Genetic prognostic factors in uveal melanoma

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Chapter 1

General introduction

Cancer in general

Cancer is characterized by an abnormal expansion of cells, which leads to invasion and destruction of surrounding tissues. A single precursor will divide without respect of normal limits, and this behavior differentiates a malignant tumor from a benign one, which is self-limited in its growth, and does not invade or metastasize (although some benign tumors may be precursors to malignant ones).

The most significant difference between benign and malignant tumors is the metastatic potential of the latter. Individual tumor cells may dislodge from the primary tumor and spread via the blood (hematogenous spread) or lymphatic vessels (lymphatic spread) and form secondary tumors at distant anatomic locations. Cancer may affect people at all ages, but the risk of developing one of the more common varieties tends to increase with age. Together they are the second leading cause of death in the Netherlands, affecting over 95.000 people annually. In 2009, over 42.500 people died from cancer, which has serious psychological, social and financial consequences (Integraal Kankercentrum Nederland, 2010).

Cancer is caused by abnormalities in the genetic material of the tumor cells (Knudson, 2002; Duesberg et al., 2005). This genetic damage may be inherited in the germline, acquired by the action of chemicals, radiation, or micro-organisms, or a combination of these. New aspects of the genetics of cancer pathogenesis, such as epigenetic alterations in the genes responsible for sensing, interpreting, and responding to tissue-specific homeostatic signals are increasingly being recognized as important (Laird and Jaenisch, 1996; Gopalakrishnan et al., 2008; Howell Jr. et al., 2009). The phenotype of the cancerous cell may arise either from genetic alterations that disrupt gene function through sequence modifications (mutations or deletions) or epigenetic modifications that may alter the gene expression (without changing the nucleotide sequence of the genome).

Genes involved in cancer

Several processes are involved in cancer, but because a fundamental characteristic of cancer cells is their uncontrolled proliferation, it is not surprising that many cancer-related genes are involved in the normal cell-cycle regulation (Hanahan and Weinberg 2000; Hanahan and Weinberg, 2011). Some of these genes control cell growth, or stop excessive cell growth, while others control a cell's blood supply or its metastatic spread. Genetic abnormalities found in cancer typically affect two classes of genes. The classes are often referred to as oncogenes and tumor-suppressor genes, depending on whether cancer-causing mutations result in gain or loss of function, respectively. Cancer-promoting oncogenes are often activated in cancer cells, giving those cells new properties, such as continued growth and division, protection against programmed cell death, disruption of tissue boundaries, and the ability to become established in diverse tissue environments. Oncogenes can encode signaling molecules such as growth factors, or components of the signaling cascades that regulate cellular responses. Tumor-suppressor

genes are often inactivated in cancer cells, resulting in the loss of normal function in those cells, such as accurate DNA replication, control over the cell cycle, orientation and adhesion within tissues, and their interaction with protective cells of the immune system. Mutations can knock out a cell-surface receptor for inhibiting factors, or a critical component of the cascades inside the cell that receive and process the signal. Other mutations can disable proteins such as *p53*, which trigger the cell to undergo apoptosis (commit suicide) if the cell's DNA becomes damaged, or when the cell's signaling cascades go out of control. It is commonly assumed that cancer results from a stepwise accumulation of acquired and uncorrected somatic mutations, which is probably also the case in uveal melanoma.

Introduction to uveal melanoma

Melanocytes in the human eye

Melanocytes are melanin-producing cells that provide important physiological functions in the skin, eye, inner ear and meninges (Tolleson, 2005). Recognized functions associated with melanin production in humans include photo-protection, trapping of reactive oxygen species, sequestering metal ions, and binding certain drugs and organic chemicals (Riley, 1992; Riley, 1997). The largest numbers of melanocytes are found in the skin and hair follicles. Another large population resides within the uveal tract of the eye, which embryologically consists of the choroid, ciliary body and iris. Melanocytes from the uveal tract are derived from neural crest cells of the neurectoderm, which migrate following neural tube closure. Neural crest cells migrate to the uveal tract, where they develop into melanocytes. Ocular melanocyte cell numbers vary with age, race and general pigmentation: they are most numerous around the optic disc, less so in the periphery and the inner choroid. Melanocytes determine the pigmentation of the choroid in the mature eye and occur in the choroidal stroma, providing its brown color. The major determinant of color is not the number but rather the activity of the melanocytes. Melanin production takes place in unique organelles known as melanosomes. Darkly pigmented choroids have melanosomes that contain more eumelanin, the most common form of melanin. Choroidal melanocytes form an almost continuous layer in the outer choroid, spreading in the plane of the choroidal space and forming a thin three-dimensional network. This provides melanocytes within the highly vascularized uveal tract with a markedly different microenvironment compared to cutaneous melanocytes that are distributed among clusters of keratinocytes.

