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Malignant Melanoma–a Genetic Overview

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Abstract. Malignant melanoma, a potentially lethal skin neoplasm, is characterized by a complex and heterogeneous etiology. Both incidences and deaths associated with melanoma are increasing in Caucasian populations. While exposure to ultraviolet radiation through sun-exposure is the major risk factor; the host factors including skin type and number of moles are critical in predisposition. The CDKN2A is a high penetrance melanoma susceptibility gene as carriers of the mutations are predisposed to the disease within familial settings. The gene is also somatically altered to varying degrees in sporadic melanoma. The CDK4 gene due to occurrence of activation mutations in a few families worldwide represents another melanoma susceptibility locus. The variants within the melanocortin receptor 1 (MC1R) gene, which encodes a melanocyte specific surface receptor with a key role in pigmentation, are associated with high risk phenotypes and increased risk of melanoma. Melanoma tumors are characterized by activation of the RAS-RAF-MEK-ERK pathway through either autocrine growth factor stimulation or oncogenic mutations in the B-RAF or N-RAS genes. Somatic mutations in the B-RAF gene are complemented by those in the N-RAS gene and represent the major genetic alterations. The mutations in the B-RAF gene in melanoma due to occurrence in melanocytic nevi represent early events that additionally require loss of cell cycle inhibitors like CDKN2A for melanoma progression and development. The sequence of events points to the cooperative collaboration between different genetic pathways in tumor development that can be and are being used as targets for developing specific therapeutic agents.

Key words: melanoma, genetics, CDKN2A, B-RAF.

MELANOMA MALIGNO: UNA VISIÓN DE CONJUNTO SOBRE LA GENÉTICA

Resumen. El melanoma maligno, una neoplasia cutánea potencialmente mortal, se caracteriza por una etiología compleja y heterogénea. Tanto la incidencia como las muertes asociadas al melanoma están aumentando en la población caucásica. Aunque la exposición a la radiación ultravioleta a través de la exposición solar es el principal factor de riesgo, los factores que dependen del huésped, como el fototipo y el número de nevus, son críticos en la predisposición. El CDKN2A es un gen de susceptiblidad para el melanoma de alta penetrancia, ya que los portadores de mutaciones están predispuestos a la enfermedad en el entorno familiar. El gen también está alterado somáticamente, en grados variables, en el melanoma esporádico. El gen CDK4, debido a la activación de mutaciones en algunas familias a nivel mundial, representa otro locus de susceptibilidad para el melanoma. Las variaciones dentro del gen del receptor de la melanocortina 1, que codifica un receptor de superficie específico de los melanocitos con un papel clave en la pigmentación, están asociadas con fenotipos de alto riesgo y un riesgo aumentado de melanoma. Los tumores de melanoma se caracterizan por la activación de la vía RAS-RAF-MEK-ERK a través de la estimulación por factor de crecimiento autocrino o por mutaciones oncógenas en los genes B-RAF o N-RAS. Las mutaciones somáticas en el gen B-RAF se complementan por aquellas en el gen N-RAS y representan las principales alteraciones genéticas. Las mutaciones en el gen B-RAF en el melanoma, que tienen lugar en los nevus melanocíticos, representan eventos iniciales que requieren, además, la pérdida de inhibidores del ciclo celular como CDKN2A para la progresión y el desarrollo del melanoma. La secuencia de eventos apunta hacia una colaboración entre las diferentes vías genéticas en el desarrollo tumoral, que pueden y están siendo empleadas como dianas para desarrollar agentes terapéuticos específicos.

Palabras clave: melanoma, genética, CDKN2A, B-RAF.

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Introduction

Melanoma is the most aggressive and potentially lethal skin tumor. It originates in pigment-producing melano-

cytes that are found in the basal layer of the epidermis and in the eye[1]. The number of melanoma cases and deaths worldwide has increased faster than many other cancers though, lately, the trend has stabilized[2-4]. The annual increase in incidence rates has been 3-7 % per year for white-skinned Caucasian populations[5,6]. Estimates suggested a doubling of melanoma incidence every 10-20 years[7]. The highest annual incidence rates are found in Australia and New Zealand with about 56 cases per 100,000 inhabitants with no statistically significant differences between males and females[2,8]. Other countries with high melanoma incidence rates are USA and Canada[9,10]. In Europe, the highest incidence rates have been reported in Scandinavia with about 15 cases per 100,000 inhabitants[11,12]. In Germany, the incidence rate is about 10-12 cases per 100,000 inhabitants[11]. The lowest incidence rates in Europe are reported in Mediterranean countries with about 5-7 cases per 100,000 inhabitants[11,13,14]. The north-south gradient in melanoma incidence in Europe has been explained by the differences in skin pigmentation between populations[11]. At a global level, the lowest annual incidence rates are found in Asian countries with rates near 0.5 cases per 100,000 inhabitants[15,16]. Melanoma is rare in individuals below 20 years of age and frequent in young and middle-aged adults[6,17,18].

