Genetic dependencies in hereditary and sporadic melanoma

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Chapter 1

General Introduction

CUTANEOUS MALIGNANT MELANOMA PREFACE

The word 'melanoma', according to Hippocrates back in the fifth century BC, originates from the ancient Greek adjective 'μέλᾱς', meaning 'black' and the suffix 'ώμᾱ' referring to a tumor; although it was first described as a disease entity by René Theophile Hyacinthe Laënnec in 1812 [8].

Cutaneous Melanoma (CM) develops from malignant transformation of melanocytes, the pigment producing cells residing in our skin. CM is one of the deadliest types of skin cancer due to its high metastatic propensity. Although considerable efforts have been employed to effectively eliminate the disease, incidence rates of CM are increasing considerably worldwide. Approximately 232,100 CM cases are diagnosed and about 55,500 deaths are reported annually [9]. The incidence and mortality rates of CM vary per geographic location although the highest incidence rates are reported for Caucasian populations due to fair skin color [10].

Specifically in the Netherlands, melanoma of the skin is the 5th most common cancer type with 6,189 cases reported in 2017 as well as 796 deaths according to the Dutch Cancer Registry [11, 12]. Most melanoma cases are diagnosed early, at a localized stage and are reported with a two-year survival rate of 96% [11]. Survival of metastatic melanoma however, remains poor in spite of introduction of novel immune and targeted therapies [13].

ENVIRONMENTAL RISK FACTORS AND CLASSIFICATION

To better understand CM development we may consider melanoma as a multi-factorial disease arising from an interplay of genetic and environmental risk factors. An important environmental risk factor for melanoma development is strong intermittent exposure to ultraviolet (UV) radiation and sunburn at a young age [14]. Artificial exposure to UV radiation through tanning bed sessions for cosmetic purposes may also be associated with an increased risk for CM development [15, 16]. UV radiation mainly causes genetic alterations in the skin through direct DNA damage, mainly formation of pyrimidine dimers, resulting in mutations that may drive malignant transformation of expanding keratinocytes and melanocytes [17]. At the molecular level, UV increases skin pigmentation through stimulation of the melanocortin 1 receptor (MC1R) on the surface of melanocytes by its ligand α-melanocyte-stimulating (α-MSH). This mediates production of melanin, the main defense mechanism against UV radiation-induced damage [18, 19]. Germline variants in the *MC1R* gene are associated with fair skin and these individuals have a lower capacity of activating *MC1R*, associated with increased susceptibility to melanoma [20, 21].

CM may be classified into two types depending on UV-exposure duration and genetic signatures; The chronically affected sun-damaged areas such as head and neck and non-chronically affected sun damaged areas such as the trunk, legs and arms (CSD and non-CSD respectively) [3, 22, 23]. The most common non-CSD subtypes are superficial spreading melanoma (SSM) and nodular melanoma (NM) whereas lentigo maligna melanoma (LMM) is a common CSD subtype [24, 25]. An un-common sub-type of melanoma in Caucasian populations is Acral Lentiginous Melanoma (ALM), with only 5% reported cases. ALM is a frequent subtype of melanoma in Asian, African and Hispanic populations [26].

MUTATIONAL SIGNATURE OF CM

Melanocytes are neural crest-derived cells which not only migrate to the skin but also different parts of the body such as the eyes and mucosal areas during development. [27, 28]. These melanocytes can give rise to different types of melanoma, including mucosal and uveal in addition to cutaneous melanoma.

Primary CMs are not only found *de novo* but can develop from precursor lesions such as a common melanocytic nevus, an atypical melanocytic nevus or a lentigo maligna (Figure 1). Approximately 30% of CMs are derived from a common melanocytic nevus, although the percentage in high-risk individuals is higher, reaching 50% [29, 30].

