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Genetic prognostic factors in uveal melanoma

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Genetic Prognostic Factors in Uveal Melanoma



Willem Maat

Genetic Prognostic Factors in Uveal Melanoma

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Genetic Prognostic Factors in Uveal Melanoma

Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof. mr. P.F. van der Heijden,
volgens besluit van het College voor Promoties
te verdedigen op dinsdag 4 september 2012
klokke 15.00 uur
door

Willem Maat

geboren te Gorinchem
in 1978



Universiteit Leiden

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Dr. N.A. Gruis

U spoor ik aan beste jongelui, de geneeskunde aan het hart te drukken,
en zich met alles wat u in zich hebt aan haar over te geven,
daar zij u aanzien, roem, gezag en rijkdom zal doen toekomen,
en u op uw beurt met haar hulp aan uw vrienden, aan uw vaderland,
ja aan de gehele mensheid geen geringe voordelen zult schenken.

Desiderius Erasmus, 1500 AD

Uit: *Lof der Geneeskunde*

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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 Gulberg, 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*.

References

- Aalto Y, Eriksson L, Seregard S, Larsson O, Knuutila S. Concomitant loss of chromosome 3 and whole arm losses and gains of chromosome 1, 6, or 8 in metastasizing primary uveal melanoma. *Invest Ophthalmol Vis Sci.* 2001 Feb;42(2):313-7.
- Ashton N, Wybar K. Primary tumours of the iris. *Ophthalmologica.* 1966;151(1):97-113.
- Augsburger JJ, Corrêa ZM, Schneider S, Yassin RS, Robinson-Smith T, Ehya H, Trichopoulos N. Diagnostic transvitreal fine-needle aspiration biopsy of small melanocytic choroidal tumors in nevus versus melanoma category. *Trans Am Ophthalmol Soc.* 2002;100:225-32; discussion 232-4.
- Augsburger JJ, Gamel JW. Clinical prognostic factors in patients with posterior uveal malignant melanoma. *Cancer.* 1990 Oct 1;66(7):1596-600.
- Barlow DP. Gametic imprinting in mammals. *Science.* 1995 Dec 8;270(5242):1610-3. Review.
- Bartek J, Bartkova J, Lukas J. The retinoblastoma protein pathway in cell cycle control and cancer. *Exp Cell Res.* 1997 Nov 25;237(1):1-6. Review.
- Becker JC, Terheyden P, Kämpgen E, Wagner S, Neumann C, Schadendorf D, Steinmann A, Wittenberg G, Lieb W, Bröcker EB. Treatment of disseminated ocular melanoma with sequential fotemustine, interferon alpha, and interleukin 2. *Br J Cancer.* 2002 Oct 7;87(8):840-5.
- Bedikian AY, Johnson MM, Warneke CL, Papadopoulos NE, Kim K, Hwu WJ, McIntyre S, Hwu P. Prognostic factors that determine the long-term survival of patients with unresectable metastatic melanoma. *Cancer Invest.* 2008 Jul;26(6):624-33.
- Bedikian AY, Kantarjian H, Young SE, Bodey GP. Prognosis in metastatic choroidal melanoma. *South Med J.* 1981 May;74(5):574-7.
- Bedikian AY, Legha SS, Mavligit G, Carrasco CH, Khorana S, Plager C, Papadopoulos N, Benjamin RS. Treatment of uveal melanoma metastatic to the liver: a review of the M. D. Anderson Cancer Center experience and prognostic factors. *Cancer.* 1995 Nov 1;76(9):1665-70.
- Bergman L, Seregard S, Nilsson B, Lundell G, Ringborg U, Ragnarsson-Olding B. Uveal melanoma survival in Sweden from 1960 to 1998. *Invest Ophthalmol Vis Sci.* 2003 Aug;44(8):3282-7.
- Bestor TH, Verdine GL. DNA methyltransferases. *Curr Opin Cell Biol.* 1994 Jun;6(3):380-9. Review.
- Bird A. Perceptions of epigenetics. *Nature.* 2007 May 24;447(7143):396-8.
- Blom DJ, Luyten GP, Mooy C, Kerkvliet S, Zwinderman AH, Jager MJ. Human leukocyte antigen class I expression. Marker of poor prognosis in uveal melanoma. *Invest Ophthalmol Vis Sci.* 1997 Aug;38(9):1865-72.
- Brantley MA Jr, Harbour JW. Deregulation of the Rb and p53 pathways in uveal melanoma. *Am J Pathol.* 2000 Dec;157(6):1795-801.
- Brantley MA Jr, Harbour JW. Inactivation of retinoblastoma protein in uveal melanoma by phosphorylation of sites in the COOH-terminal region. *Cancer Res.* 2000 Aug 15;60(16):4320-3.
- Brose MS, Volpe P, Feldman M, Kumar M, Rishi I, Gerrero R, Einhorn E, Herlyn M, Minna J, Nicholson A, Roth JA, Albelda SM, Davies H, Cox C, Brignell G, Stephens P, Futreal PA, Wooster R, Stratton MR, Weber BL. BRAF and RAS mutations in human lung cancer and melanoma. *Cancer Res.* 2002 Dec 1;62(23):6997-7000.
- Calipel A, Mouriaux F, Glotin AL, Malecize F, Faussat AM, Mascarelli F. Extracellular signal-regulated kinase-dependent proliferation is mediated through the protein kinase A/B-Raf pathway in human uveal melanoma cells. *J Biol Chem.* 2006 Apr 7;281(14):9238-50.
- Callender GR. Malignant melanotic tumors of the eye: a study of histologic types in 11 cases. *Trans Am Acad Ophthalmol Otolaryn.* 1931 (36):131-142.
- Chang AE, Karnell LH, Menck HR. The National Cancer Data Base report on cutaneous and noncutaneous melanoma: a summary of 84,836 cases from the past decade. The American College of Surgeons Commission on Cancer and the American Cancer Society. *Cancer.* 1998 Oct 15;83(8):1664-78.
- Chang SH, Worley LA, Onken MD, Harbour JW. Prognostic biomarkers in uveal melanoma: evidence for a stem cell-like phenotype associated with metastasis. *Melanoma Res.* 2008 Jun;18(3):191-200.
- Char DH. Metastatic choroidal melanoma. *Am J Ophthalmol.* 1978 Jul;86(1):76-80.

