathway have been associated with immunotherapy resistance, but the role of non-genomic mechanisms is poorly understood. We postulated that non-genomic mechanisms could protect melanoma cells from IFNγ-induced apoptosis, thereby interfering with the efficacy of immunotherapy. To study this, we established IFNγ-resistant variants from IFNγ-sensitive parental tumor cells. The sensitive and resistant tumor cell pairs were analyzed for transcriptional signatures associated with resistance to cytokine-induced apoptosis and phenotypic alterations were confirmed at the protein level. This led to the identification of an EMT (epithelial to mesenchymal transition)-like phenotypic switch in cells with acquired resistance to IFNγ going along with the downregulation of IFNγ signaling pathway genes. Interestingly, the EMT signature has previously been reported to be associated with unresponsiveness to both treatments with targeted inhibitors and immune-modulating antibodies, suggesting a broader relevance of our findings in the field of melanoma therapy.

Introduction

Melanoma is one of the most heterogeneous and immunogenic tumors with the highest mutational load among different cancer types (1). Immunotherapy has significantly improved the clinical outcome of patients. This therapy, as the treatment with immune checkpoint modulating antibodies, reinvigorates tumor-reactive CD8+ T cells, which in turn attack tumor cells (2,3). Still, many patients do not respond or benefit from therapy only for a short time, due to intrinsic and acquired resistance mechanisms (4-6). Upon tumor cell recognition, CD8+ T cells release cytolytic granules containing perforins and granzymes that directly kill the target cells. Moreover, CD8+ T cells release cytokines, predominantly IFNγ, which induce cell cycle arrest and apoptosis also of neighboring cells (7,8). Binding of IFNγ to its receptor IFNGR1/IFNGR2 on the tumor cells causes the activation of the JAK1 and JAK2 receptor-associated kinases, which in turn phosphorylate the transcription factor STAT1. Phosphorylated STAT1 is able to form homodimers which directly activate the transcription of primary response genes. This includes IRF1, a transcriptional activator that triggers the expression of secondary response genes causing growth arrest and cell death. Recent studies associated genomic defects in the IFNγ signaling pathway, in particular those affecting the JAK1/2 kinases, with resistance to immune checkpoint blockade. *JAK1/2* deficiency is caused by inactivating mutations affecting on allele and chromosomal alterations causing loss of the second allele (9-11).

How far non-genomic mechanisms, in contrast to genetic alterations, affect the responsiveness of melanoma cells to IFNγ is poorly understood. Here, by continuously culturing melanoma cells in the presence of IFNγ, we established IFNγ-resistant variants from IFN-sensitive parental tumor cells. The sensitive and resistant tumor cell pairs were studied for transcriptional signatures associated with cytokine resistance and phenotypic alterations were confirmed at protein level. This led to the identification of an EMT-like phenotypic switch in cells with acquired resistance to IFNγ.

Results

Melanoma cells show intratumor heterogeneity in IFNγ-induced apoptosis

To get insights into cellular response pattern of melanoma cells to IFNγ, we first selected cell lines established from two distinct metastasis of patient Ma-Mel-86 for our analysis (**Figure 1a**). These two cell lines show distinct differentiation phenotypes as demonstrated by Western blot (**Figure 1b**). Ma-Mel-86a cells show a dedifferentiated $MITF^{low}$/$MLANA^{low}$ phenotype, whereas Ma-Mel-86c is highly differentiated, with high expression of the lineage transcription factor *MITF* and its target gene *MLANA*. Treating both cell lines with IFNγ (one dose, 500U/ml) we observed the induction of apoptosis for Ma-Mel-86c cells, while Ma-Mel-86a did not show any sign of enhanced cell death (**Figure 1c**). To exclude that the heterogenous response to IFNγ was caused by a defect in the IFNγ signaling pathway, we analyzed its activation upon cytokine addition. Both, Ma-Mel-86a and Ma-Mel-86c cells upregulated STAT1, pSTAT1, and IRF1 upon IFNγ treatment (**Figure 1d**). Therefore, we excluded the presence of mutations in the IFNγ signaling pathway impairing the response to IFNγ in Ma-Mel-86a. Interestingly, $BRAF^{V600E}$-mutant Ma-Mel-86a cells were intrinsically resistant not only to IFNγ-mediated cell death but also to apoptosis induced by BRAF-V600E inhibitor (BRAFi) Vemurafenib, while Ma-Mel-86c were killed by both cytokine and targeted inhibitor treatment (**Figure 1e**). Melanoma cell dedifferentiation, as observed for Ma-Mel-86a cells, has been linked to BRAFi resistance, suggesting similar non-genomic mechanisms could be involved also in resistance to IFNγ.

Identifying gene expression signature associated with IFNγ resistance

To determine if non-genomic mechanisms contribute to IFNγ resistance, we cultured IFNγ sensitive cells over time in the presence of the cytokine in order to generate IFNγ-resistant variants. In addition to Ma-Mel-86c cells, we selected Ma-Mel-61a, UKE-Mel-103a, UKE-Mel-105b, UKE-Mel-118c, UKE-Mel-130b. Culturing the cells in the presence of IFNγ over 7 days leads to significant apoptosis in all cases (**Figure 2a**), with some cells adapting to the treatment. Persisting tumor cells were continuously cultured in the presence of the cytokine. After several weeks or months proliferative IFNγ resistant cells grew out. IFNγ-resistant cells were kept continuously under IFNγ 500 U/ml pressure and expanded for RNA-seq (**Figure 2b**).

We performed RNA-seq on the parental IFNγ-sensitive cell lines, treated for 7 days with IFNγ (500U/ml) and untreated, and on the IFNγ-resistant cell lines, to determine resistance-associated gene signatures. Firstly, as a control, we analyzed by DESeq2 the differential expressed genes (DEGs) in parental cells treated with IFNγ compared to untreated cells, to confirm that the IFNγ signature was dominant in this comparison. Analyzing the DEGs in GSEA (**Figure 2c**) we found the HALLMARK_INTERFERON_GAMMA_RESPONSE, together with the of HALLMARK_ALLOGRAFT_REJECTION, and HALLMARK_INTERFERON_ALPHA_RESPONSE to be highly and significantly upregulated in the IFNγ-treated cells (padj < 0.05). These hallmarks match genes that are upregulated by IFNγ in the process of T cell activation, including *HLA* and cytokine genes.

Next, we aimed to identify the transcriptional program associated with IFNγ resistance, analyzing all the cell lines together. As DESeq2 is based on the comparison of samples between two groups, we decided to integrate the results as described in the method section and schematically shown in Figure 3a. This way, we obtained the final resistance gene signature, contained 820 DEGs, with log2FoldChange > I1.5I & padj <= 0.05. The 820 DEGs were run through Gene Set Enrichment Analysis (GSEA). We identified an enrichment of the transcriptional program associated with the hallmark of epithelial-mesenchymal transition, accounting for 44 genes matching this signature (**Figure 3b, c**). GSEA also revealed a significant downregulation of the hallmark of IFNγ response genes in the resistant cells (**Figure 3b, c**).

The same list of 820 resistance genes was run through Reactome (http://reactome.org) (12,13) (**Figure S1**), to validate the GSEA results. Interestingly, the significant hits (False Discovery Rate or FDR < 0.05) are associated to collagen degradation, which is an essential step in EMT, and Interleukin-4 and Interleukin-13 signaling, playing an important role in immune response and activation of the STAT6 pathway (14,15).

Melanoma cells with acquired IFNγ resistance show an upregulation of EMT, a downregulation of IFNγ response genes and cross resistance to BRAF inhibitor

Transcriptome analyses revealed a downregulation of IFNγ response genes in melanoma cells with acquired IFNγ-resistance. To confirm this at the protein level, we

first analyzed the IFNγ pathway activation in IFNγ-resistant cells compared to naïve cells treated IFNγ for 7 days. As shown in Figure 3d, all parental IFNγ-sensitive cell lines treated with IFNγ for 7 days upregulated the IFNγ pathway components pSTAT1, STAT1, and IRF1, whereas IFNγ-resistant cell lines, continuously treated with IFNγ, blocked, partially or completely, the activation of the pathway (**Figure 3d**). This result is in agreement with the downregulation of IFNγ response genes identified by GSEA.

Next, we studied the upregulation of the EMT genes in IFNγ-resistant cells. Here we focused on the cell lines Ma-Mel-86c and UKE-Mel-103a, both showing the strongest enrichment for EMT genes (**Figure 4a**). During EMT, cells undergo cytoskeletal reorganization, change their morphology and acquire a more spread surface. Thus, we analyzed the morphology of the IFNγ-resistant cell lines, compared to the parental ones. Both, the IFNγ-resistant Ma-Mel-86c and UKE-Mel-103a cells showed an elongated, mesenchymal-like shape compared to the untreated or short-term treated parental cells (**Figure 4b**).

The EMT switch is known to be associated with a more invasive phenotype. Therefore, we investigated in a real-time xCELLigence invasion assay the invasive properties of the two IFNγ-resistant cell lines. As shown in Figure 5a, both the IFNγ-resistant Ma-Mel-86c and UKE-Mel-103a cells were more invasive compared to their corresponding parental cell lines (**Figure 5a**). Furthermore, on protein level, IFNγ-resistant Ma-Mel-86c and UKE-Mel-103a cells showed a downregulation of E-cadherin, considered to be one of the hallmarks of EMT. At the same time, EGFR and WNT5A, known as markers of therapy resistance and involved in the EMT process, are upregulated. The parental cell lines also show high levels of lineage-specific transcription factors SOX10, while the IFNγ-resistant cells express SOX9, a marker characterizing melanoma cells being dedifferentiated, mesenchymal-like and therapy resistant (16,17). These changes are consistent in both cell lines (**Figure 5b**). By which mechanisms EMT-like phenotype switching leads to the downregulation of IFNγ signaling pathway activation remains to be determined.

Having demonstrated that this dynamic switch is a general mechanism for acquired resistance to IFNγ, we wondered if the IFNγ-resistant *BRAF*^{V600E} mutant Ma-Mel-86c cells would also be resistant to the BRAFi Vemurafenib. The crystal violet cell viability assay demonstrated that the IFNγ-resistant Ma-Mel-86c cells proliferated not only in the presence of IFNγ but also in the presence of BRAFi and

under combined treatment, while the parental cells died in response to single and combination treatment (**Figure S2**).

Finally, we asked whether the IFNγ-resistant melanoma cells were addicted to the cytokine. As depicted in Figure 5c, the xCELLigence real-time proliferation assay revealed continuous proliferation of IFNγ-resistant Ma-Mel-86c and UKE-Mel-103a cells in the presence or absence of IFNγ, excluding a dependency of the IFNγ-resistant cell lines to the cytokines (**Figure 5c**). As expected, the IFNγ-treated parental cell lines show a decrease in proliferation compatible with the level of IFNγ-induced apoptosis measured in Figure 2a.