Risk Factors

An individual's risk of developing melanoma depends on two sets of factors: a) host-related factors such as pigmentation and skin reaction to sunlight; and b) environmental factors (table 1)[19-21].

The main environmental risk factor is exposure to ultraviolet (UV) light. Epidemiological evidence suggests that high-intensity intermittent sun exposure is the key factor in inducing of the majority of melanomas[22,23]. The risk of melanoma is higher in fair-skinned people, especially those with blond or red hair who sunburn and freckle easily, than in people with darker complexions[24]. In addition to UV exposure, age at exposure is an important determinant of the risk of melanoma. Several studies have suggested a strong link between sunburn in childhood and development of melanocytic neoplasia later in life[25]. High sun exposure during adult life constitutes a significant risk factor for melanoma only if there had been substantial sun exposure during childhood[26]. The presence of atypical or dysplastic melanocytic nevi are major markers for melanoma risk across all continents, both in high risk families and in the general population[27,28]. Also the presence of multiple, non-dysplastic moles points to increased melanoma risk[29,30].

One of the most significant risk factors for melanoma is a family history of the disease. It is estimated that aproximately 10 % of melanoma cases report a first- or second-degree relative with melanoma. Epidemiological studies suggest that the estimated genetic component in malignant melanoma is around 18 %[31,32]. Analysis of familial cancer risk of melanoma has shown a risk of roughly 2.5 for an offspring when a parent had melanoma[33]. Genetic predisposition in families is in part attributed to two melanoma susceptibility genes. Germline alterations in the major melanoma susceptibility gene, CDKN2A on chromosome 9p21, occur in 25-40 % of melanoma families[34,35]. The CDK4 oncogene on chromosome 12q14 is considered to be another melanoma susceptibility gene. However, to date, only four families worldwide with melanoma-prone kindreds have been reported to carry mutations in CDK4[36-38].

Table 1. Melanoma risk factors

Constitutional predisposition
Fair skin and hair color
Number of benign nevi (moles)
Freckles
Presence of three or more atypical nevi
Propensity to burn in the sun rather than tan
Prior therapy with psoralen and ultraviolet (UV) A light
History of solar keratoses, squamous cell carcinoma, or xeroderma pigmentosum
Family history of dysplastic nevi or melanoma
Risk behaviors
History of three or more episodes of sunburn, especially during childhood
Episodic excessive sunlight exposure (e.g. recreational tanning)
Long term continuous sunlight exposure (e.g. outdoor workers)
UV exposure at tanning salons
Environmental factors
Stratospheric ozone depletion, latitudes closer to equator (e.g., higher UV radiation)

Genetics of Melanoma

The CDKN2A Gene Locus

The tumor suppressor $p16^{INK4A}$ (henceforth called p16) was identified through two independent lines of research.

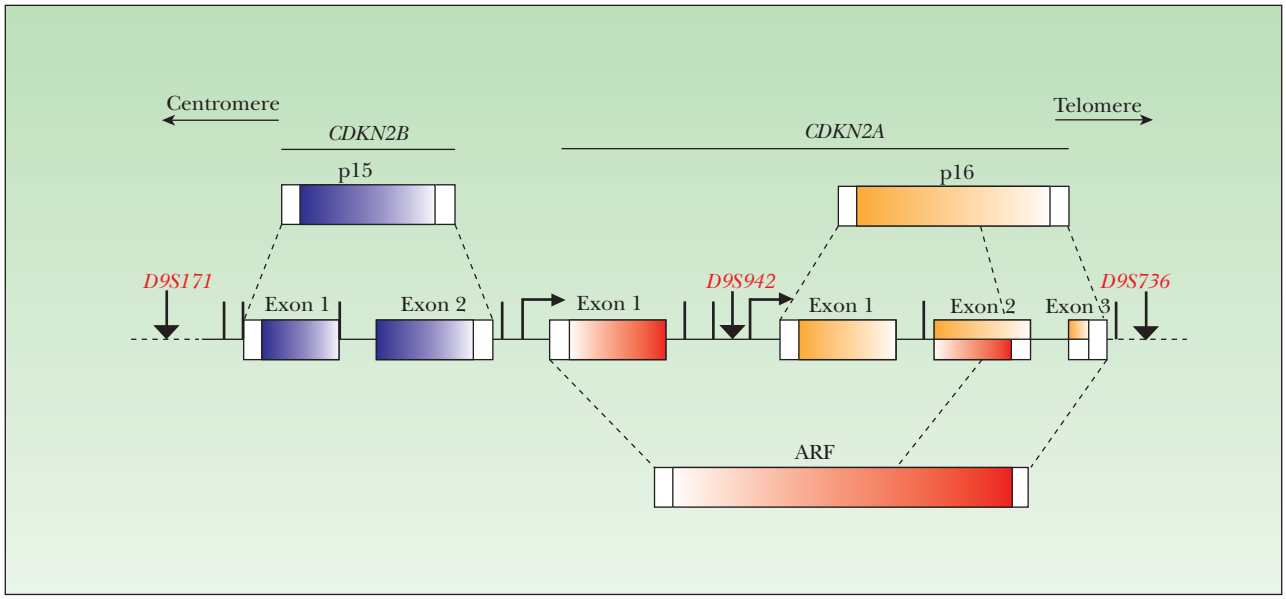

Figure 1. Genomic organization of the CDKN2A locus.