- Cohen C, Zavala-Pompa A, Sequeira JH, Shoji M, Sexton DG, Cotsonis G, Cerimele F, Govindarajan B, Macaron N, Arbiser JL. Mitogen-activated protein kinase activation is an early event in melanoma progression. *Clin Cancer Res*. 2002 Dec;8(12):3728-33.
- Cohen Y, Goldenberg-Cohen N, Parrella P, Chowers I, Merbs SL, Pe'er J, Sidransky D. Lack of *BRAF* mutation in primary uveal melanoma. *Invest Ophthalmol Vis Sci*. 2003 Jul;44(7):2876-8.
- Coleman K, Baak JP, Van Diest P, Mullaney J, Farrell M, Fenton M. Prognostic factors following enucleation of 111 uveal melanomas. *Br J Ophthalmol*. 1993 Nov;77(11):688-92.
- Collaborative Ocular Melanoma Study Group. COMS report no. 1. Accuracy of diagnosis of choroidal melanomas in the Collaborative Ocular Melanoma Study. *Arch Ophthalmol*. 1990 Sep;108(9):1268-73.
- Collaborative Ocular Melanoma Study Group. COMS Report No. 5. Factors predictive of growth and treatment of small choroidal melanoma. The Collaborative Ocular Melanoma Study Group. *Arch Ophthalmol*. 1997 Dec;115(12):1537-44.
- Collaborative Ocular Melanoma Study Group. COMS report no. 20. Trends in size and treatment of recently diagnosed choroidal melanoma, 1987-1997: findings from patients examined at collaborative ocular melanoma study (COMS) centers. *Arch Ophthalmol*. 2003 Aug;121(8):1156-62.
- Coupland SE, Anastassiou G, Stang A, Schilling H, Anagnostopoulos I, Bornfeld N, Stein H. The prognostic value of cyclin D1, p53, and MDM2 protein expression in uveal melanoma. *J Pathol*. 2000 Jun;191(2):120-6.
- Cruz F 3rd, Rubin BP, Wilson D, Town A, Schroeder A, Haley A, Bainbridge T, Heinrich MC, Corless CL. Absence of *BRAF* and *NRAS* mutations in uveal melanoma. *Cancer Res*. 2003 Sep 15;63(18):5761-6.
- Dahl C, Guldberg P. The genome and epigenome of malignant melanoma. *APMIS*. 2007 Oct;115(10):1161-76. Review.
- Damato B, Duke C, Coupland SE, Hiscott P, Smith PA, Campbell I, Douglas A, Howard P. Cytogenetics of uveal melanoma: a 7-year clinical experience. *Ophthalmology*. 2007 Oct;114(10):1925-31.
- Damato B. Treatment of primary intraocular melanoma. *Expert Rev Anticancer Ther*. 2006 Apr;6(4):493-506. Review.
- Damato B. Detection of uveal melanoma by optometrists in the United Kingdom. *Ophthalmic Physiol Opt*. 2001 Jul;21(4):268-71.
- Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, Teague J, Woffendin H, Garnett MJ, Bottomley W, Davis N, Dicks E, Ewing R, Floyd Y, Gray K, Hall S, Hawes R, Hughes J, Kosmidou V, Menzies A, Mould C, Parker A, Stevens C, Watt S, Hooper S, Wilson R, Jayatilake H, Gusterson BA, Cooper C, Shipley J, Hargrave D, Pritchard-Jones K, Maitland N, Chenevix-Trench G, Riggins GJ, Bigner DD, Palmieri G, Cossu A, Flanagan A, Nicholson A, Ho JW, Leung SY, Yuen ST, Weber BL, Seigler HF, Darrow TL, Paterson H, Marais R, Marshall CJ, Wooster R, Stratton MR, Futreal PA. Mutations of the *BRAF* gene in human cancer. *Nature*. 2002 Jun 27;417(6892):949-54.
- de la Cruz PO Jr, Specht CS, McLean IW. Lymphocytic infiltration in uveal malignant melanoma. *Cancer*. 1990 Jan 1;65(1):112-5.
- de Waard-Siebinga I, Hilders CG, Hansen BE, van Delft JL, Jager MJ. HLA expression and tumor-infiltrating immune cells in uveal melanoma. *Graefes Arch Clin Exp Ophthalmol*. 1996 Jan;234(1):34-42.
- Delston RB, Harbour JW. Rb at the interface between cell cycle and apoptotic decisions. *Curr Mol Med*. 2006 Nov;6(7):713-8. Review.
- Dieckhues B, Jünemann G, Küchle HJ, Püttmann T. [HLA-antigens in eye diseases (author's transl)]. *Klin Monbl Augenheilkd*. 1979 Nov;175(5):681-5.
- Dithmar S, Crowder J, Jager MJ, Vigniswaran N, Grossniklaus HE. [HLA class I antigen expression correlates with histological cell type in uveal melanoma]. *Ophthalmologe*. 2002 Aug;99(8):625-8.
- Dolin PJ, Foss AJ, Hungerford JL. Uveal melanoma: is solar ultraviolet radiation a risk factor? *Ophthalmic Epidemiol*. 1994 Mar;1(1):27-30.
- Duesberg P, Li R, Fabarius A, Hehlmann R. The chromosomal basis of cancer. *Cell Oncol*. 2005;27(5-6):293-318. Review.
- Edmunds SC, Cree IA, Di Nicolantonio F, Hungerford JL, Hurren JS, Kelsell DP. Absence of *BRAF* gene mutations in uveal melanomas in contrast to cutaneous melanomas. *Br J Cancer*. 2003 May 6;88(9):1403-5.
- Egan KM, Seddon JM, Glynn RJ, Gragoudas ES, Albert DM. Epidemiologic aspects of uveal melanoma. *Surv Ophthalmol*. 1988 Jan-Feb;32(4):239-51. Review.
- Ehlers JP, Harbour JW. Molecular pathobiology of uveal melanoma. *Int Ophthalmol Clin*. 2006 Winter;46(1):167-80. Review.