Overall, these data suggest IFNγ can induce EMT-like phenotype switching in melanoma, associated with a resistance to IFNγ-induced apoptosis. The EMT phenotype might be involved in the attenuation of the IFNγ signaling pathway, which requires further investigation.

Discussion

We report here that non-genomic mechanisms shifting melanoma cells towards a dedifferentiated cell phenotype are associated with resistance to IFNγ-induced apoptosis. IFNγ has been demonstrated to be a major mediator of anti-tumor immune responses by induction of cell cycle arrest and apoptosis of melanoma cells (18). Its critical role in anti-tumor immunity indicates the importance to understand mechanisms of resistance to IFNγ.

Starting our analysis with cell lines derived from two different lesions of patient Ma-Mel-86, we observed that dedifferentiated $MITF^{low}$ Ma-Mel-86a cells with an EMT-like phenotype were intrinsically resistant to IFNγ-induced apoptosis, while the differentiated $MITF^{high}$ Ma-Mel-86c cells were highly sensitive. Interestingly, resistance to the BRAFi Vemurafenib followed the same pattern. Of the two $BRAF^{V600E}$ mutant melanoma cell lines, Ma-Mel-86a was resistant to BRAFi-induced cell death whereas Ma-Mel-86c died under inhibitor treatment. Phenotype-dependent resistance to targeted therapy has previously been described (19), suggesting that this could potentially be a mechanism of non-genomic resistance towards IFNγ in melanoma cell lines.

To test this hypothesis, we induced IFNγ-resistance in six patient-derived cell lines. The parental cells were IFNγ sensitive, i.e. the majority of the cells went into apoptosis in the presence of IFNγ while some cells survived. The persisting cells were continuously cultured in the presence of IFNγ and resumed proliferation after weeks or months. By RNA-seq we determined the transcriptomes of the parental IFNγ-sensitive cells, the parental cells treated short-term with IFNγ and the corresponding IFNγ-resistant cells (permanently under IFNγ). An integrated data analysis revealed that IFNγ signaling was downregulated in the IFNγ-resistant cell lines while an EMT-like program was upregulated. These findings are in line with previously shown resistance signatures form patients receiving immune checkpoint blocking therapy. Hugo et al., determined the so called IPRES (Innate PD-1 Resistance) signature, which defines a transcriptional reprogrammed cell state characterized by an elevated expression of genes related to mesenchymal transition, extracellular matrix remodeling, angiogenesis, and wound healing in the resistant tumors (5). In fact, the hallmark of EMT gene signature of our IFNγ-resistant melanoma cells partially matches the mesenchymal transition gene signature from Hugo et al. (AXL, ROR2, **WNT5A**, LOXL2, **TWIST2**, TAGLN, **FAP**, matching genes in

bold) (5). Our results are in line also previous publications linking acquired resistance to immunotherapy and stimulation with TNFα to inflammation-induced dedifferentiation of malignant cells (20,21). TNFα together with interleukin-6 (IL-6) and transforming growth factor-beta (TGF-β), can promote dedifferentiation and EMT of melanoma and multiple cancers entities (20-23). Reactome pathway analysis (12,13) also revealed an enrichment of genes involved in collagen degradation and Interleukin-4 and Interleukin-13 signaling, the first being essential for EMT and the latter shown to be involved in alternative activation of macrophages and wound healing mechanisms (24-26), further validating the GSEA outcome. We confirmed the phenotype switching induced by IFNγ also at the protein level, demonstrating downregulation of E-cadherin, a hallmark of EMT, together with an upregulation of WNT5A, EGFR and SOX9, which are already described to play a role in the development of drug resistance and EMT-like phenotype (5,16,17,27,28). Accordingly, the IFNγ-resistant cell lines were also more invasive than their corresponding parental cell lines *in vitro*.

The switching of melanoma cells from a proliferative towards an invasive cell state has previously been associated with resistance to targeted therapy (19,29,30). Interestingly, acquired IFNγ resistance of *BRAF*^{V600E} mutant Ma-Mel-86c cells was linked also to acquired BRAFi resistance, but this preliminary result requires further validation in additional model systems. Moreover, we cannot absolutely exclude the contribution of genetic alterations to IFNγ-induced melanoma phenotype switching which will be addressed in future studies.

While the specific mechanisms underlying this switch between a differentiated to a more dedifferentiated phenotype are still unclear, our results describe a so far unknown role of IFNγ in the induction of dedifferentiation in melanoma cell lines, to gain resistance and invasive properties.

In the future, we will also need to confirm our results on large patient cohorts under immunotherapy. This would show whether a more dedifferentiated phenotype of tumor cells is associated with lack of response to immunotherapy. Our findings might contribute to the definition of a predictive signature for immunotherapy, currently needed to stratify patients in responders and non-responders.

Figure Legend

Figure 1. Differences in IFNγ- and BRAFi-induced apoptosis among distinct melanoma cell lines.

(a) Clinical history of patient Ma-Mel-86. Horizontal line, time axis; arrows indicates cell lines established from metastases

(b) Melanoma cell Ma-Mel-86a and -86c analyzed by Western blot for expression of differentiation antigens MITF, Melan-A, AXL, c-Jun; GAPDH, loading control. Representative data from 3 independent experiments.

(c) IFNγ-induced apoptosis in Ma-Mel-86a and -86c cells determined by AnnexinV/PI staining after 7 days treatment. Percentage of early (AnnV+) and late apoptotic (AnnV+/PI+) cells shown. Mean values (+ s.e.m.) from n=3 independent experiments. Statistical differences defined by paired Student's t-test, ** $P \leq 0.01$

(d) Ma-Mel-86a and -86c cells analyzed by Western blot for expression of the IFNγ signaling pathway components STAT1, pSTAT1 (Y), IRF1 after IFNγ treatment (7 days); GAPDH, loading control. Representative data from 3 independent experiments.

(e) Vemurafenib-induced apoptosis in Ma-Mel-86a and -86c cells determined by AnnexinV/PI staining after 7days treatment. Percentage of early (AnnV+) and late apoptotic (AnnV+/PI+) cells shown. Mean values (+ s.e.m.) from n=3 independent experiments. Statistical differences defined by paired Student's t-test, * $P \leq 0.05$.

Figure 2. Transcriptional IFNγ-signaling pathway activation in melanoma cells.

(a) IFNγ-induced apoptosis in Ma-Mel-61a, Ma-Mel-86c, UKE-Mel-103a, UKE-Mel-105, UKE-Mel-118c and UKE-Mel-130b cells determined by AnnexinV/PI staining after 7 days of treatment. Percentage of early (AnnV+) and late apoptotic (AnnV+/PI+) cells shown. Mean values (+ s.e.m.) from n=3 independent experiments. Statistical differences defined by paired Student's t-test, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, *** $P \leq 0.0001$

(b) Schematic of the generation of IFNγ-resistant melanoma cell variants. IFNγ-sensitive cell lines were treated with IFNγ (500 U/ml) for 7 days (short-term treatment). Surviving cells (intermediate state) were cultured under continuous IFNγ (500 U/ml) treatment (long-term treatment) to obtain IFNγ-resistant cells. Transcriptomes from IFNγ-sensitive cells, intermediate state-surviving cells and resistant cells were analyzed by RNA sequencing.

(c) Hallmark pathways from GSEA analysis defined by fgsea R package. Ranked differentially expressed gene sets detected in sensitive non-treated versus short-term (7 days) treated cells (Sens_7d_IFNg vs Sens_untr sets). Normalized enrichment scores given as colored bar indicating whether or not the pathway is significantly altered. Blue = True, significant with padj < 0.05; Red= False, non-significant with padj > 0.05. List of hallmark pathways from GSEA, ordered by NES (normalized enrichment score) and showing p-values (pval), adjusted p-values (padj), NES and the number of genes matching the hallmark pathway (size).

Figure 3. EMT signature genes enriched and IFNγ-signaling pathway genes downregulated in transcriptomes of melanoma cells with acquired IFNγ-resistance.

(a) Schematic and formula (see Material and Methods for further information) applied to determine the gene signatures associated with IFNγ resistance. Hallmark pathways from GSEA analysis defined by fgsea R package. Ranked resistance genes run in fgsea. Normalized enrichment scores given as colored bar indicating whether or not the pathway is significantly altered. Blue = True, significant with padj < 0.05; Red= False, non-significant with padj > 0.05.
(b) List of hallmarks significantly altered pathways (padj < 0.05) from GSEA, ordered by normalized enrichment score (NES) and showing p-values (pval), adjusted p-values (padj), NES and the number of genes matching the hallmark pathway (size).
(c) List of resistance genes matching the GSEA EMT hallmark
(d) Melanoma cells Ma-Mel-61a, Ma-Mel-86c, UKE-Mel-103a, UKE-Mel-105, UKE-Mel-118c and UKE-Mel-130b analyzed by Western blot for expression of the IFNγ signaling pathway components pSTAT1 (Y), STAT1, IRF1; GAPDH, loading control. Parental cell lines were treated with IFNγ for 7 days or left untreated; IFNγ-resistant cells are always kept continuously under IFNγ. Representative data from 3 independent experiments.

Figure 4. IFNγ-resistant Ma-Mel-86c and UKE-Mel-103a cells show a mesenchymal-like cell shape.

(a) Hallmark pathways from GSEA analysis defined by fgsea R package and found altered in Ma-Mel-86c and UKE-Mel-103a cells separately analyzed. NES given as colored bar indicating whether or not the pathway is significantly altered Blue = True, significant with padj < 0.05; Red= False, non-significant with padj > 0.05.

(b) Morphology of Ma-Mel-86c and UKE-Mel-103a cells documents by light microscopy. Parental cell lines were treated with IFNγ for 7 days or left untreated; IFNγ-resistant cells are continuously kept under IFNγ.

Figure 5. Ma-Mel-86c and UKE-Mel-103a IFNγ-resistant cell lines are more invasive and show EMT-specific markers.

(a) Real-time invasion of Ma-Mel-86c and UKE-Mel-103a cells determined on the xCELLigence platform. Representative data from n=2 independent experiments.

(b) Ma-Mel-86c and UKE-Mel-103a cells analyzed by Western blot for expression of the EMT and differentiation markers: Fibronectin, E-cadherin, c-Jun, AXL, MITF, MelanA (MLANA), EGFR, WNT5A, SOX9, SOX10; GAPDH, loading control. Parental cell lines were treated with IFNγ for 7 days or left untreated; IFNγ-resistant cells were kept continuously under IFNγ stimulation. Representative data from 3 independent experiments.