Uveal melanoma

A normal uveal melanocyte that acquires malignant properties can develop into a uveal melanoma. Although uveal melanocytes are of the same embryological origin as cutaneous melanocytes, uveal melanomas seem to have a totally different incidence, prevalence and metastatic behavior as skin melanoma. It should be noted that wherever the term uveal melanoma is used in this thesis, it refers to melanoma of the choroid and/or ciliary body. Melanomas of the iris, the third part of the uvea, are excluded from our studies, as these tumors are distinguished from melanomas of the choroid and ciliary

body by their smaller size and relatively benign pathogenesis (Ashton and Wybar, 1966; Jakobiec and Silbert, 1981). Also, their clinical appearance and treatment are totally different (Rones and Zimmermann, 1958).

Epidemiology

Approximately 5% of all malignant melanomas arise in ocular and adnexal structures (Singh and Topham, 2003a). Most ocular melanomas are uveal in origin, whereas primary conjunctival and orbital melanomas are very rare (Chang et al., 1998; Singh and Topham, 2003a). Uveal melanoma accounts for 70% of all primary eye tumors and occurs at an annual incidence of 6 to 8 cases per million people in Caucasian populations (Egan et al., 1988). The age-adjusted annual incidence rate for uveal melanoma in the United States (4.3 per million) has remained stable for the past 25 years and is similar to that reported in European countries (Singh and Topham, 2003a).

Recently, a European analysis of incidence of uveal melanoma in Europe between 1983 and 1994, as well as its geographic and temporal variation, using cancer registry data collected in the framework of the EUROCARE project, also reported a stable incidence of uveal melanoma (Virgili et al., 2008). Interestingly, a north-to south decreasing gradient was found, with standardized incidence rates from a minimum of less than 2 per million in Spain and southern Italy to more than 8 per million in Norway and Denmark. Other studies in France and Denmark have found an incidence of about 7 per million (Vidal et al., 1995; Isager et al., 2005). This incidence is 15 to 50 times lower in Africans and Orientals (Egan et al., 1988). Highest incidence rates are observed in Northern Europe and Australia and the lowest rates among Asian, Hispanic, and black populations, consistent with other observations of lower rates of uveal melanoma in pigmented people (Margo et al., 1998; Vajdic et al., 2003; Hu et al., 2008). The incidence increases between ages 30 and 70 and most often this tumor is observed in the sixth decade of life (Jensen, 1963; Singh and Topham, 2003a). Although uveal melanoma has been recognized and treated for over a century, its cause is only recently being uncovered (Van Raamsdonk et al, 2008; Van Raamsdonk et al., 2010).

Diagnosis and Treatment

About 60% of patients present with complaints such as blurred or distorted vision, visual field loss, floaters or photopsia (Damato, 2001). Sometimes uveal melanomas cause no symptoms and are discovered on routine ocular examination by ophthalmoscopy. About 10% of cases are asymptomatic, usually corresponding to small or medium-sized tumors situated close to the equator of the eye, discovered incidentally on routine ocular fundus examination, such as after cataract surgery. On visual examination, uveal melanomas typically appear as discrete solid tumors, sometimes causing a secondary serous retinal detachment, which can be responsible for visual loss. They may frequently break through Bruch's membrane, extending into the subsensory retinal space. Uveal melanomas may display a discoid, dome-shaped or mushroom-shaped growth pattern. The retina overlying the tumor may show degenerative