- Elwood JM, Jopson J. Melanoma and sun exposure: an overview of published studies. *Int J Cancer*. 1997 Oct 9;73(2):198-203. Review.
- Ericsson C, Seregard S, Bartolazzi A, Levitskaya E, Ferrone S, Kiessling R, Larsson O. Association of HLA class I and class II antigen expression and mortality in uveal melanoma. *Invest Ophthalmol Vis Sci*. 2001 Sep;42(10):2153-6.
- Eskelin S, Pyrhönen S, Summanen P, Hahka-Kemppinen M, Kivelä T. Tumor doubling times in metastatic malignant melanoma of the uvea: tumor progression before and after treatment. *Ophthalmology*. 2000 Aug;107(8):1443-9.
- Folberg R, Rummelt V, Parys-Van Ginderdeuren R, Hwang T, Woolson RF, Pe'er J, Gruman LM. The prognostic value of tumor blood vessel morphology in primary uveal melanoma. *Ophthalmology*. 1993 Sep;100(9):1389-98.
- Foss AJ, Alexander RA, Hungerford JL, Harris AL, Cree IA, Lightman S. Reassessment of the PAS patterns in uveal melanoma. *Br J Ophthalmol*. 1997 Mar;81(3):240-6; discussion 247-8.
- Foss AJ, Alexander RA, Jefferies LW, Hungerford JL, Harris AL, Lightman S. Microvessel count predicts survival in uveal melanoma. *Cancer Res*. 1996 Jul 1;56(13):2900-3.
- Fukasawa M, Kimura M, Morita S, Matsubara K, Yamanaka S, Endo C, Sakurada A, Sato M, Kondo T, Horii A, Sasaki H, Hatada I. Microarray analysis of promoter methylation in lung cancers. *J Hum Genet*. 2006;51(4):368-74.
- Ghazvini S, Char DH, Kroll S, Waldman FM, Pinkel D. Comparative genomic hybridization analysis of archival formalin-fixed paraffin-embedded uveal melanomas. *Cancer Genet Cytogenet*. 1996 Sep;90(2):95-101.
- Gilchrist BA, Eller MS, Geller AC, Yaar M. The pathogenesis of melanoma induced by ultraviolet radiation. *N Engl J Med*. 1999 Apr 29;340(17):1341-8. Review.
- Glatz-Krieger K, Pache M, Tapia C, Fuchs A, Savic S, Glatz D, Mihatsch M, Meyer P. Anatomic site-specific patterns of gene copy number gains in skin, mucosal, and uveal melanomas detected by fluorescence in situ hybridization. *Virchows Arch*. 2006 Sep;449(3):328-33.
- Goding CR. Mitf from neural crest to melanoma: signal transduction and transcription in the melanocyte lineage. *Genes Dev*. 2000 Jul 15;14(14):1712-28. Review.
- Gopalakrishnan S, Van Emburgh BO, Robertson KD. DNA methylation in development and human disease. *Mutat Res*. 2008 Dec 1;647(1-2):30-8.
- Gragoudas ES, Egan KM, Seddon JM, Glynn RJ, Walsh SM, Finn SM, Munzenrider JE, Spar MD. Survival of patients with metastases from uveal melanoma. *Ophthalmology*. 1991 Mar;98(3):383-9; discussion 390.
- Griffin CA, Long PP, Schachat AP. Trisomy 6p in an ocular melanoma. *Cancer Genet Cytogenet*. 1988 May;32(1):129-32.
- Gruis NA, Weaver-Feldhaus J, Liu Q, Frye C, Eeles R, Orlow I, Lacombe L, Ponce-Castaneda V, Lianes P, Latres E, et al. Genetic evidence in melanoma and bladder cancers that p16 and p53 function in separate pathways of tumor suppression. *Am J Pathol*. 1995 May;146(5):1199-206.
- Hammer H, Oláh J, Tóth-Molnár E. Dysplastic nevi are a risk factor for uveal melanoma. *Eur J Ophthalmol*. 1996 Oct-Dec;6(4):472-4.
- Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell*. 2000 Jan 7;100(1):57-70. Review.
- Harland M, Meloni R, Gruis N, Pinney E, Brookes S, Spurr NK, Frischauf AM, Bataille V, Peters G, Cuzick J, Selby P, Bishop DT, Bishop JN. Germline mutations of the CDKN2 gene in UK melanoma families. *Hum Mol Genet*. 1997 Nov;6(12):2061-7.
- Hofmann UB, Kauczok-Vetter CS, Houben R, Becker JC. Overexpression of the KIT/SCF in uveal melanoma does not translate into clinical efficacy of imatinib mesylate. *Clin Cancer Res*. 2009 Jan 1;15(1):324-9.
- Horsman DE, Sroka H, Rootman J, White VA. Monosomy 3 and isochromosome 8q in a uveal melanoma. *Cancer Genet Cytogenet*. 1990 Apr;45(2):249-53.
- Horsman DE, White VA. Cytogenetic analysis of uveal melanoma. Consistent occurrence of monosomy 3 and trisomy 8q. *Cancer*. 1993 Feb 1;71(3):811-9.
- Howell PM Jr, Liu S, Ren S, Behlen C, Fodstad O, Riker AI. Epigenetics in human melanoma. *Cancer Control*. 2009 Jul;16(3):200-18. Review.
- Hu DN, Yu G, McCormick SA, Finger PT. Population-based incidence of conjunctival melanoma in various races and ethnic groups and comparison with other melanomas. *Am J Ophthalmol*. 2008 Mar;145(3):418-423.
- Hussussian CJ, Struwing JP, Goldstein AM, Higgins PA, Ally DS, Sheahan MD, Clark WH Jr, Tucker MA, Dracopoli NC. Germline p16 mutations in familial melanoma. *Nat Genet*. 1994 Sep;8(1):15-21.

- Häusler T, Stang A, Anastassiou G, Jöckel KH, Mrzyk S, Horsthemke B, Lohmann DR, Zeschnigk M. Loss of heterozygosity of 1p in uveal melanomas with monosomy 3. *Int J Cancer*. 2005 Oct 10;116(6):909-13.
- Isager P, Østerlind A, Engholm G, Heegaard S, Lindegaard J, Overgaard J, Storm HH. Uveal and conjunctival malignant melanoma in Denmark, 1943-97: incidence and validation study. *Ophthalmic Epidemiol*. 2005 Aug;12(4):223-32.
- Jager MJ, Hurks HM, Levitskaya J, Kiessling R. HLA expression in uveal melanoma: there is no rule without some exception. *Hum Immunol*. 2002 Jun;63(6):444-51. Review.
- Jakobiec FA, Silbert G. Are most iris "melanomas" really nevi? A clinicopathologic study of 189 lesions. *Arch Ophthalmol*. 1981 Dec;99(12):2117-32.
- Janssen CS, Sibbett R, Henriquez FL, McKay IC, Kemp EG, Roberts F. The T1799A point mutation is present in posterior uveal melanoma. *Br J Cancer*. 2008 Nov 18;99(10):1673-7.
- Jensen OA. Malignant melanomas of the uvea in Denmark 1943-1952. A clinical, histopathological, and prognostic study. *Acta Ophthalmol (Copenh)*. 1963;43:SUPPL 75:1-220.
- Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. *Nat Rev Genet*. 2002 Jun;3(6):415-28. Review.
- Kang GH, Lee S, Lee HJ, Hwang KS. Aberrant CpG island hypermethylation of multiple genes in prostate cancer and prostatic intraepithelial neoplasia. *J Pathol*. 2004 Feb;202(2):233-40.
- Keunen JE, Bleeker JC. [Eye-preserving treatment of uveal melanoma. Leidse Oogmelanoom Groep]. *Ned Tijdschr Geneesk*. 1997 Oct 18;141(42):2005-9. Review.
- Keunen JE, Journée-de Korver JG, Oosterhuis JA. Transpupillary thermotherapy of choroidal melanoma with or without brachytherapy: a dilemma. *Br J Ophthalmol*. 1999 Nov;83(11):1212-3.
- Kiliç E, Brüggewirth HT, Verbiest MM, Zwarthoff EC, Mooy NM, Luyten GP, de Klein A. The RAS-BRAF kinase pathway is not involved in uveal melanoma. *Melanoma Res*. 2004 Jun;14(3):203-5.
- Knudson AG. Cancer genetics. *Am J Med Genet*. 2002 Jul 22;111(1):96-102. Review.
- Kochanek S, Renz D, Doerfler W. Transcriptional silencing of human Alu sequences and inhibition of protein binding in the box B regulatory elements by 5'-CG-3' methylation. *FEBS Lett*. 1995 Feb 27;360(2):115-20.
- Ksander BR, Chen PW. Immunotherapy of uveal melanoma. *Dev Ophthalmol*. 1999;30:220-30. Review.
- Kujala E, Mäkitie T, Kivelä T. Very long-term prognosis of patients with malignant uveal melanoma. *Invest Ophthalmol Vis Sci*. 2003 Nov;44(11):4651-9.
- Laird PW, Jaenisch R. The role of DNA methylation in cancer genetic and epigenetics. *Annu Rev Genet*. 1996;30:441-64. Review.
- Lee JE, Abdalla J, Porter GA, Bradford L, Grimm EA, Reveille JD, Mansfield PF, Gershenwald JE, Ross MI. Presence of the human leukocyte antigen class II gene DRB1*1101 predicts interferon gamma levels and disease recurrence in melanoma patients. *Ann Surg Oncol*. 2002 Jul;9(6):587-93.
- Lefevre G, Glotin AL, Calipel A, Mouriaux F, Tran T, Kherrouche Z, Maurage CA, Auclair C, Mascarelli F. Roles of stem cell factor/c-Kit and effects of Glivec/STI571 in human uveal melanoma cell tumorigenesis. *J Biol Chem*. 2004 Jul 23;279(30):31769-79.
- Loercher AE, Harbour JW. Molecular genetics of uveal melanoma. *Curr Eye Res*. 2003 Aug;27(2):69-74. Review.
- López-Larrea C, González S, Martínez-Borra J. The role of HLA-B27 polymorphism and molecular mimicry in spondylarthropathy. *Mol Med Today*. 1998 Dec;4(12):540-9. Review.
- Lorigan JG, Wallace S, Mavligit GM. The prevalence and location of metastases from ocular melanoma: imaging study in 110 patients. *AJR Am J Roentgenol*. 1991 Dec;157(6):1279-81.
- Maat W, Haasnoot GW, Claas FH, Schalijs-Delfos NE, Schreuder GM, Jager MJ. HLA Class I and II genotype in uveal melanoma: relation to occurrence and prognosis. *Invest Ophthalmol Vis Sci*. 2006 Jan;47(1):3-6.
- Maat W, Jordanova ES, van Zelderen-Bhola SL, Barthen ER, Wessels HW, Schalijs-Delfos NE, Jager MJ. The heterogeneous distribution of monosomy 3 in uveal melanomas: implications for prognostication based on fine-needle aspiration biopsies. *Arch Pathol Lab Med*. 2007 Jan;131(1):91-6.
- Maat W, Kilic E, Luyten GP, de Klein A, Jager MJ, Gruis NA, Van der Velden PA. Pyrophosphorolysis detects B-RAF mutations in primary uveal melanoma. *Invest Ophthalmol Vis Sci*. 2008 Jan;49(1):23-7.
- Maat W, Ly LV, Jordanova ES, de Wolff-Rouendaal D, Schalijs-Delfos NE, Jager MJ. Monosomy of chromosome 3 and an inflammatory phenotype occur together in uveal melanoma. *Invest Ophthalmol Vis Sci*. 2008 Feb;49(2):505-10.