(c) Real-time proliferation of Ma-Mel-86c and UKE-Mel-103a cells determined on the xCELLigence platform. Both IFNγ-sensitive and IFNγ-resistant cell lines were cultured in the presence or absence of IFNγ. Addition of IFNγ after 24 h from seeding. Representative data from n=2 independent experiments.

Figure 1

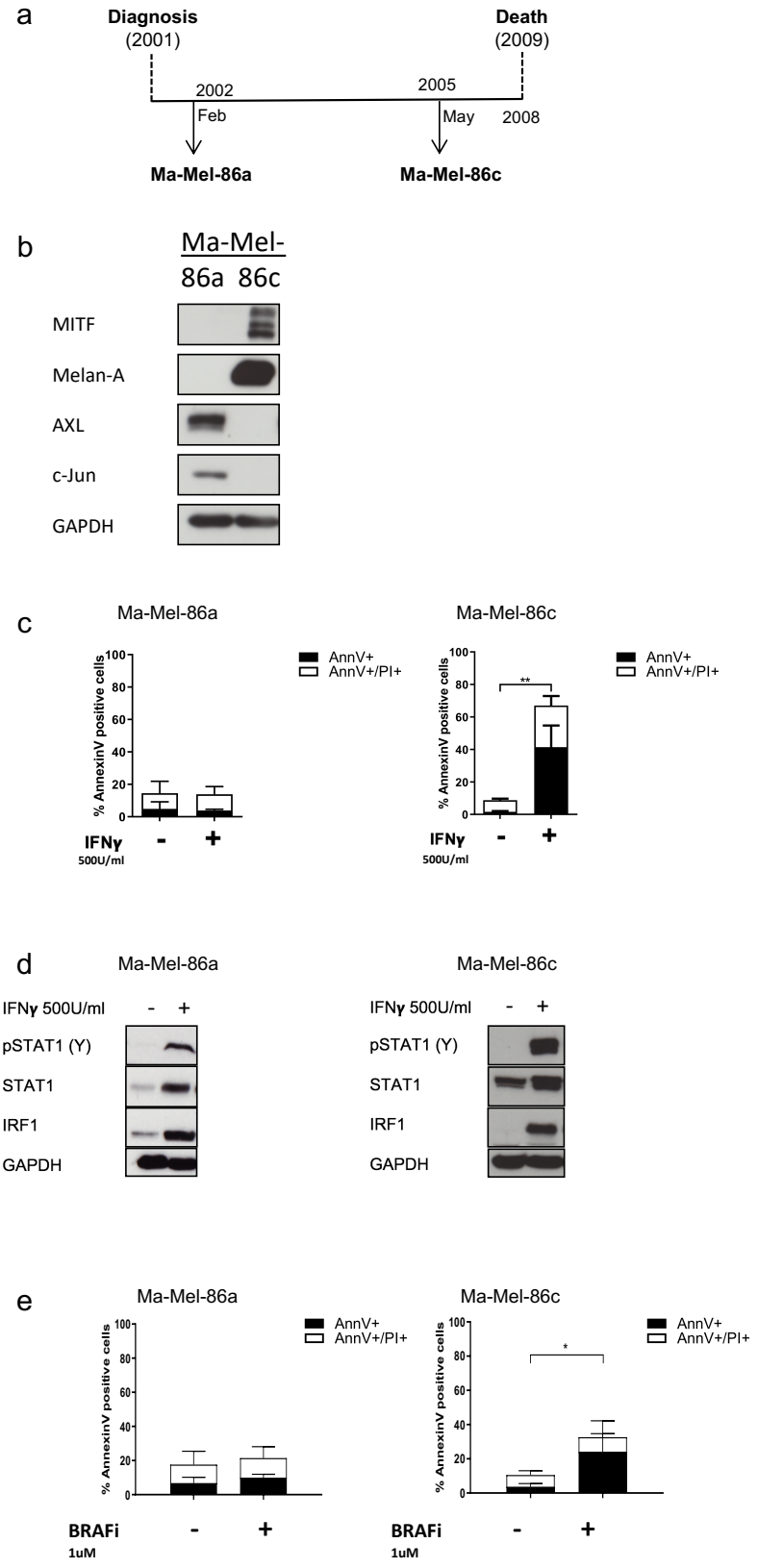
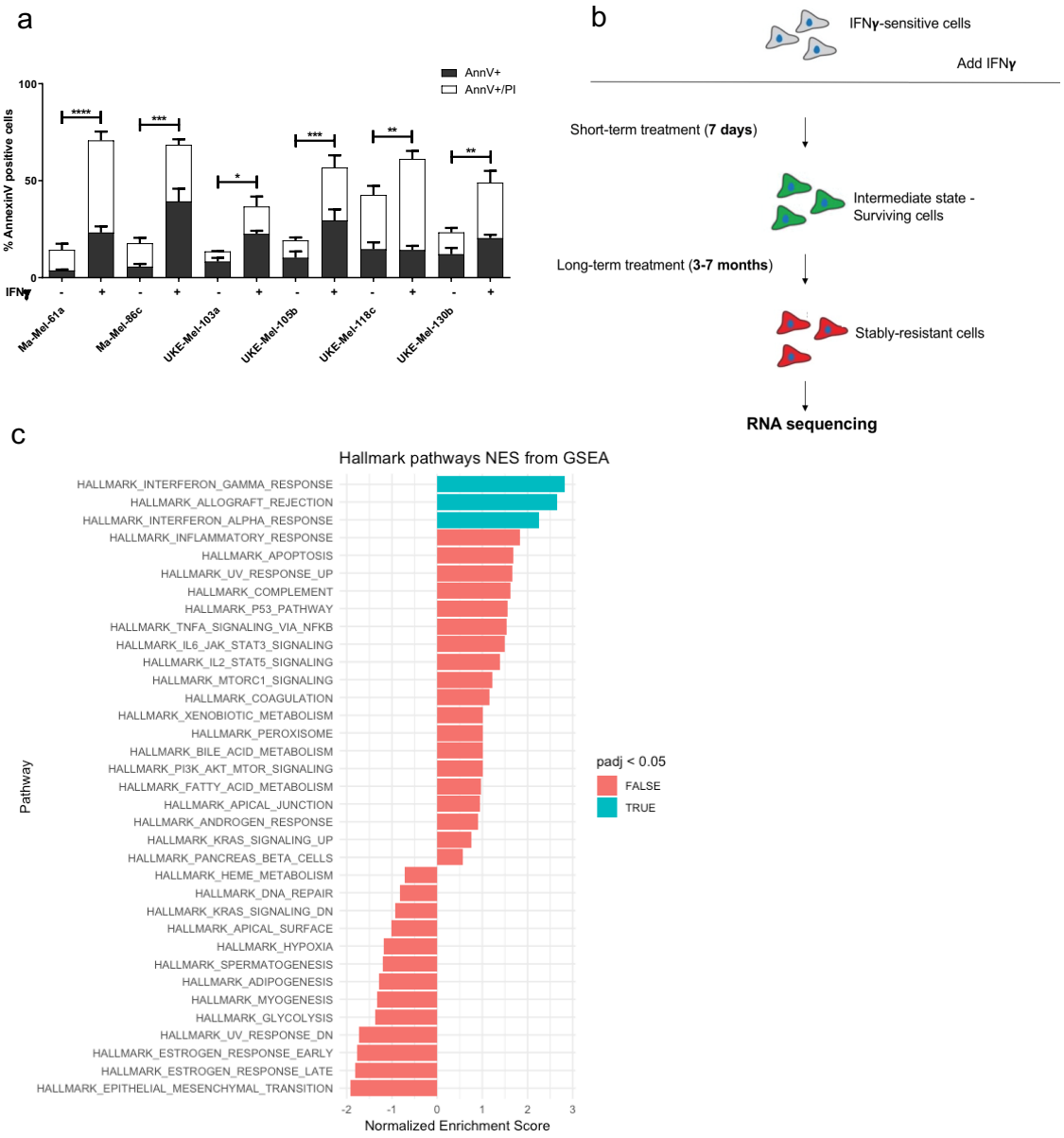

a
Diagnosis
(2001)
Death
(2009)
2002
Feb
2005
May
2008
Ma-Mel-86a
Ma-Mel-86c
b
Ma-Mel-
86a 86c
MITF
Melan-A
AXL
c-Jun
GAPDH
c
Ma-Mel-86a
Ma-Mel-86c
% AnnexinV positive cells
AnnV+
AnnV+/PI+
IFNγ
500U/ml
-
+
**
d
Ma-Mel-86a
Ma-Mel-86c
IFNγ 500U/ml
pSTAT1 (Y)
STAT1
IRF1
GAPDH
e
Ma-Mel-86a
Ma-Mel-86c
BRAFi
1uM
*

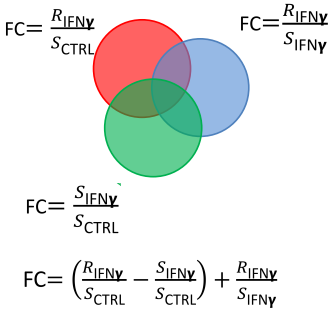
Figure 2

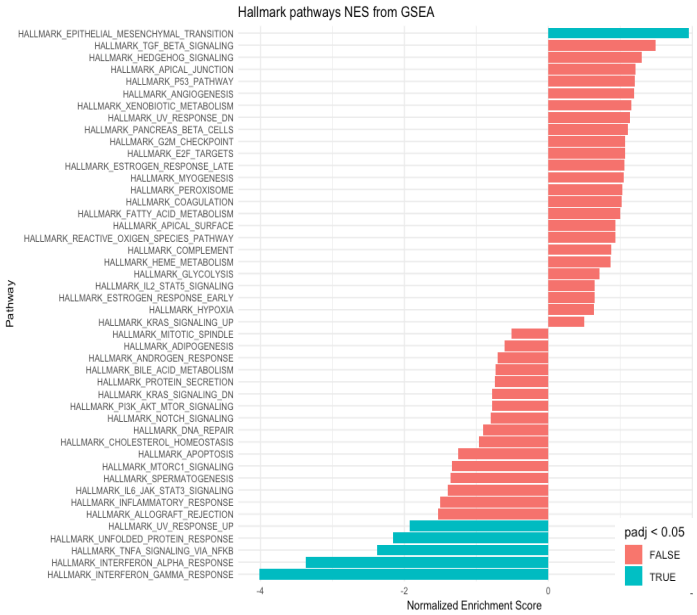


	pathway	pval	padj	NES	size
1	HALLMARK_INTERFERON_GAMMA_RESPONSE	0.0011111111111111	0.0138888888888889	2.82758481820799	50
2	HALLMARK_ALLOGRAFT_REJECTION	0.00119047619047619	0.0138888888888889	2.64709588296239	29
3	HALLMARK_INTERFERON_ALPHA_RESPONSE	0.00119047619047619	0.0138888888888889	2.25789068200807	29
4	HALLMARK_INFLAMMATORY_RESPONSE	0.00670241286863271	0.0586461126005362	1.82560006958604	12
5	HALLMARK_APOPTOSIS	0.0125195618153365	0.0756961340902947	1.69563713238681	4
6	HALLMARK_UV_RESPONSE_DN	0.0194647201946472	0.0756961340902947	-1.73669959356241	3
7	HALLMARK_ESTROGEN_RESPONSE_EARLY	0.0170316301703163	0.0756961340902947	-1.76150958775616	3
8	HALLMARK_ESTROGEN_RESPONSE_LATE	0.0165289256198347	0.0756961340902947	-1.80680592636847	4
9	HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	0.0179856115107914	0.0756961340902947	-1.92498728033167	10
10	HALLMARK_UV_RESPONSE_UP	0.0273141122913505	0.0955993930197269	1.65671725968666	5