In cell biology experiments, it was detected through its interaction with CDK4 in a yeast two-hybrid screen[39]. Simultaneously, the gene *CDKN2A (MTS1)* was mapped to the frequently altered chromosome 9p21 locus by positional cloning[40,41]. The p16 protein consists of 156 amino acids encoded by three exons.

Subsequent to the discovery of p16, a second transcript arising from the *CDKN2A* locus was discovered, which comprised of an alternate exon 1 located about 20 kb upstream the regular exon 1 . Exon 1 splices with exons 2 and 3 to transcribe $p14^{ARF}$ (henceforth called ARF) from a separate promoter ($p19^{ARF}$ in the mouse). The ARF transcript is translated in an alternate reading frame (fig. 1). The human ARF protein consists of 132 amino acids. The two proteins, p16 and ARF, encoded from a partially shared genomic sequence are structurally unrelated. Incidentally, both function as cell cycle inhibitors[42,43]; p16 functions in the retinoblastoma pathway and ARF in the p53 pathway of cell cycle regulation. Adjacent to *CDKN2A*, two exons of *CDKN2B* encode $p15^{INK4B}$ which is homologous and functionally similar to $p16^{INK4A}$ (fig. 1)[44,45].

The CDKN2A gene locus represents a unique structure in the mammalian genome. Overlapping gene structures are common in viral and bacterial genomes. In the small-sized viral genomes, this structure type represents an important mechanism to maximize the usage of the coding sequence[43]. The unique genomic organization of the *CDKN2A* gene locus may explain why p16/ARF is a frequent target of inactivation in tumorigenesis. A single genetic hit might result in simultaneous disruption of two key anti-oncogenic mechanisms.

p16 and the Retinoblastoma Pathway

In hypophosphorylated state, the retinoblastoma protein (Rb) binds to E2F transcription factors, which are necessary for cell cycle progression from G1 to S phase[46,47]. The enzymatic complex of CDK4/6 and cyclin D positively regulates cell cycle by phosphorylating Rb. However, p16 disrupts the kinase complex of CDK4/6 and cyclin D by binding to CDK4. It inhibits phosphorylation of Rb and therefore negatively regulates cell cycle progression[48]. Ankyrin-like repeats in the protein sequence motif of p16 are involved in binding to CDK4[49,50].

ARF and the p53 Pathway

The ARF protein acts as a cell cycle inhibitor by antagonizing MDM2-mediated degradation of p53, thereby stabilizing this tumor suppressor protein[51,52]. The exact mechanism whereby ARF stabilizes p53 is not entirely clear. Three models have been proposed to explain the mechanism of p53 stabilization by ARF[53]. One model suggests that ARF localizes to the nucleolus and sequesters MDM2 to that compartment, resulting in release of p53 from MDM2 inhibition[54,55]. Another proposed mechanism is the formation of ternary complexes of ARF, MDM2 and p53 in the nucleoplasm, which prevents nuclear export of both MDM2 and p53[56]. A third possible mechanism is that ARF need not to relocate MDM2 to the nucleolus for proper function; but rather only inhibits E3-ligase activity of MDM2 to stabilize p53[57,58]. Expression of c-myc, E2F, mutated RAS or loss of Rb induces

ARF[59-62]. This response to oncogene expression depends on the cellular context; RAS potently induces ARF in murine, but not human, cells[62-64]. In murine embryonic fibroblasts (MEFs), ARF expression correlates with onset of senescence, and cells lacking ARF do not senesce in culture[65,66]. In contrast, ARF does not play a major role in replicative senescence of human cells[67,68]. Recently, interactions of ARF with SUMO-E2, AP-1 dimers, BCL6, p63, DP1 and nucleophosmin (NPM)/B23 have been reported[69-74]. By degradation of B23, ARF decreases the processing of ribosomal RNA, thereby limiting cell growth and inducing cell cycle arrest[74,75]. Repressor molecules such as Twist, TBX2, and Pokemon have been shown to inhibit ARF expression[76-78].