- Mao L, Merlo A, Bedi G, Shapiro GI, Edwards CD, Rollins BJ, Sidransky D. A novel p16INK4A transcript. *Cancer Res.* 1995 Jul 15;55(14):2995-7.
- Margo CE, Mulla Z, Billiris K. Incidence of surgically treated uveal melanoma by race and ethnicity. *Ophthalmology.* 1998 Jun;105(6):1087-90.
- McCubrey JA, Steelman LS, Chappell WH, Abrams SL, Wong EW, Chang F, Lehmann B, Terrian DM, Milella M, Tafuri A, Stivala F, Libra M, Basecke J, Evangelisti C, Martelli AM, Franklin RA. Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance. *Biochim Biophys Acta.* 2007 Aug;1773(8):1263-84. Review.
- McLean IW, Foster WD, Zimmerman LE, Gamel JW. Modifications of Callender's classification of uveal melanoma at the Armed Forces Institute of Pathology. *Am J Ophthalmol.* 1983 Oct;96(4):502-9.
- McLean IW, Zimmerman LE, Evans RM. Reappraisal of Callender's spindle a type of malignant melanoma of choroid and ciliary body. *Am J Ophthalmol.* 1978 Oct;86(4):557-64.
- Merbs SL, Sidransky D. Analysis of p16 (CDKN2/MTS-1/INK4A) alterations in primary sporadic uveal melanoma. *Invest Ophthalmol Vis Sci.* 1999 Mar;40(3):779-83.
- Missotten GS, Keunen JE. Screening for uveal melanoma metastasis. Literature review. *Bull Soc Belge Ophtalmol.* 2004;(294):13-22. Review.
- Moan J, Porojnicu AC, Dahlback A. Ultraviolet radiation and malignant melanoma. *Adv Exp Med Biol.* 2008;624:104-16. Review.
- Mooy CM, De Jong PT. Prognostic parameters in uveal melanoma: a review. *Surv Ophthalmol.* 1996 Nov-Dec;41(3):215-28. Review.
- Mooy CM, Van der Helm MJ, Van der Kwast TH, De Jong PT, Ruiter DJ, Zwarthoff EC. No N-ras mutations in human uveal melanoma: the role of ultraviolet light revisited. *Br J Cancer.* 1991 Aug;64(2):411-3.
- Mukai S, Dryja TP. Loss of alleles at polymorphic loci on chromosome 2 in uveal melanoma. *Cancer Genet Cytogenet.* 1986 May;22(1):45-53.
- Naus NC, Verhoeven AC, van Drunen E, Slater R, Mooy CM, Paridaens DA, Luyten GP, de Klein A. Detection of genetic prognostic markers in uveal melanoma biopsies using fluorescence in situ hybridization. *Clin Cancer Res.* 2002 Feb;8(2):534-9.
- Netherlands Cancer Registry (NCR) 2010. Nederlandse Kanker Registratie / kerncijfers. Website: <http://www.cijfersoverkanker.nl/kerncijfers-over-kanker-49.html>
- Noter SL, Rothbarth J, Pijl ME, Keunen JE, Hartgrink HH, Tijl FG, Kuppen PJ, van de Velde CJ, Tollenaar RA. Isolated hepatic perfusion with high-dose melphalan for the treatment of uveal melanoma metastases confined to the liver. *Melanoma Res.* 2004 Feb;14(1):67-72.
- Okano M, Xie S, Li E. Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. *Nat Genet.* 1998 Jul;19(3):219-20.
- Onken MD, Lin AY, Worley LA, Folberg R, Harbour JW. Association between microarray gene expression signature and extravascular matrix patterns in primary uveal melanomas. *Am J Ophthalmol.* 2005 Oct;140(4):748-9.
- Onken MD, Worley LA, Ehlers JP, Harbour JW. Gene expression profiling in uveal melanoma reveals two molecular classes and predicts metastatic death. *Cancer Res.* 2004 Oct 15;64(20):7205-9.
- Peters S, Voelter V, Zografos L, Pampallona S, Popescu R, Gillet M, Bosshard W, Fiorentini G, Lotem M, Weitzen R, Keilholz U, Humblet Y, Piperno-Neumann S, Stupp R, Leyvraz S. Intra-arterial hepatic fotemustine for the treatment of liver metastases from uveal melanoma: experience in 101 patients. *Ann Oncol.* 2006 Apr;17(4):578-83.
- Petrausch U, Martus P, Tönnies H, Bechrakis NE, Lenze D, Wansel S, Hummel M, Bornfeld N, Thiel E, Foerster MH, Keilholz U. Significance of gene expression analysis in uveal melanoma in comparison to standard risk factors for risk assessment of subsequent metastases. *Eye (Lond).* 2008 Aug;22(8):997-1007.
- Pollack MS, Livingston PO. HLA and DR antigen frequencies in melanoma patients: possible relation to disease prognosis. *Tissue Antigens.* 1985 Oct;26(4):262-5.
- Pollock PM, Meltzer PS. A genome-based strategy uncovers frequent BRAF mutations in melanoma. *Cancer Cell.* 2002 Jul;2(1):5-7.
- Prescher G, Bornfeld N, Becher R. Two subclones in a case of uveal melanoma. Relevance of monosomy 3 and multiplication of chromosome 8q. *Cancer Genet Cytogenet.* 1994 Oct 15;77(2):144-6.