Figure 3

a





b

pathway	pval	padj	NES	size
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	0.001312335958005 25	0.0343283582089552	1.95534298404269	44
HALLMARK_INTERFERON_GAMMA_RESPONSE	0.00373134328358209	0.0343283582089552	-4.01070079139651	32

c

COL3A1	LUM	POSTN	SDC1	MYL9	NTM	VCAN	BGN	SFRP4
COL5A2	LOXL2	THY1	PCOLCE	TGFB1	CXCL12	TGFBI	FSTL1	TGM2
COL5A1	THBS2	TFPI2	TNFRSF11B	APLP1	PDGFRB	MGP	FBN2	EDIL3
FBN1	NNMT	SPOCK1	EFEMP2	PTX3	SLIT2	WNT5A	ANPEP	SLIT3
COL1A1	COL11A1	VCAM1	PCOLCE2	DCN	RGS4	COL1A2	FAP	

d

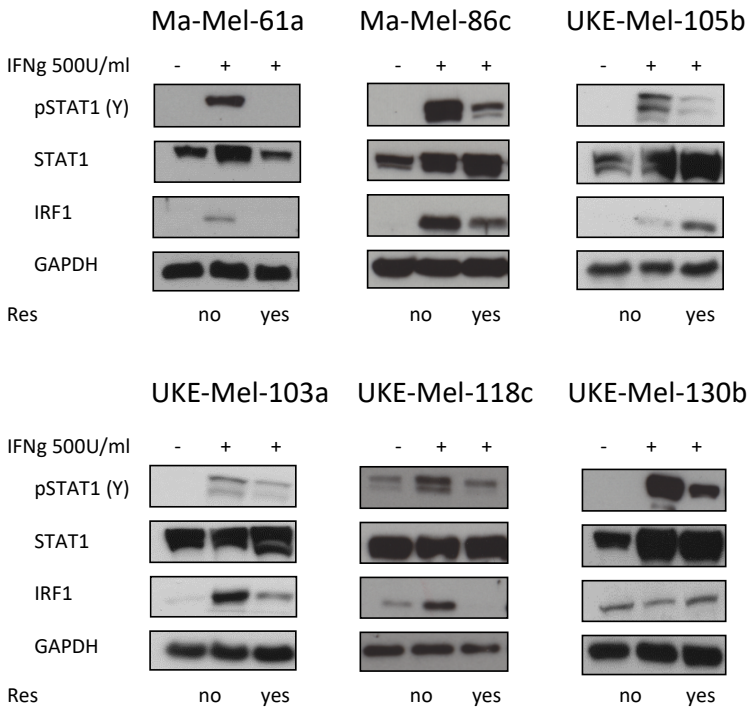
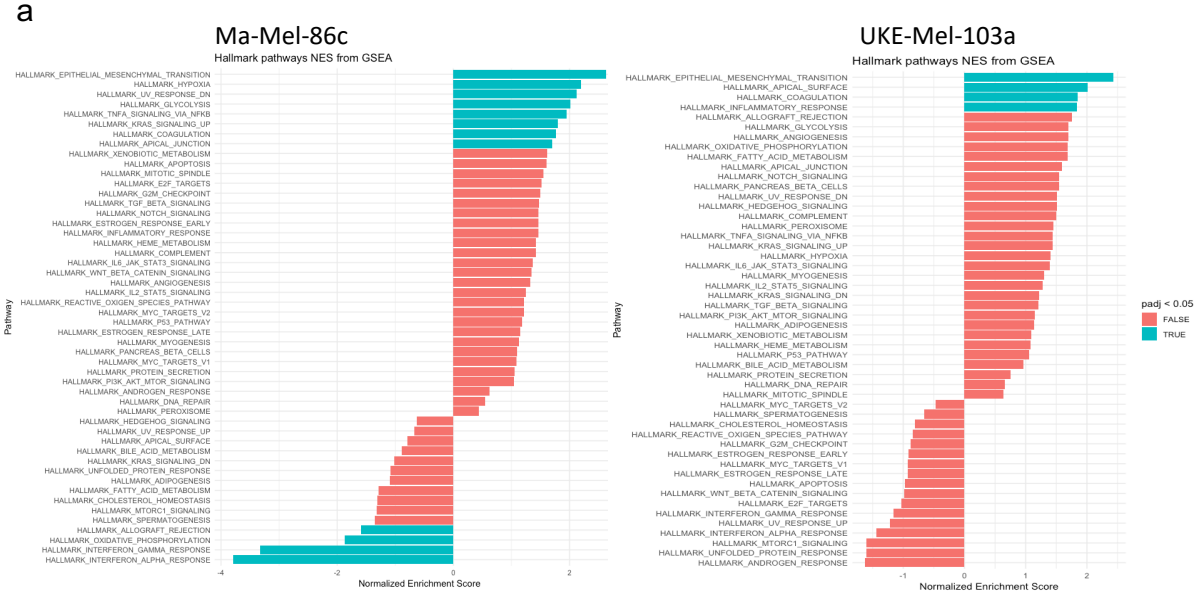


Figure 4

a


Ma-Mel-86c
Hallmark pathways NES from GSEA
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION
HALLMARK_HYPOXIA
HALLMARK_UV_RESPONSE_DN
HALLMARK_GLYCOLYSIS
HALLMARK_TNFA_SIGNALING_VIA_NFKB
HALLMARK_KRAS_SIGNALING_UP
HALLMARK_COAGULATION
HALLMARK_APICAL_JUNCTION
HALLMARK_XENOBIOTIC_METABOLISM
HALLMARK_APOPTOSIS
HALLMARK_MITOTIC_SPINDLE
HALLMARK_E2F_TARGETS
HALLMARK_G2M_CHECKPOINT
HALLMARK_TGF_BETA_SIGNALING
HALLMARK_NOTCH_SIGNALING
HALLMARK_ESTROGEN_RESPONSE_EARLY
HALLMARK_INFLAMMATORY_RESPONSE
HALLMARK_HEME_METABOLISM
HALLMARK_COMPLEMENT
HALLMARK_IL6_JAK_STAT3_SIGNALING
HALLMARK_WNT_BETA_CATENIN_SIGNALING
HALLMARK_ANGIOGENESIS
HALLMARK_IL2_STAT5_SIGNALING
HALLMARK_REACTIVE_OXIGEN_SPECIES_PATHWAY
HALLMARK_MYC_TARGETS_V2
HALLMARK_P53_PATHWAY
HALLMARK_ESTROGEN_RESPONSE_LATE
HALLMARK_MYOGENESIS
HALLMARK_PANCREAS_BETA_CELLS
HALLMARK_MYC_TARGETS_V1
HALLMARK_PROTEIN_SECRETION
HALLMARK_PI3K_AKT_MTOR_SIGNALING
HALLMARK_ANDROGEN_RESPONSE
HALLMARK_DNA_REPAIR
HALLMARK_PEROXISOME
HALLMARK_HEDGEHOG_SIGNALING
HALLMARK_UV_RESPONSE_UP
HALLMARK_APICAL_SURFACE
HALLMARK_BILE_ACID_METABOLISM
HALLMARK_KRAS_SIGNALING_DN
HALLMARK_UNFOLDED_PROTEIN_RESPONSE
HALLMARK_ADIPOGENESIS
HALLMARK_FATTY_ACID_METABOLISM
HALLMARK_CHOLESTEROL_HOMEOSTASIS
HALLMARK_MTORC1_SIGNALING
HALLMARK_SPERMATOGENESIS
HALLMARK_ALLOGRAFT_REJECTION
HALLMARK_OXIDATIVE_PHOSPHORYLATION
HALLMARK_INTERFERON_GAMMA_RESPONSE
HALLMARK_INTERFERON_ALPHA_RESPONSE
Pathway
-4
-2
0
2
Normalized Enrichment Score
UKE-Mel-103a
Hallmark pathways NES from GSEA
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION
HALLMARK_APICAL_SURFACE
HALLMARK_COAGULATION
HALLMARK_INFLAMMATORY_RESPONSE
HALLMARK_ALLOGRAFT_REJECTION
HALLMARK_GLYCOLYSIS
HALLMARK_ANGIOGENESIS
HALLMARK_OXIDATIVE_PHOSPHORYLATION
HALLMARK_FATTY_ACID_METABOLISM
HALLMARK_APICAL_JUNCTION
HALLMARK_NOTCH_SIGNALING
HALLMARK_PANCREAS_BETA_CELLS
HALLMARK_UV_RESPONSE_DN
HALLMARK_HEDGEHOG_SIGNALING
HALLMARK_COMPLEMENT
HALLMARK_PEROXISOME
HALLMARK_TNFA_SIGNALING_VIA_NFKB
HALLMARK_KRAS_SIGNALING_UP
HALLMARK_HYPOXIA
HALLMARK_IL6_JAK_STAT3_SIGNALING
HALLMARK_MYOGENESIS
HALLMARK_IL2_STAT5_SIGNALING
HALLMARK_KRAS_SIGNALING_DN
HALLMARK_TGF_BETA_SIGNALING
HALLMARK_PI3K_AKT_MTOR_SIGNALING
HALLMARK_ADIPOGENESIS
HALLMARK_XENOBIOTIC_METABOLISM
HALLMARK_HEME_METABOLISM
HALLMARK_P53_PATHWAY
HALLMARK_BILE_ACID_METABOLISM
HALLMARK_PROTEIN_SECRETION
HALLMARK_DNA_REPAIR
HALLMARK_MITOTIC_SPINDLE
HALLMARK_MYC_TARGETS_V2
HALLMARK_SPERMATOGENESIS
HALLMARK_CHOLESTEROL_HOMEOSTASIS
HALLMARK_REACTIVE_OXIGEN_SPECIES_PATHWAY
HALLMARK_G2M_CHECKPOINT
HALLMARK_ESTROGEN_RESPONSE_EARLY
HALLMARK_MYC_TARGETS_V1
HALLMARK_ESTROGEN_RESPONSE_LATE
HALLMARK_APOPTOSIS
HALLMARK_WNT_BETA_CATENIN_SIGNALING
HALLMARK_E2F_TARGETS
HALLMARK_INTERFERON_GAMMA_RESPONSE
HALLMARK_UV_RESPONSE_UP
HALLMARK_INTERFERON_ALPHA_RESPONSE
HALLMARK_MTORC1_SIGNALING
HALLMARK_UNFOLDED_PROTEIN_RESPONSE
HALLMARK_ANDROGEN_RESPONSE
Pathway
-1
0
1
2
Normalized Enrichment Score
padj < 0.05
FALSE
TRUE