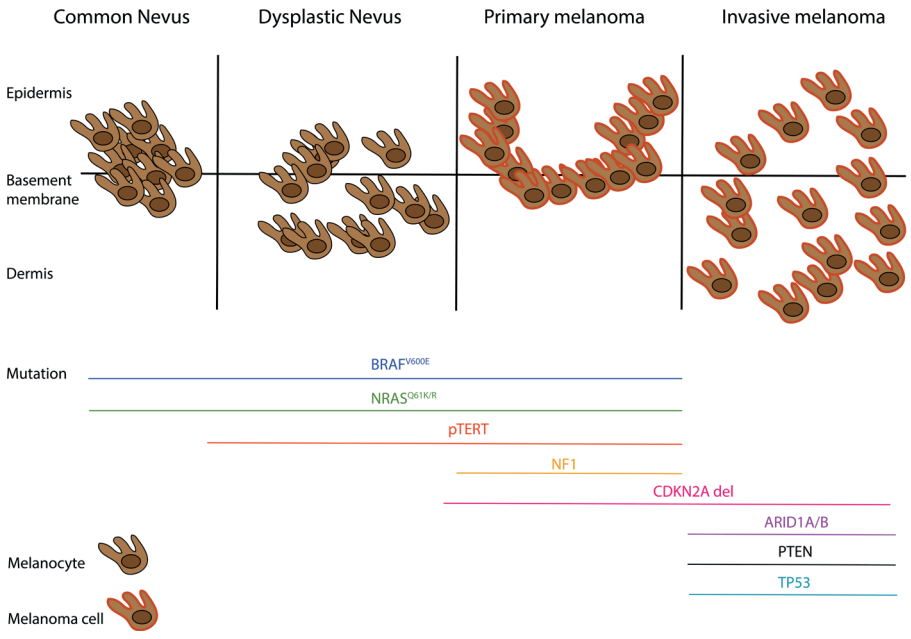

Figure 1 Genetic evolution of cutaneous melanoma. Simplistic model of genetic evolution of cutaneous melanoma (CM) and the underlying common genetic alterations describing precursor lesions (common nevus), intermediate lesions (dysplastic nevus), primary and invasive melanoma. Source data were adapted from the following resources: [1-4].

A complex network of events contributes to CM development and considerable efforts have been employed to enhance our understanding of the different molecular pathways involved. The most frequently hyperactivated signaling pathway in melanoma development is the mitogen-activated protein kinase (MAPK) pathway that mediates transcription of proliferative genes and cell growth. Several oncogenes and tumor suppressor genes are involved in melanoma pathogenesis. The three most common MAPK-activating mutations in two oncogenes are *BRAF*^{V600E} and *NRAS*$^{Q61K/R}$ known to exist in a mutually exclusive pattern. Following these two oncogenes, *NF1*, seems to be the third most commonly mutated tumor suppressor gene and negative regulator of the MAPK-pathway according to whole exome sequencing (WES) analysis and functional validations (Figure 1) [31]. Also, *KIT* is a driver oncogene activating MAPK signaling in a small percentage of melanomas [32, 33].

Collectively, based on the significantly mutated oncogenic driver genes in melanoma, genomic classification reveals four sub-types and these include the BRAF subtype (presence of *BRAF* hotspot mutations), RAS Subtype (presence of *RAS* hotspot mutations), NF1 subtype (presence of *NF1* loss-of-function mutations) and a triple wild-type (WT) subtype that lacks hot-spot *BRAF, NRAS* or *NF1* mutations [34].

Starting from the common melanocytic nevus phase, recent studies provide evidence for clonality of *BRAF*^{V600E} mutation (Figure 1) [2]. A distinct feature that distinguishes benign nevi from melanomas is that nevi eventually stabilize and undergo cellular senescence. Activation of senescence pathways in benign nevi prevents further cell growth. The G1/S checkpoint pathway appears to be the main mediator of senescence in nevi [35]. The concept of oncogene activation in nevus cells that does not result in tumor formation, is known as Oncogene-Induced Senescence. Benign nevi in the current instance enter a permanent cell-cycle arrest following the first *BRAF* mutation [36]. Several lines of evidence show that the immune system plays a role in regulating the apoptotic potential of benign nevi [37, 38]. A clinical study demonstrated a three-fold increased risk of malignant melanoma development in immunosuppressed transplant recipients compared to matched controls, suggesting a role of the immune system in preventing progression into a melanoma [39].

The atypical or dysplastic nevus is a genetically intermediate melanocytic lesion that may be difficult to distinguish from a malignant melanoma [40]. In contrast to benign nevi, those melanocytic lesions not only have a single activating mutation in *BRAF* but multiple driver mutations such as *NRAS*, *TERT* promoter (p*TERT*) mutations and also heterozygous alterations for tumor suppressor genes such as *CDKN2A* (Figure 1) [2]. Collectively, these data suggest that dysplastic nevi are indeed a distinct entity from benign nevi and melanomas based on their genetic make-up. It is worth noting that individuals with increased numbers of dysplastic nevi are also at increased risk of developing melanoma [41].