Cdkn2a Knockout Studies

The major support for tumor suppressor function of the *CDKN2A* gene locus came from knockout studies. The construction of different types of knockout mice provided comparisons of *p16*- and *Arf*-null phenotypes, respectively. In the first *Cdkn2a* knockout mice exon 2 was ablated, resulting in inactivation of both *p16* and *Arf* transcripts (100). These animals did not develop melanoma, but were prone to the development of other tumors, like fibrosarcomas and lymphomas. To assess the effects of deleting the *Arf* transcript alone, exon 1 of the *Cdkn2a* locus was knocked out in mice[65]. The animals expressed a phenotype similar to *p16/Arf*-null mice, suggesting that inactivation of *Arf*, rather than *p16*, may be responsible for the tumor susceptibility phenotype in the first model. Mice with deletion of one copy of *Arf* combined with complete inactivation of *p16* developed melanomas with very high penetrance, compared to *p16* knockout mice that retained both *Arf* alleles. *Arf* appears to be haploinsufficient in this context, suggesting cooperation between the p16 and Arf pathways in melanoma development[79]. Crossing *p16/Arf*-null mice with mice overexpressing oncogenic *H-ras* (Tyr-*H-ras* transgenic mice) resulted in offspring that developed cutaneous malignant melanoma spontaneously with high penetrance[80]. These data support the hypothesis that further genetic events in addition to *CDKN2A* inactivation are required for melanocyte tumorigenesis. Two groups later generated specific, "pure" p16 knockout mice. In one model (designated Ink4a $^{*/*}$), a stop signal at codon 101 in exon 2 was introduced, producing a truncated and unstable p16 protein[79]. In the other type (termed Ink4a ^{exon1}), exon 1 was deleted[63]. In both knockouts the expression of *Arf* was unaffected. The "pure" *p16*-null mice had a lower frequency of spontaneous tumor development compared to the *Arf*-null mice. Importantly, like mice with deletion of one copy of *Arf* and *p16* inactivation, the "pure" *p16*-null mice were susceptible to spontaneous melanoma development, albeit at a lower frequency. Altogether, knockout studies indicate that both, *p16* and *Arf*, function as tumor suppressors in mice. Significantly, both types of "pure" *p16* knockout mice developed melanoma, which was not detected neither in *p16/Arf*-null mice nor in *Arf*-null mice.

Data from other non-murine model systems also support the notion that p16 functions as a tumor suppressor gene. Canine primary melanomas and osteosarcoma cell lines from harbor frequent *p16* inactivation[81,82]. Rat carcinogen models show a high incidence of *p16* promoter methylation and *p16/Arf* deletion[83,84]. Strains of the fish Xiphophorus are prone to melanoma. Susceptibility in this species maps to the *DIFF* locus, which is tightly linked to the Xiphophorus *INK4A* locus[85].

Alterations of the CDKN2A Gene Locus in Melanoma

Germline alterations

The *CDKN2A* has been identified as a high penetrance melanoma susceptibility gene. Around 50 % of melanoma-prone kindreds show genetic linkage to markers within the 9p21 region, and of those, approximately 40 % carried germline mutations in *CDKN2A*[35,38,86]. Data from families studied worldwide indicate that the frequency of *CDKN2A* mutations increases with a) the number of melanoma cases in the family; b) the presence of individuals with multiple melanomas and c) an age at diagnosis less than 50 years[87]. In addition to melanoma, mutation carriers are at an increased risk of pancreatic cancer. Several studies have reported the occurrence of pancreatic cancer in families with *CDKN2A* mutations[88,89].

Recently, *ARF* mutations have been suggested to predispose to melanoma, as well as to nervous system tumors (NSTs)[90-92]. This combination of tumors has been proposed as a discrete syndrome by several investigators[93,94]. A specific germline deletion of *ARF* in the absence of concomitant loss of *p16* was found in a family segregating melanomas and NSTs[90]. It has been concluded that exon 1 alone is sufficient for ARF function[90]. A deletion of *ARF* exon 1 was found in a family where the mother and daughter had melanoma[95]. A germline 16 bp insertion in exon 1 was detected in a patient with multiple melanomas but without a family history of the disease[96]. Exon 1 mutations that do not alter p16 function have been reported in kindreds with familial melanoma and astrocytoma[90,96]. A cluster of five different germline mutations at the *ARF* exon 1 splice donor site was recently identified in melanoma pedigrees; three of the variants resulted in aberrant splicing of ARF mRNA[97].