b

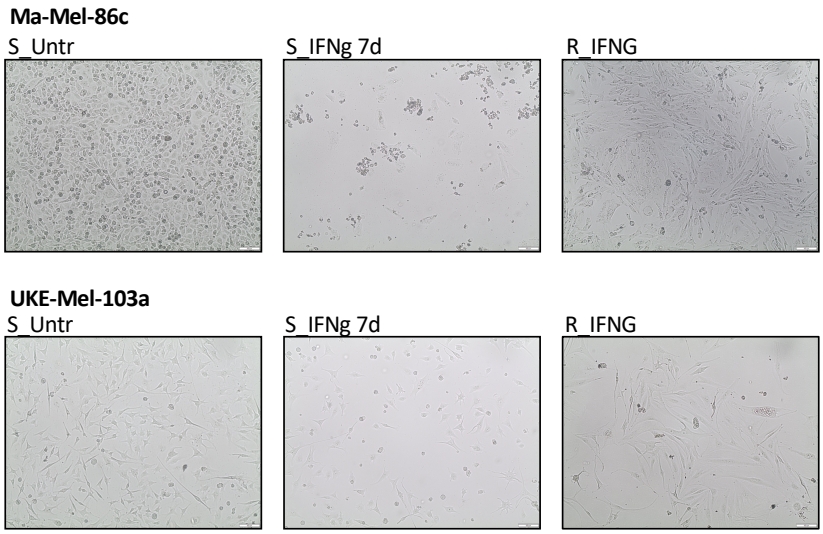
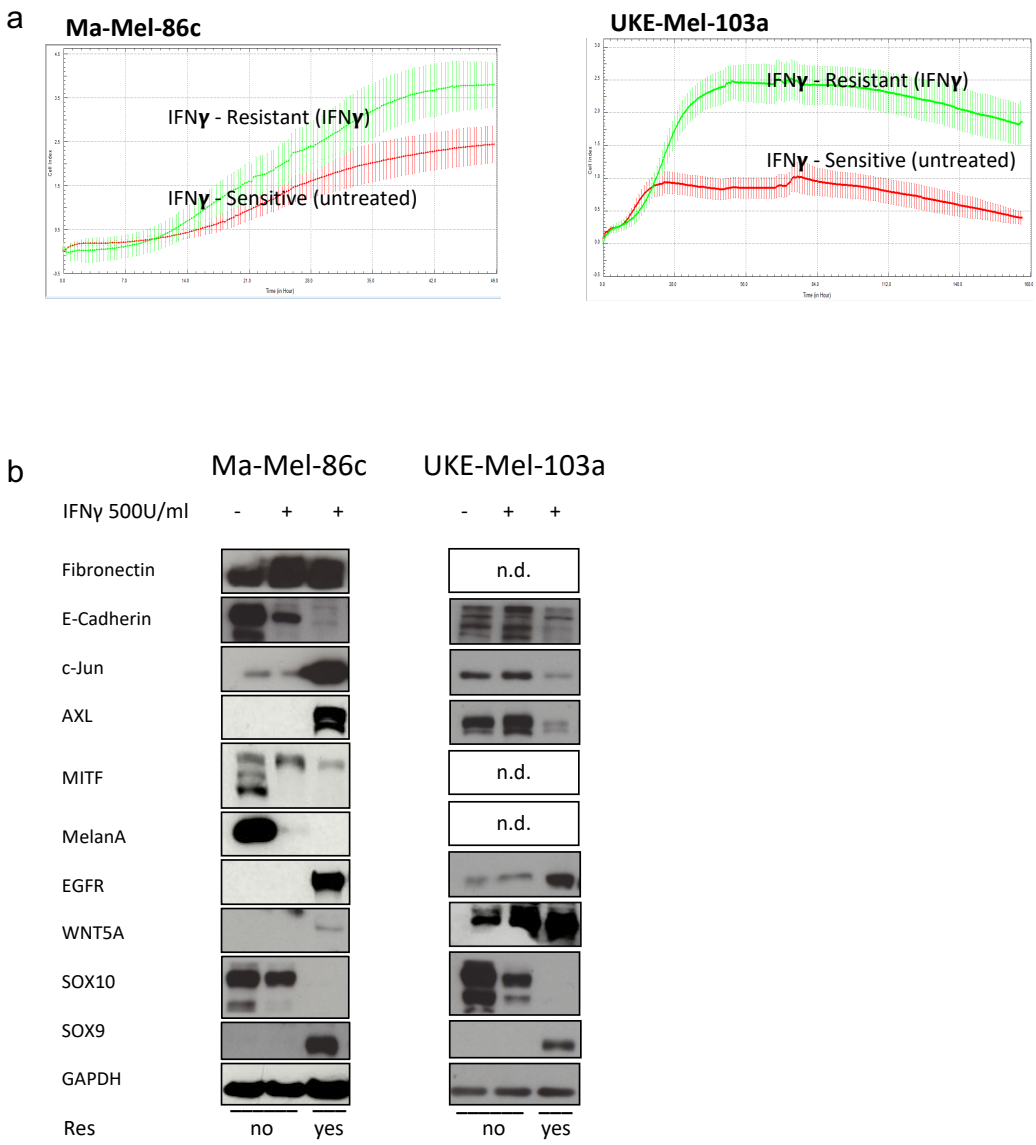

Ma-Mel-86c
S_Untr
S_IFNg 7d
R_IFNG
UKE-Mel-103a
S_Untr
S_IFNg 7d
R_IFNG

Figure 5


a
Ma-Mel-86c
IFNγ - Resistant (IFNγ)
IFNγ - Sensitive (untreated)
UKE-Mel-103a
IFNγ - Resistant (IFNγ)
IFNγ - Sensitive (untreated)
b
Ma-Mel-86c
UKE-Mel-103a
IFNγ 500U/ml - + + - + +
Fibronectin
E-Cadherin
c-Jun
AXL
MITF
MelanA
EGFR
WNT5A
SOX10
SOX9
GAPDH
n.d.
n.d.
n.d.
Res
no yes
no yes
c
Ma-Mel-86c
UKE-Mel-103a
IFNγ-S untreated
IFNγ-S IFNγ 500U 7d
IFNγ-R IFNγ 500U
IFNγ-R untreated 7d

Supplementary Figures

Figure S1. Reactome pathway analysis of IFNγ-resistance signature.

Figure S2. Ma-Mel-86c IFNγ-resistant cells show cross-resistance to BRAF inhibitor Vemurafenib.

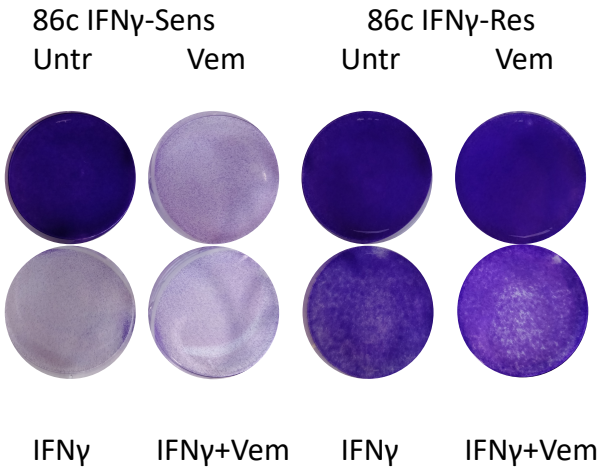
(a) Viability crystal violet assay on Ma-Mel-86c. Parental cell line and IFNγ-resistant cells were treated with IFNγ 500U/ml (IFNγ), Vemurafenib 1uM (Vem), the combination of the two treatments (IFNγ +Vem) for 7 days or left untreated (Untr).

Figure S1

The following table shows the 25 most relevant pathways sorted by p-value.

Pathway name	Entities				Reactions	
	found	ratio	p-value	FDR*	found	ratio
Collagen degradation	18 / 76	0.004	3.56e-06	0.003	21 / 34	0.003
Collagen chain trimerization	12 / 44	0.002	4.65e-06	0.003	9 / 28	0.002
Interleukin-4 and Interleukin-13 signaling	46 / 365	0.018	5.13e-06	0.003	32 / 46	0.004
Assembly of collagen fibrils and other multimeric structures	17 / 74	0.004	1.10e-05	0.004	22 / 26	0.002
ECM proteoglycans	20 / 116	0.006	5.12e-05	0.016	15 / 23	0.002
Phase 2 - plateau phase	9 / 44	0.002	5.72e-04	0.1	1 / 2	1.64e-04
Collagen formation	25 / 142	0.007	5.73e-04	0.1	54 / 77	0.006
Degradation of the extracellular matrix	27 / 205	0.01	5.78e-04	0.1	56 / 105	0.009
Collagen biosynthesis and modifying enzymes	20 / 107	0.005	5.93e-04	0.1	32 / 51	0.004
Defective B3GALT6 causes EDSP2 and SEMDJL1	6 / 21	0.001	8.85e-04	0.122	1 / 1	8.19e-05
Defective B4GALT7 causes EDS, progeroid type	6 / 21	0.001	8.85e-04	0.122	1 / 1	8.19e-05
Defective B3GAT3 causes JDSSDHD	6 / 22	0.001	0.001	0.131	1 / 1	8.19e-05
LGI-ADAM interactions	5 / 15	7.46e-04	0.001	0.131	4 / 5	4.10e-04
Anchoring fibril formation	5 / 15	7.46e-04	0.001	0.131	1 / 4	3.28e-04
Molecules associated with elastic fibres	8 / 40	0.002	0.001	0.132	8 / 10	8.19e-04
Crosslinking of collagen fibrils	6 / 24	0.001	0.002	0.165	13 / 13	0.001
Presynaptic depolarization and calcium channel opening	5 / 17	8.45e-04	0.002	0.185	1 / 2	1.64e-04
Elastic fibre formation	9 / 55	0.003	0.003	0.219	15 / 17	0.001
Phase 0 - rapid depolarisation	10 / 70	0.003	0.004	0.322	2 / 2	1.64e-04
Dermatan sulfate biosynthesis	4 / 13	6.46e-04	0.005	0.375	4 / 4	3.28e-04
Neutrophil degranulation	38 / 480	0.024	0.007	0.524	10 / 10	8.19e-04
Prostanoid ligand receptors	4 / 15	7.46e-04	0.008	0.557	3 / 8	6.55e-04
Transport of glycerol from adipocytes to the liver by Aquaporins	2 / 3	1.49e-04	0.011	0.695	2 / 2	1.64e-04
Defective CHST3 causes SEDCJD	3 / 9	4.48e-04	0.012	0.695	1 / 1	8.19e-05
Defective CHST14 causes EDS, musculocontractural type	3 / 9	4.48e-04	0.012	0.695	1 / 1	8.19e-05