Regulation of telomerase activity and telomere length has been a contributing factor not only for underlying features of intermediate and primary melanoma lesions but also in determining melanoma risk. The telomerase reverse transcriptase (*TERT*) gene encodes for a ribonucleoprotein that regulates telomere length and cell integrity [42-45]. The wild-type p*TERT* contains binding sites for c-Myc (E-Box), SP1, and ETS transcription factors [46]. Genetic and transcriptomic data suggest that increased telomere length is associated with higher melanoma risk and is correlated with disturbed homeostasis of telomere regulation [47]. The majority of *pTERT* mutations are found at two hotspots, in a mutually exclusive pattern, at −124 bp (c.1-124C>T) and -146bp (c.1-146C>T) upstream from the ATG start site. These mutations create ETS/TCF transcription factor binding motifs causing increased *TERT* expression [48]. Upregulation of *TERT* is correlated with presence of mutations in the promoter region and is mainly observed in primary and invasive stages of melanoma but not in the benign nevus phase [4].

The primary stage of melanoma requires additional genetic alterations and these are mainly centered around the impairment of G1/S checkpoint pathway resulting in senescence escape of melanocytic cells [4]. Specifically, loss of *CDKN2A* mainly by deletions, is a significant contributing factor leading to loss of $p16^{INK4A}$ expression in melanomas [22]. Some novel driver genes identified for CM by application of WGS *include DDX3X, RASA2, PPP6C, RAC1* or *RB1* all found to be specific for CM but not acral or mucosal melanomas [49]. Loss of *Phosphatase and tensin homolog (PTEN)*, a key tumor suppressor gene regulating cell growth, is critical in facilitating melanoma development through deregulation of the PI3K/AKT/mTOR pathway, reduction of apoptosis and promotion of cell survival [50]. In addition, deregulation of p53-dependent apoptotic pathways and mutations within *tumor protein 53* (TP53) are correlated to a more advanced progressed state and metastatic melanoma behavior (Figure 1) [4].

Collectively, improved knowledge on the molecular pathways enhanced the identification of novel biomarkers to improve CM diagnosis and treatment, although there is still more to be uncovered.

PATHWAYS TO CM TREATMENT

BRAF, is a protein kinase and key regulator of the MAPK signaling pathway that is mutated in about 50% of CMs but also in other types of cancer including colorectal, leukemia and thyroid [51]. About 90% of mutations within *BRAF* are specific to position V600E, a gain of function mutation leading to a constitutively active state of BRAF and hyperphosphorylation of MEK thereby stimulating cancer cell growth [52].

Vemurafenib, dabrafenib and encorafenib are FDA-approved BRAF inhibitors that initially presented promising results in melanoma targeted therapy through inhibition of hyperactivation of MAPK signaling and suppression of tumor growth [53-55]. Nevertheless, combinatory treatment using a MEK inhibitor, trametinib, cobimetinib and binimetinib, against downstream components of MAPK pathway, showed delay in the onset of resistance and improved overall survival (OS) in phase 3 clinical trials [56-59]. Even though tumor reduction was observed in more than 50% of $BRAF^{V600E}$ mutated patients, in the majority of cases there was development of tumor resistance within 4-9 months after treatment through re-activation of MAPK pathway [60-63]. Still, there is little clinical evidence about guidance for the best targeted treatment of metastatic melanoma with limited toxic events and no relapse development [64].

Since targeted therapy through *BRAF* inhibition can only be applied in about 50% of patients, immunotherapies can provide effective treatment with long-term responses independent of the mutational status of patients [65]. This has been successful through the development of antibodies against immune checkpoints such as ipilimumab, a monoclonal antibody against cytotoxic T-lymphocyte antigen 4 (CTLA-4) which downregulates immune responses [66]. In addition, nivolumab and pembrolizumab target the programmed cell death protein 1 (PD-1), a T cell inflammatory activity suppressor, and showed improved OS in patients with progressed melanoma. The combination of CTLA-4 and PD-1 inhibitors have been proven superior to monotherapy in patients with PD-L1 negative tumors [67-69]. Even though there has been success in targeting the immune system, still future studies are required to determine the optimal conditions and combinations but also possibly new targets to further improve the outcome of patients with metastatic melanoma [70].

INSIGHT INTO GENETIC SUSCEPTIBILITY- WHAT IS KNOWN SO FAR

A family history of melanoma has a significant role in determining an individual's risk of developing the disease. About 10-12% of reported CM cases occur in familial kindreds (Figure 2), therefore, familial (or hereditary) melanoma is arbitrarily defined by the clustering of at least two or more melanomas in first degree relatives [71]. High-penetrance genes have low population frequency and a higher impact on cancer development while, low-penetrance genes have high population frequencies but with a reduced effect size [72]. Several methods have been employed to identify high-penetrance genes that may predispose to familial melanoma.