changes, occasionally to the point of complete attenuation with tumor perforation into the vitreous cavity. On top of the choroidal melanoma, orange pigment, lipofuscin, may be present. Pigment is due to naturally occurring melanin that comes from melanocytes in the choroidal layer. Choroidal melanomas are usually pigmented, but they can be variably pigmented and even amelanotic (non-pigmented). Non-pigmented choroidal melanoma is due to a proliferation of melanocytes that have lost their ability to make melanin pigment. While this configuration is not diagnostic and limited to uveal melanoma, it is highly characteristic. The Collaborative Ocular Melanoma Study (COMS) showed that the accuracy of correct clinical diagnosis in uveal melanoma is extremely high and can be made based on fundus examination and echography (COMS report no.1., 1990).

Increased understanding and awareness of the disease appears to have led to enhanced diagnosis of patients with smaller size lesions (COMS report no.20., 2003). However, patient survival has remained poor, presumably due to silent hematogenous spreading of micro-metastases prior to the diagnosis of clinically evident disease (Eskelin et al., 2000; Singh and Topham, 2003b; Virgili et al., 2008). At present, it is considered acceptable to delay treatment of melanocytic tumors of indeterminate malignancy until growth is documented (Shields et al., 1995; COMS report no.5., 1997). Fine-needle aspiration biopsy (FNAB) is used in some centers to establish a diagnosis (Sisley et al., 1998; Augsburger et al., 2002). However, the use of FNAB's for prognosis is still under debate (Sandinha et al., 2006; Maat et al., 2007; Schoenfield et al., 2009).

Once the diagnosis of primary uveal melanoma has been made, several treatment modalities are available. In the past, enucleation of the tumor-bearing eye was the only treatment option for uveal melanoma. During the last decades, more eye-conserving treatment modalities have become available. Nowadays, the primary uveal melanoma can often be managed successfully with preservation of the eye and its remaining visual function. Plaque-brachytherapy, particle beam radiotherapy, stereotactic radiotherapy, thermotherapy, transscleral local resection, transretinal resection and diode laser phototherapy are treatment modalities used around the world (Rousseau, 2004; Damato, 2006). In Leiden, plaque-brachytherapy with ruthenium-106 is the current mode of treatment for small to medium-sized tumors. Large or recurrent tumors may undergo proton-beam therapy or enucleation (Keunen and Bleeker, 1997; Keunen et al., 1999).

It should be noted that despite the introduction of these new treatment modalities and diagnostic advances, the rate of metastatic disease has not substantially declined. Untreated, uveal melanomas tend to cause severe loss of vision and eventually an inflamed, unsightly and painful eye. However, a large tumor size in choroidal melanoma decreases the chance that vision-sparing treatments will be successful. In general, the larger the choroidal melanoma, the worse the prognosis for both vision and metastasis (Shields et al., 2009). For uveal melanoma metastases, no effective treatment has been found yet, although isolated liver perfusion and resection of liver metastases has shown some promising

results in selected cases (Pyrhonen, 1998; Missotten and Keunen, 2004). Other treatment modalities are general chemotherapy, chemo-immunotherapy, intra-arterial liver chemotherapy, isolated liver perfusion and immunotherapy (Ksander and Chen, 1999; Becker et al., 2002; Noter et al., 2004; Peters et al., 2006; Schmittel et al., 2006).

Metastases and survival

Approximately 40% to 50% of patients with uveal melanoma will ultimately develop metastases. At the time of diagnosis, over 99% have disease limited to the eye, but at least 30% of these patients will die of systemic metastases at 5 years and 45% at 15 years follow up (Kujala et al., 2003). Metastasis is by vascular spread, as the eye lacks lymphatic vessels and, consequently, the liver is involved first in up to 95% of patients who develop metastatic disease (Char, 1978; Lorigan et al., 1991; Gragoudas et al., 1991). Metastases to the liver remain the primary cause of most morbidity and nearly all mortality in patients with advanced uveal melanoma. When dissemination is diagnosed, 60% of patients have liver metastases and post-mortem examination revealed a more than 90% incidence of metastatic disease (Lorigan et al., 1991). The presence of hepatic metastases is associated with a poor survival with an average median survival of only 6 to 8 months (Bedikian et al., 1981; Bedikian et al., 1995; Kujala et al., 2003).