Figure S2


86c IFNγ-Sens
Untr
Vem
86c IFNγ-Res
Untr
Vem
IFNγ
IFNγ+Vem
IFNγ
IFNγ+Vem

Methods

Cell culture and treatment

Melanoma cell lines (Ma-Mel-61a, Ma-Mel-86a, Ma-Mel-86c, UKE-Mel-103a, UKE-Mel-105b, UKE-Mel-118c, UKE-Mel-130b) were cultured in RPMI1640 or DMEM medium with L-glutamine (Gibco/Life technologies), 10% fetal calf serum and 1% Penicillin/Streptomycin. Cell lines were confirmed to be mycoplasma-free in monthly intervals. Cells were seeded and rested overnight followed by the addition of IFNγ (500U/ml, Boehringer Ingelheim), or left untreated. To obtain the resistant strains, media was changed, and cytokine was given every 3 days for several months, before obtaining cell lines being able to grow under continuous IFNγ (500U/ml) treatment. Cells were also treated with Vemurafenib (1 µM BRAFi, Selleckchem) for 7 days for apoptosis assay and crystal violet assay.

Western blot

Proteins from tumor cell lysates were separated by SDS–polyacrylamide gel electrophoresis, blotted on nitrocellulose membranes and probed with the following primary antibodies: anti-AXL (Cell Signaling, clone C89E7, 1:5000), anti-c-JUN (Cell Signaling, clone 60A8, 1:1000), anti-E-Cadherin (Cell Signaling, clone 24E10, 1:500), anti-EGFR (Cell Signaling, clone D28B1, 1:1000), anti-Fibronectin (Santa Cruz, clone 59824, 1:200), anti-GAPDH (Cell Signaling, clone 14C10, 1:5000), anti-IRF1 (Santa Cruz, clone H-205, 1:500), anti-MITF (Sigma-Aldrich, clone C5, 1:1000), anti-Melan-A (Zytomed, anti-MART-1, clone M2-7C10, 1:5000), anti-pSTAT1 (Y701) (Cell Signaling, clone 58D6, 1:1.000), anti-SOX10 (Cell Signaling, clone D5V9L, 1:1000), anti-STAT1 (Santa Cruz, clone M-22, 1:1000), anti-SOX9 (Cell Signaling, clone D8G8H, 1:1000), anti-WNT5A (Cell Signaling, clone C27E8, 1:1000). After washing with PBS-Tween, membranes were incubated with the corresponding secondary antibodies linked to horseradish peroxidase. Antibody binding was visualized with the enhanced chemiluminescence (ECL) system.

Flow cytometry

Floating and adherent cells were harvested and resuspended in binding buffer, with Annexin-V-APC (BD Biosciences, Franklin Lakes, USA, 1:20) and Propidium Iodide (BD Biosciences, Franklin Lakes, USA, 1:10). Cells were incubated for 15 min at room temperature and the apoptosis was measured on a Gallios flow cytometer

(Beckmann Coulter, Brea, USA) and data were analyzed applying the Kaluza software (Beckmann Coulter, Brea, USA).

RNA sequencing and analysis

Messenger RNA was sequenced from all cell lines above mentioned. RNA was sequenced in triplicate, from untreated samples, treated samples with 500U/ml IFNγ for 7 days, resistant samples continuously in 500U/ml IFNγ. RNA was extracted from snap-frozen cell pellets using the QIAGEN RNeasy Mini Kit. 1 µg of total RNA per sample was submitted for sequencing. HiSeq 4000 75bp Paired-end sequencing was carried out at the Wellcome Trust Sanger Institute, Hinxton, Cambridgeshire, UK. All samples ran across 12 lanes. Kallisto (Version v0.44.0) pseudo-aligner (31) was used for quantification of transcript abundance estimates and aligned on human reference genome assembly GRCh37. R statistical software was used to perform differential expression analysis with DESeq2 to identify the resistance gene signature (RGS), using this formula:

$$\text{RGS} = \left(\frac{\mathrm{R}_{IFNg}}{\mathrm{S}_{\mathrm{CTRL}}} - \frac{\mathrm{S}_{IFNg}}{\mathrm{S}_{\mathrm{CTRL}}}\right) + \frac{\mathrm{R}_{IFNg}}{\mathrm{S}_{\mathrm{IFNg}}}$$

Where:

$\frac{\mathrm{R}_{IFNg}}{\mathrm{S}_{\mathrm{CTRL}}}$ represents genes whose expression increased/decreased in IFNγ-resistant cells and upon IFNγ administration in IFNγ-sensitive

$\frac{\mathrm{S}_{IFNg}}{\mathrm{S}_{\mathrm{CTRL}}}$ represents genes whose expression increased/decreased upon drug administration only in IFNγ-sensitive

$\frac{\mathrm{R}_{IFNg}}{\mathrm{S}_{\mathrm{IFNg}}}$ represents genes whose expression increased/decreased in IFNγ-resistant cells. In this group, the information about the initial level of untreated IFNγ-sensitive cell lines gene expression is missing.

To define the resistance gene signature, only DEGs with padj < 0.05 and log2 fold change bigger or equal to the absolute value of 1.5 were kept. 820 unique DEGs were used to run GSEA for pathway analysis of the general resistance gene signature from of all cell lines together.

Pathways analysis

Fast Gene Set Enrichment Analysis (fgsea) (https://bioconductor.org/packages/release/bioc/html/fgsea.html) is a Bioconductor R-package for fast pre-ranked gene set enrichment analysis (GSEA), allowing more permutations and obtaining more fine-grained p-values. Reactome (http://reactome.org) is a free, open-source, manually curated and peer-reviewed knowledge-base of biomolecular pathways.

Real-time proliferation assay (xCELLigence)

50 µl medium was added in each well of an E-Plate 96 (Roche) for measuring background noise. The, 3000 melanoma cells in 100 µl medium were seeded. Attachment of the cells was monitored using the RTCA SP (Roche) instrument and the RTCA software Version 1.2.1 (Roche). After 20–24 h cells were treated with IFNγ 500U/µl by adding 50µl additional volume or left untreated, for 7 days. All experiments were performed in duplicates. Cell index value indicated the changes in electrical impedance, indicating the cellular coverage of the electrode sensors, normalized to baseline impedance values measured with medium only.

Real-time invasion assay (xCELLigence)

50 µl medium was added in each well of a CIM-Plate 96 (Roche) for measuring background noise. Approximately 4 hours before seeding the cells in the upper chamber (UC), it was coated with 1:40 solution of Matrigel™ (BD Bioscience) per well. 50 µl serum-free medium was added on top of the Matrigel™ (BD Bioscience) in each well for measuring background noise, then a total of 100.000 cells per well were seeded in 100 µl serum-free medium. 160 µl complete medium was added to the lower chamber (LC). RTCA SP (Roche) instrument and the RTCA software Version 1.2.1 (Roche) automatically measured the impedance value of each well for 7 days and expressed it as a cell index number.

Crystal Violet Assay

Adherent cells were fixed in the 6-well-plates with formaldehyde 4% for 30 min at room temperature and then incubated with 0.5% Crystal Violet working solution, previously diluted in 20% ethanol, for 1 hour. The coloring solution was then washed with distilled water and plates were left to dry overnight.

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Author contributions

SL performed all the *in vitro* experiments and sequencing analysis. BT helped with the *in vitro* experiments. FZ established the Ma-Mel-86c IFNγ-resistant cell line. DS established patient models. AP led the project. SL and AP designed experiments and wrote the manuscript.

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5. Discussion

5.1. Mutational landscape of conjunctival melanoma

Conjunctival melanoma (CoM) is a rare type of eye melanoma (0.24-0.8 cases per million population in North Europe and the USA). In the past, the rarity of this disease complicated the analysis of genetic alterations contributing to its development (31). Griewank et al. were the first to sequence 78 CoM samples, the largest cohort ever analyzed in this field at the time, finding a panel of primary mutations in *BRAF* (V600E mutation in more than 90% of *BRAF*-mutated tumors) in 29% of samples and *NRAS* mutation in 18% of the tumors analyzed (32). In the same work, Griewank et al. analyzed genome-wide DNA copy number alterations in 30 CoM with array-based comparative genomic hybridization (CGH), showing that losses of chromosome (chr.) 1p, 3, and 6q and gains of 6p and 8q resemble the ones found in cutaneous melanomas (32,180). Over the last few years, the discovery of new treatments for cutaneous melanoma patients, as targeted therapies against mutations in MAPK pathway components (BRAFi and MEKi) and immunotherapy boosting anti-tumor T cell responses greatly helped the management of this disease. (84,144,145,181). A better understanding of the development of cutaneous melanoma and its mutation profile has generated knowledge that became the basis for the development of new treatments.

In our work (182), we have thoroughly described the mutational background characterizing CoM, following and confirming the previous work from Griewank et al. (32). We analyzed 67 CoM samples, with the aim to elucidate the genetic alterations behind this rare disease. By using targeted next-generation sequencing (NGS), we discovered *NF1* mutations in 21 of 63 (33%) CoM. *NF1* mutations in CoM are described in this work for the first time. Mutations in the *NF1* gene occur also in 14% of skin melanomas (13).

Activating mutations in *BRAF* were found in 25% of samples, confirming previous results (32,180). We also described activating mutations in *NRAS* in 18% of samples and discovered one sample with $KRAS^{G12A}$ mutation. The *KRAS* mutation in CoM was reported for the first time in our study. *KRAS* mutations are very rare also in cutaneous melanoma and mutually exclusive with *NRAS* mutations in cutaneous melanoma (13). Activating mutations in *BRAF*, *RAS* and loss-of-function mutations in *NF1* lead to unrestrained cell proliferation and tumor growth in tumor cells, because

of constitutive activation of the MAPK signaling pathway. From this analysis of the mutational landscape of CoM, we propose here a 4-group classification, based on the most commonly occurring mutations in CoM, into *BRAF*-mutated, *RAS*-mutated, *NF1*-mutated, and triple WT tumors, similarly to skin melanoma (13).