- Prescher G, Bornfeld N, Becher R. Nonrandom chromosomal abnormalities in primary uveal melanoma. *J Natl Cancer Inst.* 1990 Nov 21;82(22):1765-9. PubMed PMID: 2231772. 12: Prescher G, Bornfeld N, Hirsch H, Horsthemke B, Jöckel KH, Becher R. Prognostic implications of monosomy 3 in uveal melanoma. *Lancet.* 1996 May 4;347(9010):1222-5.
- Pyrhönen S. The treatment of metastatic uveal melanoma. *Eur J Cancer.* 1998 Jul;34 Suppl 3:S27-30. Review.
- Reddy KB, Nabha SM, Atanaskova N. Role of MAP kinase in tumor progression and invasion. *Cancer Metastasis Rev.* 2003 Dec;22(4):395-403. Review.
- Regan S, Judge HE, Gragoudas ES, Egan KM. Iris color as a prognostic factor in ocular melanoma. *Arch Ophthalmol.* 1999 Jun;117(6):811-4.
- Richtig E, Langmann G, Müllner K, Smolle J. Ocular melanoma: epidemiology, clinical presentation and relationship with dysplastic nevi. *Ophthalmologica.* 2004 Mar-Apr;218(2):111-4.
- Riley PA. Melanin. *Int J Biochem Cell Biol.* 1997 Nov;29(11):1235-9. Review.
- Riley PA. Materia melanica: further dark thoughts. *Pigment Cell Res.* 1992 Sep;5(3):101-6.
- Rimoldi D, Salvi S, Liénard D, Lejeune FJ, Speiser D, Zografos L, Cerottini JC. Lack of *BRAF* mutations in uveal melanoma. *Cancer Res.* 2003 Sep 15;63(18):5712-5.
- Robertson KD. DNA methylation and human disease. *Nat Rev Genet.* 2005 Aug;6(8):597-610. Review.
- Rones B, Zimmerman LE. The prognosis of primary tumors of the iris treated by iridectomy. *AMA Arch Ophthalmol.* 1958 Aug;60(2):193-205.
- Rothhammer T, Bosserhoff AK. Epigenetic events in malignant melanoma. *Pigment Cell Res.* 2007 Apr;20(2):92-111. Review.
- Rousseau AP. Uveal melanoma: update and future directions. *Can J Ophthalmol.* 2004 Jun;39(4):335-42. Review.
- Sandinha T, Farquharson M, McKay I, Roberts F. Correlation of heterogeneity for chromosome 3 copy number with cell type in choroidal melanoma of mixed-cell type. *Invest Ophthalmol Vis Sci.* 2006 Dec;47(12):5177-80.
- Satyamoorthy K, Li G, Gerrero MR, Brose MS, Volpe P, Weber BL, Van Belle P, Elder DE, Herlyn M. Constitutive mitogen-activated protein kinase activation in melanoma is mediated by both *BRAF* mutations and autocrine growth factor stimulation. *Cancer Res.* 2003 Feb 15;63(4):756-9.
- Schmidt-Pokrzywniak A, Jöckel KH, Bornfeld N, Sauerwein W, Stang A. Positive interaction between light iris color and ultraviolet radiation in relation to the risk of uveal melanoma: a case-control study. *Ophthalmology.* 2009 Feb;116(2):340-8.
- Schmittel A, Schmidt-Hieber M, Martus P, Bechrakis NE, Schuster R, Siehl JM, Foerster MH, Thiel E, Keilholz U. A randomized phase II trial of gemcitabine plus treosulfan versus treosulfan alone in patients with metastatic uveal melanoma. *Ann Oncol.* 2006 Dec;17(12):1826-9.
- Schoenfeld L, Pettay J, Tubbs RR, Singh AD. Variation of monosomy 3 status within uveal melanoma. *Arch Pathol Lab Med.* 2009 Aug;133(8):1219-22.
- Scholes AG, Damato BE, Nunn J, Hiscott P, Grierson I, Field JK. Monosomy 3 in uveal melanoma: correlation with clinical and histologic predictors of survival. *Invest Ophthalmol Vis Sci.* 2003 Mar;44(3):1008-11.
- Seddon JM, Albert DM, Lavin PT, Robinson N. A prognostic factor study of disease-free interval and survival following enucleation for uveal melanoma. *Arch Ophthalmol.* 1983 Dec;101(12):1894-9.
- Seddon JM, Gragoudas ES, Glynn RJ, Egan KM, Albert DM, Blitzer PH. Host factors, UV radiation, and risk of uveal melanoma. A case-control study. *Arch Ophthalmol.* 1990 Sep;108(9):1274-80.
- Serrano M, Hannon GJ, Beach D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature.* 1993 Dec 16;366(6456):704-7.
- Sharpless NE, DePinho RA. The *INK4A/ARF* locus and its two gene products. *Curr Opin Genet Dev.* 1999 Feb;9(1):22-30. Review.
- Sherr CJ. Mammalian G1 cyclins. *Cell.* 1993 Jun 18;73(6):1059-65. Review.
- Shields CL, Shields JA, Kirtali H, De Potter P, Cater JR. Risk factors for growth and metastasis of small choroidal melanocytic lesions. *Ophthalmology.* 1995 Sep;102(9):1351-61.