Predisposing factors

Several parameters that predispose to uveal melanoma have been described, including phenotypic risk factors. There is an elevated risk for Northern European and British ancestry as compared with Southern European or other Mediterranean heritage (Seddon et al., 1990). The racial predisposition has been explained on the basis of susceptibility of Caucasians to the oncogenic effect of sunlight. Several lines of investigation provide support both in favor and against a role for sunlight in the development of uveal melanoma (Egan et al., 1988; Dolin et al., 1994; Moan et al., 2008; Schmidt-Pokrzywniak et al., 2009). Although there is epidemiological data that support the hypothesis that ultraviolet radiation contributes to cutaneous melanoma, no conclusive judgment about the role of sunlight exposure in uveal melanoma can be made (Singh et al., 2004). Host susceptibility factors such as iris color, skin color, hair color and ability to tan are also reported to be associated with uveal melanoma. Blue or grey iris colors appear to be associated with an increased risk for uveal melanoma, as do fair skin color, red or blond hair color, the inability to tan and for the presence of more atypical naevi (Seddon et al., 1990; van Hees et al., 1994; Hammer et al., 1996; Regan et al., 1999; Stang et al., 2003; Richtig et al., 2004; Weis et al., 2006; Smith et al., 2007).

Clinical, histopathological and immunological parameters

Many clinical factors have a proven prognostic value in uveal melanoma and a wide variety of parameters have been related to survival. However, few are specific. Major well-known and established factors are age, large basal tumor diameter, tumor prominence or thickness, tumor involvement of the ciliary body,

extrascleral extension, growth rate, and initial tumor regression rate after radiotherapy (Augsburger and Gamel, 1990; Mooy et al., 1991; Bedikian et al., 2008). Histopathological prognostic factors include location (iris, ciliary body or choroid), extraocular extension, growth pattern, cell type (Callender classification; Callender, 1931) or its modification (McLean et al., 1978; McLean et al., 1983), the number of mitosis, and the presence of tumor-infiltrating lymphocytes and macrophages and specific vasculogenic mimicry patterns (Seddon et al., 1983a; Coleman et al., 1993; Folberg et al., 1993; Foss et al., 1996; Foss et al., 1997).

Other important parameters related to prognosis include immunological determinants such as Human Leukocyte Antigen (HLA) expression and leukocyte infiltration (de la Cruz Jr. et al., 1990; de Waard-Siebinga et al., 1996; Blom et al., 1997; Ericsson et al., 2001; Dithmar et al., 2002; Jager et al., 2002). Correlations between certain HLA alleles and specific diseases have been described in autoimmune disorders (Lopez-Larrea et al., 1998). Also, correlations between HLA antigens and cutaneous melanoma have been reported: HLA-B40, -DR4, and –DR5 were found to be related to cutaneous malignant melanoma (Dieckhues et al., 1979; Pollack and Livingston, 1985), whereas HLA-B40 and the Class II alleles HLA-DR11 and -DQ7 were related to local recurrences. It was suggested that Class II genes influence cytokine production and thus influence local immune responses against metastases (Lee et al., 2002). In uveal melanoma, studies failed to show that HLA antigens contributed to an increased genetic susceptibility, but this does not exclude an important role for HLA antigens in immune surveillance against uveal melanoma and their metastases (Maat et al., 2006). Increased expression of HLA Class I as well as of Class II expression of the primary tumor carries an unfavorable prognosis, occurs more frequently in epithelioid tumors, and is associated with an increased number of CD3+ and CD4+ T-lymphocytes, as well as with an increased density of CD11b+ macrophages (de Waard-Siebinga et al., 1996; Maat et al., 2008b).

In order to increase the understanding of prognostic factors and behavior of uveal melanomas, cytogenetic, genetic and epigenetic markers as prognostic factors will be discussed in the next paragraph.