Starting with genetic linkage analysis back in 1992 using DNA markers, scientists uncovered the first hint of chromosome 9p21 to be critically important in familial predisposition to melanoma [73]. Follow-up studies aiming to zoom into the chromosomal area of interest, uncovered *cyclin-dependent kinase Inhibitor 2A* (*CDKN2A)* as the first melanoma predisposition gene [74, 75]. A year later, a specific founder mutation was identified in Dutch-kindreds, a 19bp deletion in exon 2, known as the *p16^{INK4A}-Leiden* mutation (c.225_243del, p.(A76Cfs*64)) [76]. *CDKN2A* is the most common high-penetrance melanoma susceptibility gene known today, not only in The Netherlands (70%) but also world-wide (40%) (Figure 2) [77].

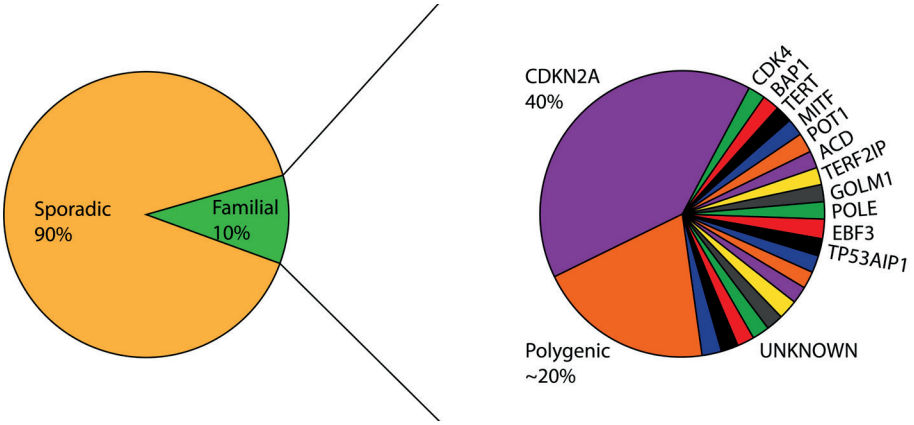

Figure 2 Summary of currently identified candidate high-penetrance melanoma susceptibility genes. The first pie chart on the left represents two settings of CM where 90% of cases are found in the general population (sporadic) and 10% report a family history of melanoma (familial). Zooming into the familial setting, several high-penetrance melanoma susceptibility genes have been identified thus far with *CDKN2A* accounting for most families (40%) and several other candidate genes, each responsible for about <1% of families. There is also a proportion of polygenic risk factors, effect of medium and low penetrance genes (20%) but also environmental risk factors. There is still a proportion of unknown genetic variability in the occurrence of hereditary melanoma.

Carriers of germline mutations in melanoma predisposition genes, such as *CDKN2A*, that may also present with increased number of atypical moles (known as dysplastic nevi) could be designated as Familial Atypical Multiple Melanoma Syndrome (FAMMM syndrome) patients [78]. FAMMM syndrome patients with germline *CDKN2A* mutations have a 70% risk of developing melanoma with the first sign of disease appearing at a relatively young age (mean <45 years) [79, 80]. Germline *CDKN2A* mutation carriers have an additional life-time risk of 15-20% to develop pancreatic ductal adenocarcinoma (pancreatic cancer; PC) [81-83]. Interestingly, clinical studies suggest variability in occurrence of melanoma and PC within families indicating that modifying factors may contribute to the risk of developing these two tumor types in patients with/without germline *CDKN2A* mutations [81, 84]. An example is the genetic variation in the *MC1R* gene, found to modify the risk of developing melanoma in *CDKN2A*-mutated families [85, 86]. Determination of genetic risk factors that modulate the risk of PC and melanoma in *CDKN2A*-mutated families, would therefore allow for a better identification of patients at increased risk that might benefit from personalized clinical management.

CDKN2A is located on chromosome 9p21.3 and encodes for two distinct proteins that are translated in alternate reading frames (ARFs) from alternatively spliced transcripts, therefore consist of different amino acid sequences (Figure 3). The α transcript encodes for $p16^{INK4A}$, a tumor suppressor protein that mediates G1 arrest by inhibiting the phosphorylation of Cyclin-D1-CDK4/6 complex [87, 88]. The alternative β transcript encodes for $p14^{ARF}$ which is also a tumor suppressor protein that inhibits MDM2-mediated ubiquitination thereby promoting p53-dependent apoptotic pathways (Figure 3) [89-92]. The *$p16^{INK4A}$-Leiden* mutation specifically causes a reading frameshift resulting in truncated $p16^{INK4A}$ protein which loses its capacity to bind to CDK4/CDK6 complex and a $p14^{ARF}$ fusion protein that seems to retain functionality (Figure 3) [7].