- Shields CL, Furuta M, Thangappan A, Nagori S, Mashayekhi A, Lally DR, Kelly CC, Rudich DS, Nagori AV, Wakade OA, Mehta S, Forte L, Long A, Dellacava EF, Kaplan B, Shields JA. Metastasis of uveal melanoma millimeter-by-millimeter in 8033 consecutive eyes. *Arch Ophthalmol*. 2009 Aug;127(8):989-98.
- Singh AD, Boghosian-Sell L, Wary KK, Shields CL, De Potter P, Donoso LA, Shields JA, Cannizzaro LA. Cytogenetic findings in primary uveal melanoma. *Cancer Genet Cytogenet*. 1994 Feb;72(2):109-15.
- Singh AD, Rennie IG, Seregard S, Giblin M, McKenzie J. Sunlight exposure and pathogenesis of uveal melanoma. *Surv Ophthalmol*. 2004 Jul-Aug;49(4):419-28. Review.
- Singh AD, Topham A. Survival rates with uveal melanoma in the United States: 1973-1997. *Ophthalmology*. 2003 May;110(5):962-5.
- Singh AD, Topham A. Incidence of uveal melanoma in the United States: 1973-1997. *Ophthalmology*. 2003 May;110(5):956-61.
- Sisley K, Cottam DW, Rennie IG, Parsons MA, Potter AM, Potter CW, Rees RC. Non-random abnormalities of chromosomes 3, 6, and 8 associated with posterior uveal melanoma. *Genes Chromosomes Cancer*. 1992 Oct;5(3):197-200.
- Sisley K, Nichols C, Parsons MA, Farr R, Rees RC, Rennie IG. Clinical applications of chromosome analysis, from fine needle aspiration biopsies, of posterior uveal melanomas. *Eye (Lond)*. 1998;12 (Pt 2):203-7.
- Sisley K, Rennie IG, Cottam DW, Potter AM, Potter CW, Rees RC. Cytogenetic findings in six posterior uveal melanomas: involvement of chromosomes 3, 6, and 8. *Genes Chromosomes Cancer*. 1990 Sep;2(3):205-9.
- Sisley K, Rennie IG, Parsons MA, Jacques R, Hammond DW, Bell SM, Potter AM, Rees RC. Abnormalities of chromosomes 3 and 8 in posterior uveal melanoma correlate with prognosis. *Genes Chromosomes Cancer*. 1997 May;19(1):22-8.
- Smith JH, Padnick-Silver L, Newlin A, Rhodes K, Rubinstein WS. Genetic study of familial uveal melanoma: association of uveal and cutaneous melanoma with cutaneous and ocular nevi. *Ophthalmology*. 2007 Apr;114(4):774-9.
- Soparker CN, O'Brien JM, Albert DM. Investigation of the role of the ras protooncogene point mutation in human uveal melanomas. *Invest Ophthalmol Vis Sci*. 1993 Jun;34(7):2203-9.
- Speicher MR, Prescher G, du Manoir S, Jauch A, Horsthemke B, Bornfeld N, Becher R, Cremer T. Chromosomal gains and losses in uveal melanomas detected by comparative genomic hybridization. *Cancer Res*. 1994 Jul 15;54(14):3817-23.
- Spugnardi M, Tommasi S, Dammann R, Pfeifer GP, Hoon DS. Epigenetic inactivation of RAS association domain family protein 1 (RASSF1A) in malignant cutaneous melanoma. *Cancer Res*. 2003 Apr 1;63(7):1639-43.
- Stang A, Ahrens W, Anastassiou G, Jöckel KH. Phenotypical characteristics, lifestyle, social class and uveal melanoma. *Ophthalmic Epidemiol*. 2003 Dec;10(5):293-302.
- Tolleson WH. Human melanocyte biology, toxicology, and pathology. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev*. 2005;23(2):105-61. Review.
- Tschentscher F, Prescher G, Zeschnigk M, Horsthemke B, Lohmann DR. Identification of chromosomes 3, 6, and 8 aberrations in uveal melanoma by microsatellite analysis in comparison to comparative genomic hybridization. *Cancer Genet Cytogenet*. 2000 Oct 1;122(1):13-7.
- Vajdic CM, Krickler A, Giblin M, McKenzie J, Aitken J, Giles GG, Armstrong BK. Incidence of ocular melanoma in Australia from 1990 to 1998. *Int J Cancer*. 2003 May 20;105(1):117-22.
- van der Velden PA, Metzelaar-Blok JA, Bergman W, Monique H, Hurks H, Frants RR, Gruis NA, Jager MJ. Promoter hypermethylation: a common cause of reduced p16(INK4a) expression in uveal melanoma. *Cancer Res*. 2001 Jul 1;61(13):5303-6.
- van Doorn R, Gruis NA, Willemze R, van der Velden PA, Tensen CP. Aberrant DNA methylation in cutaneous malignancies. *Semin Oncol*. 2005 Oct;32(5):479-87. Review.
- van Elsas A, Zerp S, van der Flier S, Krüse-Wolters M, Vacca A, Ruiter DJ, Schrier P. Analysis of N-ras mutations in human cutaneous melanoma: tumor heterogeneity detected by polymerase chain reaction/single-stranded conformation polymorphism analysis. *Recent Results Cancer Res*. 1995;139:57-67.
- van Gils W, Lodder EM, Mensink HW, Kiliç E, Naus NC, Brüggewirth HT, van Ijcken W, Paridaens D, Luyten GP, de Klein A. Gene expression profiling in uveal melanoma: two regions on 3p related to prognosis. *Invest Ophthalmol Vis Sci*. 2008 Oct;49(10):4254-62.
- van Hees CL, de Boer A, Jager MJ, Bleeker JC, Kakebeeke HM, Crijns MB, Vandenbroucke JP, Bergman W. Are atypical nevi a risk factor for uveal melanoma? A case-control study. *J Invest Dermatol*. 1994 Aug;103(2):202-5.

- Van Raamsdonk CD, Bezrookove V, Green G, Bauer J, Gaugler L, O'Brien JM, Simpson EM, Barsh GS, Bastian BC. Frequent somatic mutations of GNAQ in uveal melanoma and blue naevi. *Nature*. 2009 Jan 29;457(7229):599-602.
- Van Raamsdonk CD, Griewank KG, Crosby MB, Garrido MC, Vemula S, Wiesner T, Obenauf AC, Wackernagel W, Green G, Bouvier N, Sozen MM, Baimukanova G, Roy R, Heguy A, Dolgalev I, Khanin R, Busam K, Speicher MR, O'Brien J, Bastian BC. Mutations in GNA11 in uveal melanoma. *N Engl J Med*. 2010 Dec 2;363(23):2191-9.
- Vidal JL, Bacin F, Albuissou E, Rozan R, Desjardins L, D'Hermies F, Grange JD, Chauvel P, Caujolle JP, Sahel J, et al. ["Melanoma 92": epidemiological study of uveal melanoma in France]. *J Fr Ophtalmol*. 1995;18(8-9):520-8. Review.
- Virgili G, Gatta G, Ciccolallo L, Capocaccia R, Biggeri A, Crocetti E, Lutz JM, Paci E; EURO CARE Working Group. Survival in patients with uveal melanoma in Europe. *Arch Ophthalmol*. 2008 Oct;126(10):1413-8.
- Weber A, Hengge UR, Urbanik D, Markwart A, Mirmohammadsaegh A, Reichel MB, Wittekind C, Wiedemann P, Tannapfel A. Absence of mutations of the *BRAF* gene and constitutive activation of extracellular-regulated kinase in malignant melanomas of the uvea. *Lab Invest*. 2003 Dec;83(12):1771-6.
- Weis E, Shah CP, Lajous M, Shields JA, Shields CL. The association between host susceptibility factors and uveal melanoma: a meta-analysis. *Arch Ophthalmol*. 2006 Jan;124(1):54-60.
- Wellbrock C, Karasarides M, Marais R. The RAF proteins take centre stage. *Nat Rev Mol Cell Biol*. 2004 Nov;5(11):875-85. Review.
- White VA, Chambers JD, Courtright PD, Chang WY, Horsman DE. Correlation of cytogenetic abnormalities with the outcome of patients with uveal melanoma. *Cancer*. 1998 Jul 15;83(2):354-9.
- Wiltshire RN, Elner VM, Dennis T, Vine AK, Trent JM. Cytogenetic analysis of posterior uveal melanoma. *Cancer Genet Cytogenet*. 1993 Mar;66(1):47-53.
- Wolf SF, Migeon BR. Studies of X chromosome DNA methylation in normal human cells. *Nature*. 1982 Feb 25;295(5851):667-71.
- Worley LA, Onken MD, Person E, Robirds D, Branson J, Char DH, Perry A, Harbour JW. Transcriptomic versus chromosomal prognostic markers and clinical outcome in uveal melanoma. *Clin Cancer Res*. 2007 Mar 1;13(5):1466-71.
- Yeo W, Wong WL, Wong N, Law BK, Tse GM, Zhong S. High frequency of promoter hypermethylation of RASSF1A in tumorous and non-tumorous tissue of breast cancer. *Pathology*. 2005 Apr;37(2):125-30.
- Zhang BH, Guan KL. Activation of B-Raf kinase requires phosphorylation of the conserved residues Thr598 and Ser601. *EMBO J*. 2000 Oct 16;19(20):5429-39.
- Zuidervaart W, van Nieuwpoort F, Stark M, Dijkman R, Packer L, Borgstein AM, Pavey S, van der Velden P, Out C, Jager MJ, Hayward NK, Gruijs NA. Activation of the MAPK pathway is a common event in uveal melanomas although it rarely occurs through mutation of *BRAF* or *RAS*. *Br J Cancer*. 2005 Jun 6;92(11):2032-8.