Alterations in Sporadic Melanoma

The CDKN2A (p16) gene is involved in the development of sporadic melanoma. Monoallelic deletion of the *CDKN2A* gene locus is found in roughly 50 % of primary tumors and nearly all melanoma cell lines[98-100]. However, some reports have not found frequent alterations, thereby the role of CDKN2A (p16) in sporadic melanoma appears inconsistent[101,102]. Genetic alterations of p16 involved in sporadic melanoma are point mutations (0-26 %), promoter methylation (0-10 %), and homozygous deletions (5-25 %)[103]. Intragenic mutations and hypermethylation of the *p16* promoter appear to be rare[104-106]. The low frequency of p16 mutations in conjunction with a high frequency of allelic losses at chromosome 9p21 has also been interpreted as an indicator of the presence of other tumor suppressor genes at this locus[100,107-109]. Loss of p16 expression is associated with advanced stages of sporadic melanomas and a high mitotic index, suggesting that loss of p16 is a late event in the progression of sporadic primary melanomas[110,111]. In another report, the degree of p16 expression was related to the histological type of tumor[112]. Increased allelic loss at 9p21 also correlated with increased patient age at diagnosis[108]. Homozygous deletions affecting p16 are more frequent in melanoma cell lines than in primary tumors, which in part is due to technical constraints in detection of homozygous deletions in tumors[98,101].

CDKN2A Polymorphisms

The *CDKN2A* gene carries several polymorphisms. Two polymorphisms in the 3'untranslated region of the *CDKN2A* gene, C500G and C540T, have been associated with melanoma[113,114]. The C500G change ablates an *Msp*I/*Hpa*II restriction site; it has an estimated allele frequency of 12-15 %[115,116]. The C540T change results in the loss of a *Hae*III site; its estimated frequency is 20-25 %[100,116]. The functional importance of these polymorphisms is not known, but carriers of either of these variants had a significantly shorter progression time from diagnosis of the primary tumor to the appearance of metastasis[117]. On the other hand, presence of the C540T polymorphism in multivariate analysis was significantly associated with improved survival in patients with vertical growth phase tumors[118].

Several additional polymorphisms of the *CDKN2A* gene are known, that do not alter the amino acid sequence of p16 or which are functionally indistinguishable from the wild-type protein[119,120]. The most extensively documented polymorphism, A148T, has previously been shown to have no effect on p16 protein function[119,121]. However, in a latter study, A148T was associated with an increased risk of melanoma development[122]. Furthermore, in a case-control study the A148T polymorphism was detected at a significantly higher proportion in multiple primary melanoma cases compared to healthy controls[123].

The CDK4 Gene

Besides CDKN2A, the CDK4 oncogene on chromosome 12q14 is considered to be another melanoma susceptibility gene; however, only four melanoma-prone kindreds have been reported to carry mutations in CDK4. All mutations involved codon 24 of the gene. Two families carried a Arg24Cys germline point mutation (33), and two other families an Arg24His substitution[37,38]. CDK4 is a key regulator of the cell cycle. Binding to p16 prevents CDK4 from forming a complex with cyclin D, thereby blocking Rb phosphorylation and cell cycle progression[124]. Both types of mutations affect the p16-binding domain of the CDK4 protein, generating an activated oncogene that is resistant to inhibition by p16[36]. Mice with knocked-in Arg24Cys mutation develop pancreatic hyperplasia and are highly susceptible to melanoma development after carcinogenic exposure to 7,12-dimethylbenz(a)anthracene (DMBA) and 12-0-tetradecanoylphorbol-13-acetate (TPA)[125,126].

Melanocortin Receptor 1 (MC1R) Gene

Another critical functional pathway with a major role in melanoma involves pigmentation genes, with a central role for *MC1R*. Human *MC1R* gene consists of a single exon located on chromosome 16q24.3 that encodes a membrane receptor. The gene encoding receptor is highly polymorphic and up to date more than 100 variants have been described, many of which are non-synonymous[127,128]. MC1R is the major contributor to human pigmentation diversity accentuated by the association of the gene variants with a) skin pigmentation variation; b) skin cancer risk including melanoma; c) influence on penetrance of germline CDKN2A mutations in carriers; and d) the frequency of somatic BRAF mutations melanoma tumors[129-133]. Particularly, five single nucleotide polymorphisms associated with red hair, fair skin and freckling are designated as RHC variants. These include the D84E, R142H, R151C, R160W and D294H polymorphisms[134]. Moreover, in Northern European population groups the variants V60L, R142H, R151C, R160W and D294H account for 60 % of all cases of red hair and at least one variant is present in 30 % of that population[135].

Functional analysis of MC1R variants revealed inefficient stimulation of the downstream cAMP pathway. Hypomorphic RHC variants diminish receptor function either due to incomplete integration of the receptor molecule into the melanocytic membrane, diminished binding capacity of the variant receptor to the -MSH ligand or because of defective G-protein activation[128,134,136]. As a consequence, low TYR activity results in synthesis of yel-

low phaeomelanin, which is responsible for the phenotype of red hair and fair skin. It was recently shown that red hair and fair skin represents the phenotype of individuals with truncated MC1R protein, thus the MC1R null genotype[137]. Up-to-date, there is plenty of evidence showing that MC1R and its variants affect more than skin or hair pigmentation variation. The association of polymorphisms in the *MC1R* gene with melanoma is not only due to reduced pigmentation capabilities[138]. Functional studies revealed that *MC1R* variants are also associated with reduced apoptosis and inefficient DNA repair in melanocytes, thereby, pointing to an effect beyond pigmentation traits[139,140].