Previously, many other studies highlighted the genetic similarities between CoM and cutaneous melanoma (32,34,35,183).

Based on the fact that both *BRAF* and *RAS* mutant tumors are dependent on the MAPK signaling, as well as *NF1* mutant melanoma (184), our classification of CoM will have important implications for therapy. We can speculate that MAPKi targeted therapies could be beneficial for around 50% of patients with CoM, carrying mutations in *BRAF* and *RAS* (32,33). Recently, a patient with metastatic CoM received for the first time combination therapy of MAPKi. The patient is reported to have achieved a clinical benefit, with no reoccurrence after 1 year of starting the targeted therapies (185). This study shows that therapeutic benefit could be achieved by treating CoM patients with MAPKi. Nevertheless, broader studies are necessary to get better insights on the effect of targeted therapied on CoM confirm the possible benefits of this therapy in CoM patients.

In our work, we report 33% of CoM having *NF1* mutations. Studies have shown that *NF1* mutant cutaneous melanomas are resistance to BRAF inhibitors both *in vitro* and *in vivo* (186,187). Nevertheless, these tumors have a higher tumor burden and respond better to immune checkpoint blockade (ICB), as they have a higher number of neoantigens (95,188–190). These data suggest that *NF1*-mutant CoM patients may benefit from ICB therapy. Last year, Sagiv et al. have reported of a small group of patients with metastatic CoM (n=5) treated with anti-PD1 inhibitor nivolumab (4/5 patients), showing complete response at 36 months after completing treatment. One patient was treated with a second anti-PD-1 antibody, pembrolizumab, and showed stable disease in the first 6 months of treatment, but disease progression after 11 months (191).

Overall, our work confirms the genetic similarities between CoM and skin melanoma. In our work, we discover new mutations, important for a reliable classification of CoM. For the first time, our study finds mutations in *NF1* in CoM patients, occurring in 33% of samples analyzed. Also, previously reported mutations in *BRAF* and *NRAS* genes were here reported with a frequency of 25% and 18%, respectively. We classify CoM in four different categories, based on the most commonly occurring mutations, *BRAF*-mutant, *RAS*-mutant, *NF1*-mutant and triple-WT tumors, further confirming the

similarities between CoM and skin melanoma. This genetic classification of CoM will hopefully favor the use of targeted therapies and immunotherapies for CoM patients in the future.

5.2. *JAK2* status as biomarker for immunotherapy resistance

When T cells are activated by the recognition of melanoma cells or by ICB therapy, they secrete IFNγ and other cytokines. As an important effector molecule of an immune response (192), IFNγ can induce apoptosis in the target and neighboring cells by binding to its receptor IFNGR1/2 on the tumor cells. The binding causes the activation of the JAK/STAT/IRF1 pathway in the cells which stimulates the expression of response genes causing cell death (102–104). However, some clones can escape cytokine-induced apoptosis (193). Previous studies have shown how different genomic aberrations can favor resistant clones against T cell-mediated killing, making immunotherapy inefficient in the long run (114,160). IFNγ-resistant melanoma cells can evolve under immunotherapy, by acquiring chromosomal alterations and mutations in genes of the IFNγ pathway, especially in *JAK1* and *JAK2* (114,160). Loss of one allelic copy of *JAK2* is not enough to block the IFNγ signaling pathway in the tumor cells, but IFNγ-resistant melanoma clones might develop under the selective pressure of an active immune response or under immunotherapy, by acquiring a mutation in the remaining functional allele.

As described in Horn, Leonardelli et al. (179), we were able to identify that chromosomal loss of the *CDKN2A* tumor suppressor gene increases the susceptibility to acquired IFNγ resistance. *CDKN2A* allele losses are found in the majority of melanomas, with a frequency of 50 to 80%. Loss of *CDKN2A*, due to large chromosomal alteration of the chr. 9p, is an early event in the development of melanoma (194–196). *JAK2* gene locus is also found on chr. 9p, close to the *CDKN2A* locus. Our work demonstrates that *CDKN2A*-associated *JAK2* loss predisposes melanoma to IFNγ resistance. We detected a 75% frequency of *CDKN2A*-associated *JAK2* losses in cell lines established from metastatic lesions of different patients and a similar frequency in the skin cutaneous melanoma cohort of the TCGA. All cancers we analyzed from TCGA (melanoma, lung squamous cell carcinoma, bladder urothelial carcinoma, breast invasive carcinoma, and colorectal adenocarcinoma) showed a significant increase in the proportion of samples carrying

losses in both *CDKN2A* and *JAK2* together, compared to the samples with only one of the two genes lost. Furthermore, in the melanoma and lung squamous cell carcinoma cohort we found mutations and homozygous deletions in *JAK2* are only present in the samples with concurrent deletions of *JAK2* and *CDKN2A*. Based on these observations, we proposed a model of development of resistance to IFNγ and immune checkpoint inhibitors following the "two-hit" hypothesis of cancer evolution (98,179,197).

We are not the first to describe the association between copy number loss, melanoma progression and ICB response. Previous studies have shown that copy number alteration (CNA) burden increases during melanoma progression (9,163,194,196). Roh et al. reported that a higher CNA burden is associated with non-responders to anti-CTLA-4 therapy. Furthermore, many tumor suppressor genes are found within the lost alleles, suggesting that loss of big chromosomal regions is a benefit for tumor development and progression (163).

Our study is in line with this work, confirming that the allelic loss of chr. 9p might help the tumor to develop resistance under immunotherapy. Given that the majority of tumor samples we analyzed in TCGA are metastatic lesions with a simultaneous and associated loss in *CDKN2A* and *JAK2*, we propose here that screening for genetic defects in *JAK2* in patients before immunotherapy and during the course of therapy might be informative. Detecting *JAK2* mutations by liquid biopsy on circulating cell-free tumor DNA (ctDNA) would be a non-invasive way to monitor the *JAK2* mutation during the course of treatment. Liquid biopsy is a method of detection of circulating tumor cells (CTCs) or nucleic acids (as ctDNA) found in the blood. CTCs are freed from the tumor mass and can be found in a very small number in the blood circulation of cancer patients, and ctDNA is released from dying normal and tumor cells. ctDNA monitoring is a plasma-based assay developed in the last few years which can provide real-time information on disease progression and treatment response (198–201). Following the somatic *JAK2* mutations by ctDNA monitoring would be a non-invasive way to track the development of resistance cells under treatment.

Our study has nevertheless limitations. Here we show data from patient-derived cell lines and TCGA samples. Analysis of patient samples obtained over time and the course of immunotherapy would be helpful to confirm our findings.

Overall, in this study we conclude that detection of *JAK2* mutations could help identify patients having a higher risk of developing resistance, possibly avoiding useless costs and inadequate therapy for unresponsive patients. Moreover, monitoring *JAK2*

status during the course of therapy (for example, by ctDNA) could give central information regarding the evolution of the tumor mutations and tumor resistance.

5.3. Phenotypic plasticity is the basis of resistance to IFNγ

Many patients with advanced cutaneous melanoma respond successfully to immunotherapy, still acquired resistance results in minimal residual disease, and, at last, tumor relapse. A minimal residual disease might arise because of mutations. Gao et al. showed that defects in the interferon pathway genes reduce the chance of response to ICB. Melanoma patients who lack respond to ICB (ipilimumab) harbor a higher rate of alterations in the genes of IFNγ signaling pathway, as *JAK2*, *IFNGR1*, *IFNGR2*, *IRF1*, compared to the responding patients. Defects and mutations in the IFNγ pathway genes are associated with the absence of response to anti-CTLA-4 treatment (98).

Nevertheless, non-genomic mechanisms are also known to contribute to immunotherapy resistance (54,202,203). Our study aimed to elucidate non-genomic resistance mechanisms to IFNγ and study the transcriptional changes of different patient models by RNA-seq. To do so, cells were treated with IFNγ over several months to obtain induced IFNγ-resistant cell lines. The obtained resistance gene signature showed enrichment of genes involved in mesenchymal transition, together with a downregulation of IFNγ-response genes.

Several studies have reported that melanoma cells can be in either a proliferative or invasive state, controlled by the transcription factor MITF (65,202). MITF was previously described as a “rheostat”, defining melanoma cell state characterized by high proliferation and differentiation, and opposing a dedifferentiated and invasive phenotype (60,168,204,205). This model is called “phenotype switching”, which is a process of phenotypic and dynamic changes resembling epithelial to mesenchymal transition (EMT) (49,68). Previously, Hugo et al. associated resistance to ICB to a transcriptional program related to EMT, angiogenesis, hypoxia and wound healing, defining the so-called innate anti-PD-1 resistance (IPRES) signature (54). Furthermore, Landsberg et al. demonstrated *in vivo* that inflammation-induced de-differentiation of tumor cells contributes to resistance to adoptive T cell transfer (ACT). This was further confirmed *in vitro* by treating melanoma cells with TNFα (122). Accordingly, Mehta et al. also showed the involvement of TNFα during

inflammation-induced de-differentiation in a metastatic melanoma patient and *in vitro* (124). Inflammatory cytokines were shown in the past to induce adaptive immune resistance in different cancers. TNFα mediates dedifferentiation of melanoma cells and, together with IL-6 and TGF-β induces EMT in multiple cancers (97,118,122,124).

Activated CD8+ T cells, which are specifically stimulated by immunotherapy, predominantly secrete IFNγ rather than TNFα (177). To our knowledge, we are the first to show that IFNγ alone can induce a dedifferentiated phenotype in association with resistance to IFNγ-mediated killing. Even though our work shows only *in vitro* the involvement of IFNγ in the induction of a dedifferentiated phenotype, our results are in line with those from Reinhardt et al. of an HCmel3 melanoma-bearing mice treated with an ACT protocol. This *in vivo* model shows that late relapsed melanomas have a downregulation of IFNγ response genes and, at the same time, a downregulation of differentiation-specific targets (123).

In the future, we aim to confirm our results in a patient cohort treated with immunotherapy. This would allow us to investigate how the IFNγ signature correlates with a dedifferentiation signature to understand whether patients with high tumor infiltration ($IFN\gamma^{high}$) and high dedifferentiation state are more likely to be non-responders to immunotherapy. Furthermore, we cannot exclude that mutations might contribute to the EMT-like switch and downregulation of response to IFNγ, in the IFNγ-resistant cell lines. In the future, we intend to perform exome sequencing to understand if any mutation acquired by the IFNγ-resistant cell lines might have a role in this switch toward dedifferentiation.