Chapter 2

The Heterogeneous Distribution of Monosomy 3 in Uveal Melanomas: Implications for Prognostication Based on Fine-Needle Aspiration Biopsies

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Abstract

PURPOSE. The detection of monosomy 3 in uveal melanomas has repeatedly been associated with adverse outcome. Fine-needle aspiration biopsy is being used to detect monosomy 3 in these tumors, based on the assumption that this chromosomal abnormality is distributed homogeneously throughout the tumor. The purpose of this study is to study the distribution of monosomy 3 in primary uveal melanoma by fluorescence in situ hybridization (FISH).

METHODS. We studied 50 enucleated eyes with uveal melanoma. In all 50 tumors we performed cytogenetic analysis and FISH using a DNA-specific probe for the centromere region of chromosome 3 on cultured tumor cells. In addition, the percentage of tumor cells with monosomy 3 was assessed by FISH on nuclei, isolated from paraffin-embedded tissue and compared to results of FISH on regular histology sections of the paraffin-embedded tissue.

RESULTS. Combining karyotyping and FISH on cultured cells identified monosomy 3 in 19 (38%) of 50 tumors, whereas FISH on nuclei isolated from paraffin-embedded tissue showed 31 (62%) of 50 as having monosomy for chromosome 3. FISH analysis on paraffin sections showed tumor heterogeneity for copy number of chromosome 3 in at least 7 cases.

CONCLUSIONS. FISH analysis on paraffin sections shows that heterogeneity of monosomy of chromosome 3 is a frequent phenomenon in uveal melanoma. FISH on nuclei isolated from paraffin-embedded tissue identifies a higher frequency of monosomy 3 than the traditional combination of karyotyping and FISH on cultured uveal melanoma cells. The practice of assigning patients to risk categories based on fine-needle aspiration biopsy samples from primary uveal melanoma may be subject to error based on the heterogeneous distribution of monosomy 3 in these tumors.

Introduction

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, 2003). Several prognostic factors based on clinical and histologic features have been correlated with an unfavorable prognosis in patients with uveal melanoma: among these parameters are tumor size, ciliary body involvement, the presence of epithelioid cells, and vasculogenic mimicry (Mooy and de Jong, 1996). The identification of patients who have a high risk of developing metastases would allow the possibility of providing adjuvant immunotherapy or chemotherapy 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 at an early stage (Missotten and Keunen, 2004). Nonrandom chromosome abnormalities, such as variation in the short arm of chromosome 1, and gain or loss of chromosomal material in chromosomes 3, 6, and 8, have been detected in uveal melanomas and are associated with metastasis (Sisley et al., 1990; Naus et al., 2001). The most frequent change, in approximately 50% of all uveal melanomas, is the loss of 1 copy, or monosomy, of chromosome 3 (Horsman et al., 1990; Sisley et al., 1990). 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., 1996). The application of fluorescence in situ hybridization (FISH) on transvitreal fine-needle aspiration biopsies (FNABs) is thought to be a reliable method for assaying genetic parameters such as chromosome 3 loss (Naus et al., 2002). This approach is based in part on the assumption that uveal melanomas with or without loss of 1 copy of chromosome 3 represent 2 distinct entities: in monosomy 3-positive tumors, the chromosomal abnormality will be detected in every cell and is thus susceptible to identification by random sampling by the FNAB technique.

To detect monosomy 3, we performed standard cytogenetic analysis by karyotyping and FISH on cultured uveal melanoma cells obtained after enucleation. We subsequently also analyzed FISH on paraffin sections and observed heterogeneity within tumors. However, in many tumors, the application of this technique was limited by the presence of necrotic areas and heavy pigmentation. We therefore analyzed monosomy 3 by FISH technique on nuclei isolated from paraffin-embedded tissue. This technique can provide additional information about clonal expansion of cells with monosomy of chromosome 3 and about tumor heterogeneity for this specific chromosomal aberration. The results were compared with the results of standard karyotyping and FISH on cultured cells.

Materials and Methods

Patients and Tissue Samples

Fresh material and formalin-fixed, paraffin-embedded specimens from 50 patients with uveal melanoma were obtained from tumors that were enucleated during the period 1999 through 2004 at the Leiden University Medical Center from which enough material was present for cytogenetic analysis. After enucleation, tumor material for cytogenetic testing was obtained immediately after the globe had been opened. Each tumor sample was further processed for conventional histopathologic diagnosis. The research protocol followed the tenets of the Declaration of Helsinki (World Medical Association Declaration of Helsinki 1964; Ethical principles for medical research involving human subjects).