Genetics and epigenetics

Genetic and cytogenetic factors

From the late eighties on, several reports on the cytogenetic analysis of uveal melanoma have been published. The first molecular abnormalities described in uveal melanoma were gross chromosomal alterations in cultured uveal melanoma cells of chromosomes 3, 6 and 8 in a series of six posterior uveal melanomas (Sisley et al., 1990). Gain or loss of chromosomal material in chromosomes 3, 6, and 8, has been validated in primary uveal melanomas and are associated with prognostic outcome (Seddon et al., 1983b; Horsman et al., 1990; Prescher et al., 1990; Sisley et al., 1990;). The most frequent change, in approximately 50% of all uveal melanomas, is the loss of one copy of chromosome 3 (Horsman et al., 1990; Sisley et al.,

1990). Loss of one of the 2 copies of chromosome 3, i.e. monosomy 3 or -3, was one of the first, and still the most important, chromosomal alteration that has been described in uveal melanoma. Follow-up of patients having tumors with monosomy 3 showed that 57% developed metastases within 3 years, in contrast to patients with tumors that retained both copies of chromosome 3, who only rarely developed metastases (Prescher et al., 1990). Furthermore, monosomy 3 is correlated with other poor prognostic indicators such as larger tumor diameter, epithelioid cell type, extravascular matrix patterns and ciliary body involvement (Scholes et al., 2003; Ehlers and Harbour, 2006). However, till today it remains unclear how monosomy 3 contributes causally to uveal melanoma development and progression. Another common chromosome abnormality found in uveal melanoma is gain of extra copies of chromosome 8q (isochromosome 8q or i8q) (Horsman et al., 1990; Aalto et al., 2001). Gain of chromosomal arm 8q is found in approximately 40-65% of the tumors and is almost as strongly linked with metastatic disease as is monosomy 3 (Horsman et al., 1990; Speicher et al., 1994; Ghazvini et al., 1996; Sisley et al., 1997). Consequently, they are often found together (Horsman and White, 1993; White et al., 1998; Damato et al., 2007). Also other chromosome changes such as loss of chromosome 1p, gain of 6p and loss of chromosome 6q, seem to be involved in survival (Prescher et al., 1990; Aalto et al., 2001; Hausler et al., 2005). Furthermore, loss of chromosome 2, 21 and the sex chromosomes have been reported (Mukai and Dryja, 1986; Griffin et al., 1988; Horsman et al., 1990; Prescher et al., 1990; Sisley et al., 1990; Sisley et al., 1992; Wiltshire et al., 1993; Singh et al., 1994; Prescher et al., 1994; Tschentscher et al., 2000).

Gene-expression profiling

As shown, the chromosomal aberrations found in uveal melanoma on chromosome 3, 6 and 8q have been linked to metastatic death (Prescher et al., 1996; Sisley et al., 1997). For many years, it remained unclear whether these chromosomal aberrations are simply markers of tumor progression or whether these are associated with deregulation of specific genes (Loercher and Harbour, 2003). In recent years, there have been important breakthroughs in unraveling the molecular basis of uveal melanoma and its tendency to metastatic disease. Recently, it was shown that primary uveal melanomas cluster into two distinct molecular classes based on gene-expression profiles of roughly equal proportions (Onken et al., 2004). Tumors with the class I gene expression profile rarely metastasize, whereas those with the class II gene expression profile have a very high rate of metastasis (Onken et al., 2004; Petrausch et al., 2007; Worley et al., 2007; van Gils et al., 2008). Genes that discriminate class I (low-grade) from class 2 (high-grade) include highly significant clusters of down-regulated genes on chromosome 3 and up-regulated genes on chromosome 8q and provide insights into the mechanism underlying metastasis (van Gils et al., 2008). Biological function annotations of the most differentiating genes included cell communication, development, cell growth, cell motility and cell death. Most of the developmental genes have been implicated in neural crest development, which gives rise to melanocytes. The expression profile of class I tumors is only slightly different than of normal uveal melanocytes, suggesting that relatively few genetic changes have occurred. In contrast, the class II expression profile is very different from melanocytes and resembles primitive stemcell-like ectodermal cells (Chang et al, 2008; Onken et al., 2010).