The implementation of mouse models to generate knock-out (KO) mice for both $p16^{INK4A}$ and $p14^{ARF}$ via conventional gene-targeting approaches has also been successful in providing evidence for cancer development including fibrosarcoma and lymphoma [93, 94].

Combination of mutant *HRAS* and *CDKN2A* KO led to CM development in mice that also showed loss-of-heterozygosity (LOH) for the remaining WT allele [94, 95]. In addition, simultaneous inactivation of *CDKN2A* and *Stk11* (*Lkb1*) loss in $BRAF^{V600E}$ mutant melanocytes induced mTORC1 and mTORC2/AKT activation leading to rapid melanoma formation in mice [96]. More recently, application of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) technology to induce genomic modifications, provided evidence that loss of $p16^{INK4A}$ protein mediates invasive behavior of melanoma cells *in-vivo* due to deregulation of the BRN2 transcription factor [97].

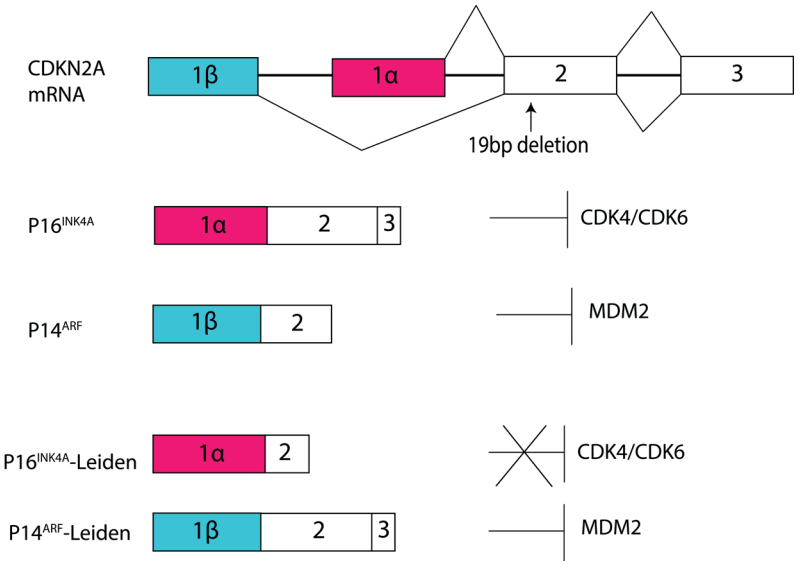

Figure 3 Schematic representation of *CDKN2A* exons and coding proteins. *CDKN2A* is located on chromosome 9p21 and encodes for two distinct tumor suppressor proteins. The 19-bp deletion of *p16^{INK4A}-Leiden* mutation is located in exon 2. The α transcript encodes p16^{INK4A} that regulates G1/S cell cycle arrest by inhibiting the CDK4/6 complex. The β transcript encodes p14^{ARF} which is involved in p53-related apoptotic pathways by inhibiting MDM2. The resulting p16^{INK4A}-Leiden truncated protein disrupts G1/S cell cycle arrest by losing the binding capacity to CDK4/CDK6 complex. The resulting p14^{ARF}-Leiden fusion protein seems to remain functional. Source data were adapted from the following resources [5-7].

Interestingly, *CDKN2A* function is lost in hereditary melanoma, but also in sporadic melanoma, commonly through deletion [22]. p16^{INK4A} expression was significantly reduced in melanomas when compared to nevi according to transcriptomic data, suggesting that p16^{INK4A} is the predominant tumor suppressor protein acting at the transition stage to invasive melanoma [4]. Bi-allelic inactivation of *CDKN2A* is mainly observed in progressed stages of the disease but about 40% of sporadic melanoma cases already carry a somatic mutation, chromosomal deletion and promoter hypermethylation in *CDKN2A* [34, 49]. Collectively, these data suggest a significant effect of both germline and somatic mutations of *CDKN2A* in melanoma development.

Following *CDKN2A*, *Cyclin-dependent kinase 4 (CDK4)* is the second high penetrance melanoma susceptibility gene identified through a candidate gene sequencing approach [98]. *CDK4* is less frequently mutated than *CDKN2A*, since it has only been reported in a total of 18 melanoma families up to date according to follow-up studies [99-102]. Besides human studies, *in-vivo* experiments with mice carrying the germline *CDK4* mutation, p.R24C, revealed susceptibility to melanoma development after exposure to carcinogen treatment [103].