The RAS-RAF-MEK-ERK Pathway

The RAS-RAF-MEK-ERK pathway is a highly conserved signalling pathway and has been found to play an important role in melanocytic neoplasia[141,142]. Activation of this pathway in cutaneous melanocytes has been shown to occur by a variety of mechanisms that include autocrine growth factor stimulation and oncogenic mutations in the *B-RAF* or *N-RAS* genes[142,143]. RAS proteins are small G-proteins that are anchored on the inner surface of the plasma membrane[144]. Those proteins are downstream of a variety of transmembrane receptors, and are activated when GDP is converted to GTP. In the active GTP-bound state, RAS activates a number of downstream signalling cascades involved in controlling cell growth and behaviour. Initially, RAS interacts with and activates B-RAF that transduces regulatory signals from RAS to MEK1/2. The signal transducer MEK1/2 phosphorylates ERK1/2, leading to activation of these kinases, which in turn activate a variety of transcription factors. ERK phosphorylates many substrates, thereby regulating numerous cellular functions, such as gene expression, metabolism and morphology. Both the duration and intensity of ERK activity are important[145]. Consequently, ERK signalling plays an important role in determining cellular fate, choosing between diverse responses such as proliferation, differentiation, senescence or survival[146].

In melanocytes, ERK is also activated in the cAMP-dependent signalling cascade as a consequence of -melanocyte-stimulating hormone binding to melanocortin-1 receptor with B-RAF as a key intermediate[147,148]. A major way by which ERK signalling promotes cell cycle progression is through transcriptional upregulation of cyclin D1[149]. Cyclin D1 forms a complex with CDK4/6, which phosphorylates the retinoblastoma protein and allows cells to progress from G1 to S phase of the cell cycle. Examples of genes that are transcriptionally induced in response to ERK activation include *VEGF*, a positive regulator of angiogenesis, and *MMP-1*, a collagenase involved in extracellular matrix degradation[150,151]. Sustained ERK activation has also been shown to induce expression of 3-integrin in certain cell types[152]. Proteins such as VEGF, MMP-1 and 3-integrin are believed to play crucial roles in RAS-mediated tumor cell invasion and metastasis[153].

The RAF Genes

Mammals carry three *RAF* genes, *A-RAF*, *B-RAF* and *C-RAF*, which reside on chromosomes Xp11, 7q34 and 3p25, respectively. RAF proteins are structurally related and share three conserved regions (CR1, CR2 and CR3). The N-terminally located CR1 contains the Ras-binding domain as well as a cysteine-rich domain, which also functions to bind Ras[154]. The C-terminally located CR3 region contains the kinase domain. Inactive cytoplasmic Raf upon binding to Ras-GTP is recruited to the cell membrane and is activated through a number of phosphorylation events.

All three RAF proteins activate MEK, but with different intensities and phenotypic differences between *A-RAF*-, *B-RAF*- and *C-RAF*-null mice suggest that individual family members perform distinct functions in development, possibly due to tissue specific differences in expression patterns[155]. Neither b-raf-null nor c-raf-null mice are viable, whereas a-raf-null mice die soon after birth[156-158]. B-raf-null mice die of vascular and neuronal defects[156]. Whereas C-Raf is ubiquitously expressed, A-Raf and B-Raf display a more restricted expression pattern[159].

The B-RAF oncogene encodes a serine/threonine kinase regulated by binding to RAS protein. B-RAF acts in the RAS/RAF/MEK/ERK pathway by transducing regulatory signals from RAS to MEK1/2. B-RAF has a substantially greater basal kinase activity than C-RAF or A-RAF[155,160]. In contrast to C-RAF or A-RAF, B-RAF possesses only two instead of four distinct RAS-GTP-dependend phosphorylation sites for maximal activation (T599 and S602)[155,160]. This structure expedites the activation of B-RAF through a single amino acid substitution. B-RAF is expressed mainly in different neuronal tissues, but also in other organs such as the testis and heart[161].

In 2002, a genome-wide screen for proto-oncogenes showed that the *B-RAF* gene is mutated in a variety of different human cancers[162]. The highest frequencies of *B-RAF* mutations were identified in melanomas (67 %), colorectal (18 %) and ovarian (14 %) cancers[162]. Over 40 different *B-RAF* mutations have been described in the literature; approximately half of those have been functionally analyzed[162-164]. The majority of *B-RAF* mutations result in increased *in vitro* kinase activities of the protein; but also mutants with impaired or no kinase activity have been identified[162,164,165]. The most common mutation found in *B-RAF* is a valine to glutamic acid change at residue 600. The V600E *B-RAF* missense mutation results in