Cell-cycle deregulation

Mutational deregulation of the cell cycle is a hallmark of tumorigenesis (Hanahan and Weinberg, 2000). The protein product of the Retinoblastoma gene (*Rb*) plays a central role as inhibitor of cellular proliferation (Bartek et al., 1997), so inactivation of the *Rb* gene leads to unregulated proliferation. Despite the absence of mutations of this gene in uveal melanoma, *Rb* is frequently mutated in many different types of cancer, such as retinoblastoma. It has been known for several years that, in uveal melanoma, disruption of the retinoblastoma tumor-suppressor pathway is common by hyperphosphorylation of *Rb*, allowing cells to re-enter the cell cycle (Brantley Jr. and Harbour, 2000a; Brantley Jr. and Harbour, 2000b). Progression of cells through the G1 phase of the cell-cycle is stimulated by the association of D-type cyclins with cyclin-dependent kinases (CDK's) that phosphorylate *Rb* (Sherr, 1993). Normally, *Rb* inhibits proliferation by arresting cells in the G1 phase of the cell cycle. For cell division to occur, *Rb* is hyperphosphorylated and inactivated by CDK's that interact with their cyclin partners to form active kinase complexes. CDK's are in turn restrained by inhibitors such as p16, which block CDK4/6 and allow hypophosphorylated *Rb* to accumulate. The result of these interactions is a tightly regulated pathway that allows cell division only under appropriate physiological circumstances (Brantley Jr. and Harbour, 2000b).

The gene that encodes for p16 (*CDKN2A*) has been identified as an inhibitor of the cyclin D/CDK complex (Serrano et al., 1993). The inhibitory activity of p16 is restricted to the cyclin D-CDK4 and cyclin D-CDK6 kinases and results in cell cycle control at the G1-S restriction point (Mao et al., 1995; van der Velden et al., 2001). *CDKN2A* is commonly inactivated in a wide range of malignancies (Sharpless and DePinho, 1999), but *CDKN2A* germ-line mutations are uniquely associated with familial cutaneous melanoma (Hussussian et al., 1994; Gruis et al., 1995; Harland et al., 1997). Whereas *CDKN2A* is the main target of inactivation in cutaneous melanoma, mutation screening and deletion mapping did not reveal such a role for *CDKN2A* in uveal melanoma (Merbs and Sidransky, 1999). Analysis of uveal melanoma cell lines and primary tumors revealed promoter methylation of *CDKN2A* as an alternative mechanism for tumor suppressor-gene inactivation (van der Velden et al., 2001). Furthermore, recent analysis revealed that other important genes such as *TIMP3* and *RASSF1a* are also inactivated by methylation, suggesting that epigenetic events are a common phenomenon in uveal melanoma.

Epigenetics

Epigenetics is a term in biology used to refer to chromatin and DNA modifications of unicellular and multicellular organisms that are stable over rounds of cell division but do not involve changes in the underlying DNA sequence of the organism (Bird, 2007). These epigenetic changes play a role in the process of cellular differentiation, allowing cells to stably maintain different characteristics despite containing the same genomic material. DNA methylation in human cells denotes the covalent addition of a methyl group to the 5' position of the cytosine ring on the DNA. In vertebrates, it typically occurs at CpG sites (cytosine-phosphate-guanine sites; that is, where a cytosine is directly followed by a guanine

in the DNA sequence). Attachment of such a methyl group to a cytosine results in conversion to a 5-methylcytosine, which process is catalyzed by a group of enzymes called DNA methyltransferases. CpG sites are uncommon in vertebrate genomes but are often found at higher density near gene promoters where they are collectively referred to as CpG islands. The methylation state of these CpG sites can have a major impact on gene activity and expression. Under physiological conditions, methylation is associated with the distinct, but mechanistically related, process of X chromosome inactivation (silencing of one X chromosome but not the other in all human female cells), genomic imprinting (silencing or activation of a gene inherited from one parent or the other) or transcriptional silencing of repetitive DNA sequences (Wolf and Migeon, 1982; Barlow, 1995; Kochanek et al., 1995).