Due to the limitation of genetic linkage and candidate gene screening to discover additional novel high penetrance melanoma susceptibility genes, it was not until 2011, that the development of new genomic sequencing technologies were implemented to discover novel genes. Germline mutations in the tumor suppressor gene, *BRCA1-associated protein 1* (*BAP1*), were identified in two families with atypical melanocytic tumors by application of sequencing technology [104, 105]. Somatic loss of the WT allele was detected in the tumors of patients. In addition, *BAP1* loss increased the predisposition for other tumor types including mesothelioma, renal cell carcinoma and basal cell carcinoma [106-109]. Overall, germline mutations in *BAP1* account for a small percentage of melanoma families.

Moreover, the *microphthalmia-associated transcription factor* gene (*MITF*) is the most well-known medium penetrance melanoma susceptibility gene. *MITF* regulates melanocyte development and differentiation, and was the first gene to be identified by next-generation sequencing (NGS) technology in melanoma susceptibility [110, 111]. The *MITF* p.E318K germline mutation alters MITF transcriptional activity through abrogation of a sumoylation motif. Germline mutation carriers have also been associated with increased risk for renal cell carcinoma and PC [112].

The application of more advanced methods such as WES analysis had a significant effect on identifying high penetrance genes for melanoma. The initial variants identified were members of the telomerase and shelterin complex including genes that protect chromosomal ends. In 2013 a variant was found within the promoter of *telomerase reverse transcriptase* gene (*TERT*). The mutation, -57bp from the translation-start site, segregated with disease in a 14-case melanoma family and functionally created a binding motif for ETS/TCF transcription factor leading to increased *TERT* expression [48]. In concordance, the *pTERT* mutation that was detected in a single melanoma-prone family, (G>A) at −246 bp upstream from the ATG start site, was previously associated with low telomerase activity in patients with non–small cell lung cancer [113]. Two-carriers with germline mutations in *TERT* developed several types of cancer, including ovarian cancer (at 27 years), melanoma (at 20 years), renal cell carcinoma, bladder cancer, breast cancer and finally lung cancer [48]. Collectively these data suggest that *TERT* constitutes an additional high penetrance gene for familial melanoma that is also mutated in sporadic cases.

Moreover, application of whole-genome sequencing (WGS), WES and targeted sequencing identified loss-of-function mutations in the *protection of telomeres 1* (*POT1*) gene in melanoma families from the UK and Australia [114]. Six families were found positive for novel *adrenocortical dysplasia homologue* (*ACD*) mutations and four families were positive for *telomeric repeat binding factor 2 interacting protein* (*TER2IP*) variants including segregating nonsense mutations for both genes by screening 510 melanoma families [115]. Collectively these data suggest that

dysregulation of telomeres is an important contributing-pathway in a proportion of high-risk families CM families.

The most recent application of WES identified additional rare variants within the *golgi membrane protein 1* (*GOLM1*) gene, *EBF Transcription Factor 3* (*EBF3*) gene, *DNA Polymerase Epsilon* (*POLE*) gene and *Tumor Protein P53 Regulated Apoptosis Inducing Protein* (*TP53AIP1*) gene, although the effect size and functional significance of these variants still requires clarification by future studies [116-119].

To summarize, with *CDKN2A* mutations accounting for about 40% of variation in familial clustering of CM world-wide, and rare mutations in *CDK4, MITF, BAP1, TERT, POT1, ACD, TERF2IP, GOLM1, EBF3, POLE* and *TP53AIP1* responsible for up to 10% of variation, there is still about 50% unexplained remaining germline variation (Figure 2). The intensive clinical follow-up data in families with proven germline mutations may reduce the number of melanoma cases. Nevertheless, the possibility for an effect of polygenic risk factors such as multiple medium and low-penetrance genes, including *MITF, MC1R, SLC45A2, ARNT* and others cannot be excluded for these families. The shared environmental exposures of affected family members could also be a contributor to melanoma development (Figure 2) [120].

Combined, other rare high-penetrance genes are very likely to exist and application of WES and WGS analysis provide the best resource in clarifying the unknown genes. The identification of alterations within the regulatory region of *TERT* suggests that WGS analysis is a promising tool in uncovering variation within the non-coding and regulatory region of our genome. Collectively, the identification of novel high penetrance melanoma susceptibility genes is still essential in order to improve genetic testing and counselling in hereditary melanoma patients.

IDENTIFICATION OF CANCER DEPENDENCIES AS NEW THERAPEUTIC TARGETS

In addition to predisposition genes and driver genes, a third class of genes is relevant for the biology and treatment of cancer and these are dependence/fitness/essential genes [121].