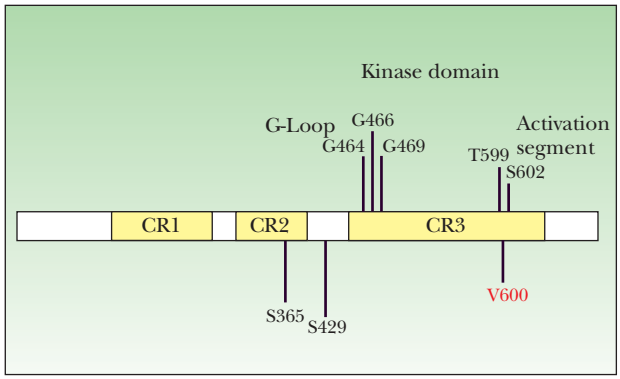

Figure 2. Structure of B-RAF with three conserved domains CR1, CR2 and CR3 that are common to all RAF proteins. The protein is activated by RAS-dependent phosphorylation of T599 and S602 in the activation segment. The G-loop contains a highly conserved glycine motif. A V600E substitution accounts for over 90 percent of all B-RAF mutations.

maximal constitutive activation of kinase activity. The mechanism involves a conformational change mimicking phosphorylation at T599/S602 residues in wild-type *B-RAF* (fig. 2)[162]. The V600E mutant possesses an up to 480-fold greater basal activity and induces transformation of cultured NIH3T3 cells with much higher efficiency compared to the wild-type[162,164].

B-RAF is mutated in about 70% of melanomas[162,166-168]. The frequency of mutations depends on histological subtype and tumor location; a higher frequency has been reported in non-chronically sun-induced damaged sites than in chronically sun-induced damaged ones[169]. Over 20 other *B-RAF* mutations described in melanoma are rather rare. *B-RAF* mutations are also found in up to 80% of melanocytic nevi, indicating that these mutations occur early during melanoma development. However, at the same time, B-RAF activation alone is insufficient to induce melanoma tumorigenesis[170-172]. Spitz nevi, with histological similarity to melanoma lack *B-RAF* mutations[171,173]. Also blue nevi with characteristic coloration do not carry *B-RAF* mutations[171,173]. Expression of V600E mutant B-raf in zebrafish results in nevi but not in melanoma formation. Expression of V600E mutant B-raf in p53-deficient fish readily resulted in invasive melanoma[174]. V600E B-Raf has been shown to transform immortalized mouse melanocytes[175]. Moreover, V600E B-Raf suppression in melanoma cell lines by small interfering RNA (siRNA) resulted in less efficient growth in nude mice compared to control cells[176,177]. Melanoma cells expressing V600E *B-RAF* showed constitutive cyclin D1 expression and downregulation of tumor suppressor $p27^{Kip1}$[178]. Other effects of increased ERK activity mediated by activated B-RAF included altered integrin expression, decreased E-cadherin expression, increased matrix metalloproteinase secretion, invasion, and the regulation of the critical melanocyte transcription factor MITF[179]. However, recent data from animal models and human melanocytes suggest that acquisition of mutations in the B-RAF gene can be a founder event in melanoma genesis without requirement for the loss of p16 for tumor progression[180,181]. In contrast to cutaneous melanoma, development of uveal melanoma also seems to occur via activation of the RAS-RAF-MEK-ERK pathway, but without involvement of mutations in the *B-RAF* or *RAS* genes[182,183].

The RAS Genes

The human RAS proto-oncogenes (H-RAS, K-RAS, and N-RAS) reside on chromosomes 11p15, 1p22 and 12p12, respectively. The three *RAS* genes encode four highly related cell membrane-associated proteins, H-Ras, N-Ras, K-Ras4A and K-Ras4B, that are involved in transduction of extracellular growth and differentiation signals[184]. The four Ras proteins carry identical initial 85 amino acids. This part includes the effector domain (residues 32-40), through which Ras proteins interact with downstream effectors. The N-terminal part also contains two mobile regions named switch I (residues 30-40) and switch II (residues 60-76) regions, both of which undergo conformational changes upon GTP binding. The most C-terminal part of Ras contains a CAAX motif. This motif is subjected to a number of post-translational modifications, which are required for proper anchoring of Ras to the cell membrane[185].

The *RAS* genes are mutated in approximately 30% of all human tumors[186]. Mutations in *K-RAS* are most common, followed by *N-RAS*, whereas mutations in *H-RAS* are rare. High frequencies of *K-RAS* alterations have been found in carcinomas of the pancreas, colon, and lung, whereas *N-RAS* mutations are frequent in myeloid leukemias and melanomas[186]. Most mutations in *RAS* genes are single base changes affecting codons 12, 13, and 61. Mutations in these codons reduce the intrinsic GTPase activity of RAS proteins and also make them insensitive to GTPase-activating proteins[187,188]. As a result, mutated RAS is locked in the GTP-bound state and continuously activates its downstream effector targets. The most frequent *N-RAS* mutations in melanoma occur in codon 61[189]. Mutations in codon 12 and 13 of the *N-RAS* gene are less common. The presence of *N-RAS* mutations in tumor associated nevi and radial growth phase lesions suggests that N-RAS activation occurs at an early stage during melanoma development[189,190]. *N-RAS* mutations are also found in 10% of common acquired nevi and 28-56% of congenital nevi[170,172,191]. *N-RAS* mutations are associated with melanoma arising in chronically sun-exposed rather than intermittently exposed skin[192-194]. Moreover, *N-RAS* mutations are rare in melanomas from sun-protected skin,