Alterations of gene expression may also be obtained during carcinogenesis through a process called de novo methylation of CpG islands in gene promoters (Bestor and Verdine, 1994; Okano et al., 1998). Changes in methylation of the promoter or the first exon may have enormous effects on the expression of tumor-suppressor genes or proto-oncogenes. Hypermethylation of promoter regions may cause transcriptional silencing of tumor suppressor genes. On the other hand, hypomethylation of regulatory DNA sequences might activate transcription of proto-oncogenes, as well as genes encoding proteins involved in genomic instability or metastatic behavior. In cancer research, DNA methylation is often regarded as the epigenetic mechanism that blocks gene expression. Methylation of promoter-associated CpG islands has recently emerged as an important epigenetic mechanism leading to the transcriptional silencing or downregulation of tumor-suppressor genes in cancer development (Jones and Baylin, 2002). Methylation of tumor-suppressor genes is now commonly analyzed in tumors and even rivals mutation and deletion as the main mechanism in tumor development in certain tumors (Robertson, 2005). For example, in cutaneous melanoma, at least 50 genes have been identified to date to be silenced during disease development and progression by promoter hypermethylation (van Doorn et al. 2005; Rothhammer and Bosserhoff, 2007). Although numerous studies have addressed the genetic events involved in the development of uveal melanoma, only a few have focused on the epigenetic events that occur during tumorigenesis (see next paragraph).

MAPK Pathway activation

Activation of the RAS-RAF-MEK-ERK, or mitogen-activated protein kinase (MAPK) pathway, is crucial for the development of melanocytic neoplasia (Cohen et al., 2002). This pathway is perhaps the most common signaling pathway affected by early oncogenic mutations. Mutations in *B-RAF*, *N-RAS*, *H-RAS* and *KIT* lead to constitutive activation of this pathway and have been associated with many different types of cancer (Goding, 2000; Reddy et al., 2003). Constitutive activation of the RAF-MEK-ERK pathway stimulates the transcription of pro-proliferative genes, such as *CCND1*, *JUN* and *MYC* (Dahl and Guldberg, 2007; McCubrey et al., 2007)

In cutaneous melanoma, activation of this pathway has been shown to occur by a variety of mechanisms, including autocrine growth factor stimulation and mutation of the *N-RAS* (20% of cases) and *B-RAF* (60% of cases) genes (van Elsas et al., 1995; Davies et al., 2002; Satyamoorthy et al., 2003). All *B-RAF* mutations in cutaneous pigmented neoplasms occur within the kinase domain, and the most frequently found mutation in *B-RAF* consists of a 1799T⟶ A transversion in exon 15, although various other mutations have been described as well (Brose et al., 2002; Davies et al., 2002; Pollock and Meltzer, 2002; Satyamoorthy et al., 2003). This T1799A mutation is located in the serine/threonine kinase domain of *B-RAF*, resulting in a valine-to-glutamic acid substitution at position 600 (*B-RAF* V600E), leading to a constitutive activation of proliferation signaling (Zhang and Guan, 2000; Wellbrock et al., 2004). Interestingly, *B-RAF* mutations occur very early in cutaneous malignant melanoma, and are even present in benign and pre-malignant nevi (Davies et al., 2002; Pollock and Meltzer, 2002).