Overall, in our work we were able to demonstrate *in vitro* that IFNγ alone can mediate a transition toward a dedifferentiated and invasive cell state of melanoma cells, as an acquired resistance mechanism to IFNγ-mediated apoptosis. Together with other studies (123,124), our data confirms the role of an inflammatory microenvironment in the induction of dedifferentiation of tumor cells. Modulating this inflammation might have a positive impact on patient response to immunotherapy (206).

5.4. Cross-resistance to immunotherapy and targeted therapy

High phenotypic heterogeneity and instability of the genome make monotherapy difficult to succeed. Lately, many clinical trials started to study the efficacy of

combined targeted therapy and immunotherapy in melanoma (https://clinicaltrials.gov). The rationale behind the combination is that targeted therapy typically achieves rapid effects on the anti-tumor immunity, and therefore could synergize with immunotherapy further boosting the immune system to attack the tumor. For example, BRAFi therapy has been shown to increase antigen and MHC expression, infiltration of T cells in the tumor and reduce immunosuppressive cytokines. These factors correlate with a better response to immunotherapy (207–210). Recently, Pieper et al. demonstrated in vitro that long-term targeted therapy with BRAFi/MEKi strongly altered antigen expression on melanoma cells. This resulted in impaired antigen recognition by T cells, making combination treatment with immunotherapies ineffective. Yet, short-time treatment with targeted therapy seems to enhance expression of differentiation antigens, allowing a better recognition by T cells, suggesting an improved outcome for patients treated simultaneously with targeted treatment and immunotherapy (211).

On the other hand, Hugo et al. showed that MAPKi-resistant melanomas lose CD8+ T cell numbers and function and loose antigen presentation; likewise, anti-PD-1 resistant melanoma display transcriptomic signature that are similarly induced in MAPKi resistant cells, suggesting the existence of a common resistance program, giving a survival advance to the cells in response of both immunotherapy and targeted therapies (54,55,114).

In line with these findings, our work confirms that IFNγ-resistant cell lines might also become resistant to BRAFi treatment (vemurafenib), and we show the Ma-Mel-86c cell line as an example (206). In the future, we will screen more *BRAF*^{V600E} cell lines for cross-resistance to both IFNγ and vemurafenib, to further validate this result.

Our current knowledge of mechanisms of cross-resistance between targeted therapy and immunotherapy is limited; large-scale analyses are needed to reveal mechanisms of adaptation and therapy resistance. Finding these mechanisms and identifying common targets will help our understanding on how to effectively target, and eventually kill, the tumors.

5.5. The role of next generation sequencing in cancer research

In the past years, NGS technologies made it possible to study in-depth the genomes and the transcriptomes of tumors, reveal their heterogeneity, follow their development and understand the effect of anti-cancer therapies.

These new technological advances have opened up new avenues in clinical practice. Mutation analysis can help physicians to better classify tumors and prescribe suitable treatment (165,212–215).

DNA of sample of interest can be sequenced in different ways. Whole-genome sequencing (WGS) studies the entire genome, whole-exome sequencing (WES) gives information only on the protein-coding regions of the genome. Targeted sequencing focuses on specific regions of the genome based on relatively few specific genes of interest. Although targeted NGS does not allow for the identification of new loci as WGS and doesn't produce a large amount of data as both WGS and WES, it can be an excellent way to study candidate genes known to be associated with the disease of interest. In Scholz et al., we exploit targeted genome profiling covering 29 genes known to be mutated in cutaneous and/or uveal melanoma. By doing so, we firstly assessed the high similarities between CoM and cutaneous melanoma, based on the mutational landscape, while confirming that CoM and uveal genetically differ. Targeted sequencing allowed us to discovery for the first time mutations in *NF1*, highly common in CoM but never reported before. Secondly, we were able to genetically define and classify CoM into *BRAF*-mutated, *RAS*-mutated, *NF1*-mutated and triple-wild-type (WT) tumors. This classification will have great impact on therapeutic-oriented decisions for CoM patients. From our analysis of CoM we can conclude that therapy available for skin melanoma could have a positive impact on the treatment and survival of CoM patients (182).

In Horn, Leonardelli et al., we were able to understand how immunotherapy resistance evolve during melanomagenesis, thanks to single nucleotide polymorphism (SNP) array for the analysis of copy number variation that we performed on our patient sample and the copy number data provided by TCGA (http://www.cbioportal.org).

SNP array was first designed to genotype DNA at different SNPs across the genome simultaneously, but it allows also the detection of changes in a number of allele copies, called copy number variations (CNVs) (216). Thanks to the application of SNP array to the study of CNVs, we are able to study the deletions of chr. 9p in

melanoma patient sample from our cohort and from TCGA melanoma cohort, to demonstrate the associated deletion of *CDKN2A* and *JAK2* allele losses. This co-deletion, happening early during melanomagenesis, renders tumors more susceptible to become resistant to IFNγ and ICB later in the course of the disease (179).

Recently, we were also able to better understand acquired resistance to IFNγ and immunotherapy at the transcriptional level, using RNA-seq on IFNγ-resistant melanoma cells (206). Bulk RNA-seq is a high-throughput technology that provides information on the whole transcriptome of the sample analyzed. It is mostly used to analyze gene expression and transcriptome changes between different conditions. We exploited this technique to define the transcriptional signature contributing to acquired resistance to IFNγ and ICB, matching an upregulation EMT-like gene signature together with a downregulation of IFNγ response in the resistant cells by Gene Set Enrichment Analysis (GSEA).

To date, many research groups are still trying to understand how and why only a subgroup of patients respond to ICB. Still, there is no appropriate biomarker available. High-throughput CRISPR screenings and single cell technologies as single cell RNA-seq are recently been employed to identify specific genes associated with ICB response and explore mechanisms driving resistance to therapy at a single-cell level (41,42,217–222). Even though these new NGS technologies are helping to understand the mechanisms behind resistance to therapy, there is still a long way ahead in order to find reliable biomarkers for response to immunotherapy in melanoma and in other cancer entities.

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List of Figures

List of Abbreviations

αMSH	Melanocyte-stimulating hormone
ACT	Adoptive cell transfer
ALL	Acute lymphoblastic leukaemia
AML	Acute myeloid leukaemia
APC	Antigen presenting cell
ARID	AT-rich interaction domain
B2M	Beta-2-microglobulin
BAP1	BRCA1-associated protein 1
BCL2	B-cell CLL／lymphoma 2
BRAF	v-Raf murine sarcoma viral oncogene homolog B1
BRAFi	BRAF inhibitor
cAMP	Cyclic adenosine monophosphate
CDK2	Cyclin-dependent kinase 2
CDK4	Cyclin-dependent Kinase 4
CDKN2A	Cyclin-dependent kinase inhibitor 2A
chr.	Chromosome
CLL	Chronic lymphocytic leukaemia
CNA	Copy number alteration
CNV	Copy number variation
CoM	Conjunctival melanoma
CREB	CAMP responsive element binding protein
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DCT	Dopachrome Tautomerase

E-cadherin	Epithelial cadherin
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EIF1AX	Eukaryotic translation initiation factor 1A
EMT	Epithelial to mesenchymal transition
FDA	Food and Drug Administration
GF	Growth factor
GNA11	G Protein Subunit Alpha 11
GNAQ	G Protein Subunit Alpha Q
gp100	Glicoprotein100
GSEA	Gene Set Enrichment Analysis
HIF1α	Hypoxia-inducible factor 1-alpha
ICB	Immune checkpoint blockade
ICGC	International cancer genome consortium
IFNGR	IFNγ-receptor
IFNγ	Interferon gamma
IL-6	Interleukin-6
IPRES	Innate PD-1 Resistance
JAK	Janus-kinase
KIT	KIT Proto-Oncogene, Receptor Tyrosine Kinase
MAPK	Mitogen-activated-protein-kinase
MAPKi	MAPK inhibitor
MC1R	Melanocortin 1 receptor
MDM2	MDM2 Proto-Oncogene

MEKi	MEK inhibitor
MET	MET Proto-Oncogene
MHC-	Major histocompatibility complex
MITF	Microphthalmia-associated transcription factor
MLANA	Melan-A
MMP	Metalloproteases
mTOR	Mammalian-target-of-rapamycin
N-cadherin	Neural cadherin
NCSC	Neural crest stem cell
NF1	Neurofibromin 1
NGS	Next-generation sequencing
NRAS	Neuroblastoma RAS viral oncogene homolog
NSCLC	Non-small-cell lung carcinoma
OS	Overall survival
PAX3	Paired Box 3
PD-1	Programmed cell death protein 1
PDGFRβ	Platelet-derived growth factor receptor
PFS	Progression-free survival
PI3K	Phosphoinositide-3-kinase
PKC	Protein kinase C
POU3F2	POU Class 3 Homeobox 2
PTEN	Phosphatase-and-tensin homologue
RCC	Renal cell carcinoma
RNA-seq	RNA sequencing

RTK	Receptor tyrosine kinases
SF3B1	Splicing factor 3b subunit 1
SOX9	Sex Determining Region Y – Box 9
SOX10	Sex Determining Region Y – Box 10
STAT	Transducer and activator of transcription
TCGA	The Cancer Genome Atlas
TERT	Telomerase reverse-transcriptase
TGFβ	Transforming growth factor beta
TIL	Tumor infiltrating lymphocyte
TKR	Tyrosine kinase receptor
TNFα	Tumor necrosis factor alpha
TP53	Tumor-protein p53
TWIST	Twist family BHLH transcription factor
TYR	Tyrosinase
UV	Ultraviolet
WES	Whole exome sequencing
WGS	Whole genome sequencing
WT	Wild-type
YAP	Yes-associated protein 1
ZEB	Zinc-finger E-box-binding

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Publications

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Eidesstattliche Erklärungen

Bestätigung Eigenanteil an Publikationen

Hiermit bestätige ich die Darstellung zu den Anteilen von Frau Sonia Leonardelli an Konzeption, Durchführung und Abfassung jeder Publikation (Chapter 3 - Publications) gemäß der Promotionsordnung der Fakultät für Biologie zur Erlangung des Doktorgrades Dr. rer. nat

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Hiermit erkläre ich, Sonia Leonardelli, dass ich mit der Veröffentlichung der Publikationen (Chapter 3) im Rahmen dieser Dissertation keine Urheberrechte verletze. Das Einverständnis der entsprechenden Journale wurde eingeholt

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Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. (2) g) der Promotionsordnung der Fakultät für Biologie zur Erlangung der Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema “Genomic and non-genomic alterations in melanoma contributing to disease progression and therapy resistance” zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von Sonia Leonardelli befürworte und die Betreuung auch im Falle eines Weggangs, wenn nicht wichtige Gründe dem entgegenstehen, weiterführen werde.

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