Application of large-scale pharmacogenomic screens across different panels of cancer cell lines provides a possible solution in un-revealing novel fitness genes as possible biomarkers for therapy [122]. Moreover, the recent advancement of CRISPR-Cas9 screening technology may provide a precise method in determining novel biomarkers with high precision and less false-positive targets when compared to previously used screens through short-hairpin RNAs (shRNAs) [123, 124]. The CRISPR-Cas9 technique consists of using a single-guide RNA (sgRNA) molecule to bind to complementary DNA sequences, which simultaneously recruits the endonuclease Cas9 to introduce double-stranded breaks in the target DNA. The resulting double-stranded break is then repaired, allowing modification or removal of specific DNA bases. The mechanism of repair usually involves non-homologous end joining, an error prone pathway that results in generation of indels within the gene [125, 126].

Cancer *in vitro* systems are now being investigated using pooled CRISPR-Cas9 screens that employ genome-scale libraries consisting of thousands of sgRNAs. Data from these systems can be used to identify and prioritize new cancer therapeutic targets firstly by infecting tumor cell lines of interest and secondly by measuring the endpoint sgRNA abundance to identify depleted or enriched genes from the screen (usually 14-21 days after infection) (Figure 4) [127-129].

A recent study aiming to identify targets which when knocked-out confer resistance to melanoma immunotherapy, showed that Apelin Receptor (APLNR) was a modulator of interferon-γ responses in tumors by application of CRISPR-Cas9 positive selection screening [130]. On a similar note, negative selection screens have a general goal in identifying genes which when lost have an effect on cell proliferation and therefore are essential for cell fitness [127].

Genes may influence the fitness of melanoma cells either because they encode proteins involved in essential cellular processes, or they are required for viability specifically of cells of the melanocytic lineage. Still, the identification of context-specific fitness genes to therapeutically target for maximal clinical benefit of melanoma patients remains a challenge [129]. Collectively, these data suggest that application of CRISPR-Cas9 screening technology may provide a precise method in determining novel vulnerabilities for melanoma targeted therapy.

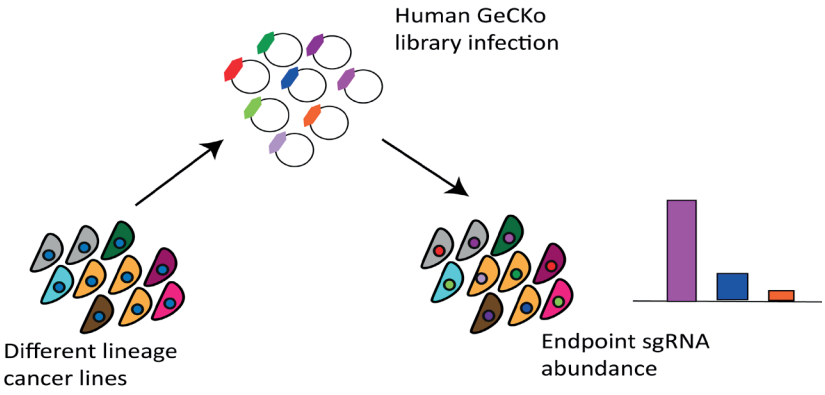

Figure 4 Schematic overview of a CRISPR-Cas9 knockout screen. The cell-lines of interest (different cancer lineage) are infected by the human library of sgRNAs knocking out all known genes (Genome Scale CRISPR Knock-Out library). The sgRNA abundance is read using Next Generation Sequencing technology at days 0 and 14 (or 21) after infection. Those sgRNAs that are depleted compared to the initial sgRNA abundance depict genes essential for cell growth whereas sgRNAs that are enriched indicate genes that may serve as possible tumor suppressors.

OBJECTIVES

This thesis sought to investigate different aspects of CM biology, mostly linked to genetic dependencies in hereditary and sporadic melanoma. Specifically, herein we have employed state of the art technologies such as WES, digital PCR (dPCR) and CRISPR-Cas9 genetic engineering to unravel the complexity of genetic events in hereditary and sporadic melanoma. Specific objectives include:

a) Identification and validation of novel high penetrance melanoma susceptibility genes.
b) Identification of genetic modifiers predicting the risk of melanoma and PC in *p16^{INK4A}-Leiden* mutation carriers.
c) Timing of *CDKN2A* loss-of-heterozygosity in melanocytic tumors of *p16^{INK4A}-Leiden* mutation carriers.
d) Determination of genetic dependencies in melanoma by analyzing and processing CRISPR-Cas9 screening technology data.

To elucidate the genetic basis of familial melanoma and discover novel high penetrance melanoma susceptibility genes, in **chapter 2**, we applied WES in a Dutch melanoma family. The results of WES analysis were also validated in available patient's tissues and functionally verified *in-vitro* using cancer cell lines. This work is absolutely essential to improve genetic testing and counselling of familial melanoma kindreds since about 50% of genetic variation underlying genetic variability remains unknown.