indicating that UV radiation may play a role in the genesis of *N-RAS* mutations in melanoma[194]. Suppression of oncogenic *N-RAS* (Q61K) in melanoma cells resulted in increased apoptosis, decreased ERK phosphorylation, and reduced expression of cyclin D1[195]. These data suggest that oncogenic *N-RAS* is important for avoiding apoptosis in melanoma, and imply a role of activating *N-RAS* mutations in melanoma development.

Both functional and genetic evidences indicate that B-RAF and N-RAS act linearly in the RAS-RAF-MEK-ERK signalling pathway, which is evidenced by almost mutual exclusiveness of mutations in these genes and consequent ERK activation[142,143]. However, activated B-RAF effects through mitogen-activated protein cascade; activated RAS effects additionally through phosphotidylinositol (PI3)-kinase and RAL guanine dissociation stimulator cascades[196].

Interaction Between the RAS-RAF-MEK-ERK and Rb/p53 Pathways

The results of several studies suggest that activated N-RAS or B-RAF alone are not able to transform human melanocytes, but require additional, cooperating events for tumor formation. Activating *B-RAF* or *N-RAS* mutations and loss of p16 expression occur at high frequencies in melanomas. In a recent study, both *B-RAF* V600E mutation and p16 inactivation have been found to accompany amplification of the major melanocyte differentiation factor MITF in melanoma cell lines. MITF amplification was more prevalent in metastatic disease and correlated with decreased patient survival[197]. These data identify *MITF* as a possible novel oncogene, which in cooperation with mutated B-RAF, can transform human melanocytes in a p16-deficient background. In human nevi, sustained V600E *B-RAF* expression induced cell cycle arrest, accompanied by both, p16 induction and senescence-associated acidic -galactosidase (SA- -GAL), a classical marker for senescence[198]. Transgenic mice overexpressing oncogenic *N-RAS* (Q61K) did not develop melanoma, but exhibited hyperpigmentation, and persistence of melanocytes in the dermis and epidermis.

Interestingly, when *N-RAS* Q61K transgenic mice were crossed with *p19*-null knockout mice, offspring developed cutaneous metastasizing melanomas within six months of birth[199]. Zebrafish expressing V600E *B-RAF* develop nevi, which require a p53-deficient background to progress to invasive melanomas[174]. Altogether, the results of these studies support the hypothesis that activated N-RAS or B-RAF require cooperating events such as p16 inactivation for melanomagenesis. Moreover, these findings underscore the importance of the interaction between RAS-RAF-MEK-ERK and Rb/p53 pathways in melanoma.

Conclusions

The ever increasing incidence of malignant melanoma makes it an important public health issue. Several risk factors associated with melanoma include exposure to ultraviolet light and a number of host factors. Family history of the disease constitutes one of the most significant risk factors, which is in part explained by germline alterations in the *CDKN2A* and *CDK4* genes. Somatic alterations at the *CDKN2A* gene locus are frequent in sporadic melanoma. Another gene that plays a crucial role in increased susceptibility to melanoma is *MC1R,* which encodes a key component of the pigmentation pathway. *MC1R* variants are associated with high risk phenotypes and melanoma. However, the major pathway with an important role in malignant melanoma is RAS-RAF-MEK-ERK, which is activated by a variety of mechanisms including autocrine growth factor stimulation and oncogenic mutations in the *B-RAF* and *N-RAS* genes. The *B-RAF* is the most frequently mutated gene in melanoma followed by *N-RAS*, and mutations in both genes occur in a mutually exclusive manner. Mutations in the *B-RAF* gene are early events, but melanoma development requires additional loss of check points that mainly occurs in the form of *CDKN2A* aberrations. The *CDKN2A* gene encodes two cell cycle inhibitors that are upstream effectors of the Rb and p53 pathways and gene aberrations may inactivate both critical cell cycle regulator mechanisms. Melanoma progression, therefore, results from active interaction between RAS-RAF-MEK and Rb/p53 pathways. However, melanoma is also characterized by considerable genetic heterogeneity and a number of subtypes can be identified, which might be important for development of therapy. Within the last years, overwhelming amounts of research have contributed to elucidate the molecular genetics of malignant melanoma. The basic module for cancer treatment requires a profound knowledge of the etiology of disease to identify new therapeutic targets.

Conflict of interest
Authors have no conflict of interest to declare.

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