Since cutaneous malignant melanoma and uveal melanoma both arise from neural-crest derived melanocytes, the MAPK pathway came also under attention in uveal melanoma research (Mooy et al., 1991; Soparker et al., 1993; Cohen et al., 2003; Cruz, III et al., 2003; Edmunds et al., 2003; Rimoldi et al., 2003; Weber et al., 2003; Kilic et al., 2004; Zuidervaart et al., 2005; Calipel et al., 2006). Activation of the MAPK pathway has been reported in uveal melanoma, although it only rarely occurs through mutations in *B-RAF* or *RAS* (Zuidervaart et al., 2005). Recently, others and we have found that uveal melanoma is heterogeneous and that, with more sensitive techniques, the percentage of mutant *B-RAF*-positive uveal melanomas may be slightly higher (Janssen et al., 2008; Maat et al., 2008a). The lack of mutations in the majority of cells is in contrast with immunohistochemistry and western blot analysis, which have shown activation of ERK1/2 in most uveal melanomas (Rimoldi et al., 2003; Weber et al., 2003; Zuidervaart et al., 2005). Furthermore, the RAF-MEK-ERK pathway target *CCND1*, which encodes cyclin D1, is over-expressed in most uveal melanomas (Brantley Jr. and Harbour, 2000a; Coupland et al., 2000), and leads to hyperphosphorylation and inactivation of the retinoblastoma tumor suppressor (Rb) in uveal melanoma (Brantley Jr. and Harbour, 2000b; Delston and Harbour, 2006). *CCND1* overexpression is likely transcriptionally mediated by activation of the RAF-MEK-ERK pathway, since amplification of Rb in uveal melanoma is rare (Glatz-Krieger et al., 2006). Nevertheless, the pharmacological inhibition of MAPK/ERK kinases 1 and 2 (MEK1/2) and the genetic targeting of *BRAF* with siRNA resulted in a reduced proliferation of uveal melanoma cell lines (Lefevre et al., 2004; Calipel et al., 2006). This indicates that although mutations are absent, the RAS-RAF-MEK-ERK pathway is essential for uveal melanoma growth and suggests that an upstream factor is involved in autonomous uveal melanoma proliferation. Recently, an alternative route to MAPK pathway activation in melanocytic neoplasia was found (see summary and general discussion).

Outline

Uveal melanoma is the most common malignancy of the eye in adults and it is the second most common form of melanoma after cutaneous melanoma (Mooy and De Jong, 1996; Bergman et al., 2003; Singh and Topham, 2003a). The identification of patients who have a high risk of developing metastases would allow the possibility of providing adjuvant therapies to prevent metastases once such therapies have been developed or may allow close monitoring for the presence of liver metastases in such individuals, who may then be offered liver resection surgery or chemotherapy at an early stage (Missotten and Keunen, 2004). The application of fluorescence in-situ hybridization (FISH) on transvitreal fine-needle aspiration biopsies (FNAB's) is thought to be a reliable method for assaying genetic parameters such as chromosome 3 loss (Naus et al., 2002). However, this is based on the assumption that this chromosomal abnormality is distributed homogeneously throughout the tumor. In *chapter 2* we investigate the distribution of monosomy 3 in primary uveal melanoma by fluorescence in-situ hybridization and show that uveal melanomas can be heterogeneous for the number of copies of chromosome 3. In *chapter 3* we investigated whether, besides for chromosomal aberrations, any evidence can be found for heterogeneity in the regulation of tumor-suppressor genes. The tumor-suppressor gene *RASSF1a*, which is located on chromosome 3p21.3, has been shown to be inactivated by hypermethylation in several human malignancies, including cutaneous melanoma (Spugnardi et al., 2003; Kang et al., 2004; Yeo et al., 2005; Fukasawa et al., 2006). Recently, a segregation study in families with uveal and cutaneous melanoma identified 9q21 as a potential locus harboring a tumor-suppressor gene. One of the genes in this area, *RASEF*, was then analyzed as a candidate tumor-suppressor gene, but lack of point mutations and copy number changes could not confirm this. In *chapter 4,* we studied whether in uveal melanoma, the *RASEF* gene was affected by mutations or gene silencing due to promoter methylation.

The RAS-RAF-MEK-ERK pathway is involved in the balance between melanocyte proliferation and differentiation. In cutaneous and uveal melanoma, the same pathway is constitutively activated and related to tumor growth and survival. Whereas mutant *B-RAF* and *N-RAS* are responsible for the activation of the RAS-RAF-MEK-ERK pathway in most cutaneous melanoma, mutations in these genes are usually absent in uveal melanoma. Nowadays, an assay with increased potential to identify mutations is available. In *chapter 5,* we set out to reanalyze uveal melanoma cell lines and primary uveal melanomas for *B-RAF* mutations by using pyrophosphorolysis-activated polymerization. In *chapter 6* we set out to explore the RAS-RAF-MEK-ERK pathway by using mitogen-activated protein kinase profiling and tyrosine kinase arrays.

Finally, conclusions drawn from above mentioned studies are summarized and put into perspective in *chapter 7*.

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