Identification of genetic risk factors, other than a germline *CDKN2A* mutation, responsible for PC and melanoma risk in *CDKN2A*-mutated families has been a challenge. In **chapter 3**, we sought to investigate a variable genomic region (SNP) within *TERT/CLPTM1L* high-cancer risk locus as a modifying genetic risk factor for PC and melanoma in *p16^{INK4A}-Leiden* mutation carriers.

Even though scientific studies provide evidence for *CDKN2A* bi-allelic loss to be an important event in the transition to invasive melanoma in sporadic cases, there is little or no evidence known about inactivation of this tumor suppressor gene in the progression stages of hereditary melanoma. Therefore, in **chapter 4**, we sought to investigate *CDKN2A* inactivation through LOH by applying dPCR in FAMMM syndrome patients, carrying a germline *CDKN2A* mutation. Utilization of dPCR assays allows for numerous applications such as quantitative detection of mutant cell fraction in a population of ad-mixed cells, LOH and quantification of T-cells in tumors (Figure 5) [131-133]. In cases where quantification of the actual mutation of interest is challenging, dPCR technology may be applied to target a common polymorphic region (SNP) that is linked to the specific mutation site. This could

be depicted by the high probability that the SNP-allele linked to the mutation, will end up in the same droplet as the mutant-allele (Figure 5). Application of SNP-based dPCR technology in melanocytic neoplasms of FAMMM syndrome patients allowed for absolute quantification of allelic imbalance within *CDKN2A*, indicative of LOH. We also attempted to deduce the order of genetic events via quantifying cells with *BRAF*^{V600E} mutation, p*TERT* mutations and chromosome 9q LOH.

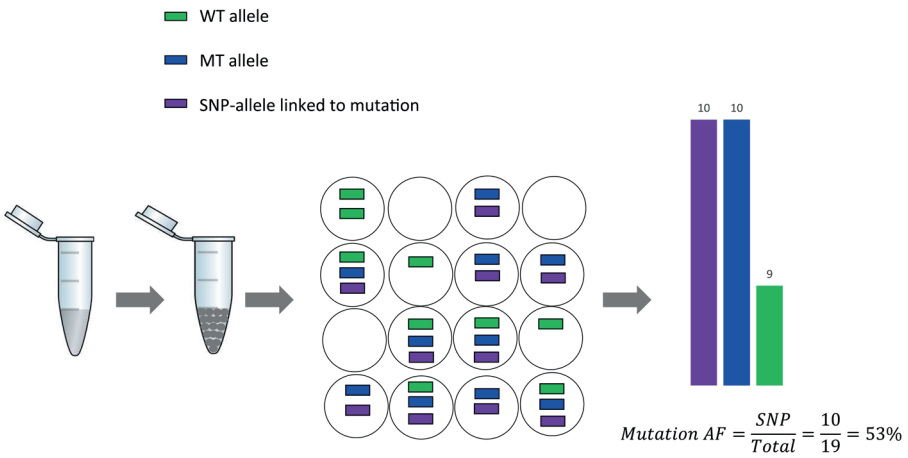

Figure 5 Diagram of SNP-based digital PCR (dPCR) analysis. The sample of interest is partitioned into 20,000 droplets which are then detected for the specific target of interest. The homozygous genotype for a wild-type (WT) sequence is depicted by a droplet positive for the green target only. The heterozygous genotype is depicted by positivity for the WT-allele (green), the mutant (MT) allele (blue) and the SNP-allele (purple) that is linked to the mutation. The homozygous genotype for the mutation is depicted by positivity for the MT allele (blue) and the SNP-allele (purple). In cases where the mutation is not the direct target of amplification, targeting of the SNP allele linked to the mutant allele allows for direct quantification of the mutation allele frequency (AF) and therefore loss-of-heterozygosity (LOH). In the example shown, mutation AF was calculated by dividing the SNP-allele counts (10) over the total allele-counts (19). The mutation AF was 53% depicting LOH in this example (>50%).

Finally, resistance to *BRAF* inhibitors warrants screening for identification of novel pathways to melanoma treatment. The application of CRISPR-Cas9 screening technology is nowadays the leading tool in revealing novel genetic vulnerabilities in cancer. Therefore, in **chapter 5** we performed comparative analysis using bioinformatic tools to study CRISPR knockout (KO) screening data and identify novel fitness genes in melanoma.

Collectively, through application of novel genomic techniques in this thesis, we hope to have explored in detail the genetic dependencies in familial and sporadic melanoma.

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