Karyotyping and Interphase FISH on Cultured Cells

After enucleation, a small part of each tumor was removed and sent out for cell culture. Following mechanical dissection of the tumor biopsy, cells were washed and placed into 1 flask with RPMI 1640 (15% fetal bovine serum [Invitrogen, Breda, The Netherlands]) medium and another flask with Amniochrome II (Cambrix Bio Science, Verviers, Belgium). The flasks were cultured at 37°C with 5% CO₂ for up to 4 weeks and harvested, according to standard protocols, when at least 75% of the surface was covered with cells (after a mean of 18 days; SD 9.4 days). When cell culturing was successful, conventional karyotyping and FISH with a DNA-specific probe was performed, to determine the presence of chromosome 3 changes according to established methods (Dauwerse et al., 1992). Probe CEN 3 (Vysis, Des Plaines, Ill) was used for all FISH experiments. The cutoff value for monosomy 3 was 10% for the cultured cells. Two independent observers assessed all evaluations and scores, each without knowledge of the results obtained by the other investigator, to ensure accuracy of quantification of the slides. In the case of a difference, consensus was reached during a simultaneous session. Cytogenetic analysis was performed on GTG-banded (G-banding with Giemsa and trypsin) metaphases. In the case of a normal karyotype, at least 20 metaphases were analyzed. When an abnormal clone was detected in the first 10 karyotyped cells, no further analysis was performed; when 3 cells with loss of 1 copy of chromosome 3 were observed, monosomy 3 was identified.

Interphase FISH on Paraffin-Embedded Tissue Sections

We performed interphase FISH on paraffin-embedded sections as described previously by Haralambieva et al. (Haralambieva et al., 2002). In brief, 4-μm paraffin-embedded sections, mounted on coated slides, were deparaffinized and rehydrated. Slides were next exposed under pressure at 100°C in Tris/ethylenediaminetetraacetic acid buffer solution, and then the slides were washed in standard saline citrate buffer solution. RNase (New England BioLabs, Ipswich, Mass) treatment was performed followed by enzymatic digestion with pepsin. Thereafter, tissue samples were washed with phosphate-buffered saline, dehydrated in ethanol, and air-dried. DNA probe CEP3 SpectrumOrange: CEN 3, specific for the centromere region of human chromosome 3 (band region 3p11.1q11.1), was used for hybridization

according to the manufacturer's protocol. The denaturation step was performed at 80°C for 12 minutes. DAPI (4'-6-diamidino-2-phenylindole) staining was performed to distinguish between tumor areas and fields containing normal cells in the sections.

Interphase FISH on Nuclei Isolated From Paraffin-Embedded Tissue

The protocol for interphase FISH on nuclei isolated from 50- μ m paraffin-embedded tissue sections was adapted from the procedure for hybridization on paraffin sections as described by Haralambieva et al. (Haralambieva et al., 2002) and optimized by Jordanova et al. (Jordanova et al., 2002). In brief, after enzymatic digestion with pepsin and 2 additional washing steps with phosphate-buffered saline, cells were filtered through a 70- μ m-pore-size nylon filter (Verseidag-Industrietextilen GmbH, Kempen, Germany). Cells were fixed with methanol-acetic acid (3:1). Prior to making cytospins, cell density was determined in a 5- μ L methanol-acetic acid suspension to ensure that at least 400 to 500 nuclei were present in each cytospins (Vaandrager et al., 2000). The slides were air dried and used for hybridization. For hybridization, the same DNA-specific probe was used as for the paraffin embedded tissue sections. Three tonsils of healthy individuals were used as controls. The tonsil sections were treated in exactly the same manner as the patient samples. The cutoff level was set at the mean of these controls plus 3 times the standard deviation and was calculated as 5% for detecting monosomy 3.

Two independent observers assessed all slides, each without knowledge of the results obtained by the other investigator, to ensure accuracy of quantification of the slides. Scoring results corresponded in 47 cases; for 3 cases with values around the cutoff value (ID UM04, UM19, and UM20) consensus could be reached during a simultaneous session. Slides were analyzed using a Leica DMRXA fluorescence microscope (Leica Imaging Systems, Cambridge, United Kingdom). Image capture was performed by a monochrome CCD camera (COHU, San Diego, Calif) attached to the fluorescence microscope and Leica Q-FISH software (Leica). The 2 observers each analyzed 100 cells, 200 interphase nuclei in total. The hybridized probes fluoresce with bright intensity both in interphase nuclei and metaphase chromosomes.

Results

Patients

In this study a substantial proportion of medium-sized and large-sized tumors (COMS Report No. 4, 1997, COMS Report No. 20, 2003) (48% and 50% of the 50 cases, respectively) were included, as relatively small tumors were treated by alternatives to enucleation such as radiation therapy. The average age of the 23 female and 27 male patients was 59 years (range, 24–85 years).

Karyotyping and FISH on Cultured Cells

Conventional karyotyping was performed on cell cultures of 50 choroidal melanomas and was unsuccessful in 4 tumors; 1 failure could be attributed to a technical problem. We did not identify significant differences in prognostic characteristics (such as tumor thickness or location) between the 4 unsuccessful tumors and the cases that were technically successful. In 2 additional cases, no conclusion could be reached regarding the absence or presence of monosomy 3, as less than 20 karyotypes could be analyzed. Karyotyping showed unambiguous monosomy 3 in 15 of 44 evaluable cases (Table 2.1). FISH analysis was also performed on cultured cells from choroidal melanomas. However, it was not performed in 4 cases that had already been shown to have monosomy 3 and in 2 cases for logistical reasons. The technique failed in 3 cases because of electrical power failure. In the 4 tumors in which karyotyping had not been successful, FISH analysis was conclusive. Of the 41 evaluable cases, FISH showed 1 copy of chromosome 3 in 15 cell cultures and identified 25 cases that carried both copies of chromosome 3. By combining karyotyping with FISH data on the cultured cells in all 50 cases, monosomy 3 was detected in 19 cases (38%), whereas 29 cases (58%) were found to be diploid for chromosome 3 and 2 tumors (4% of cases) showed 3 copies of chromosome 3 (Table 2.1).

Table 2.1. Data on Karyotyping and Fluorescence In Situ Hybridization (FISH) Analyses for 50 Tumors, Analyzed for Chromosome 3 Aberrations by Karyotyping and FISH on Cultured Cells and by FISH on Nuclei Isolated From Paraffin-Embedded Tissue. Percentages indicate the amount of cells with 2 (disomy), 1 (monosomy), 3 (trisomy), or more signals for chromosome 3. D indicates disomy; M, monosomy; T, trisomy; 4, >3 signals; F, failed; NP, not performed; and U, unclear.

Cultured cells					Isolated nuclei				
ID	Karyo- typing	FISH	FISH	D/M/T/4 (%)	ID	Karyo- typing	FISH	FISH	D/M/T/4 (%)
UM01	D	D	D	98 / 00 / 01 / 01	UM26	D	T	M	70 / 29 / 01
UM02	D	D	D	98 / 01 / 01	UM27	D	M	M	60 / 40
UM03	D	D	D	96 / 01 / 02 / 01	UM28	D	M	M	41 / 58 / 01
UM04	D	D	D	96 / 03 / 01	UM29	D	M	M	37 / 63
UM05	D	D	D	96 / 01 / 02	UM30	D	M	M	31 / 69
UM06	D	D	D	95 / 02 / 02 / 01	UM31	D	NP	M	42 / 57
UM07	D	D	D	95 / 01 / 03 / 01	UM32	F	D	M	83 / 17
UM08	D	D	D	95 / 01 / 01 / 03	UM33	F	D	M	39 / 60 / 01
UM09	D	D	D	94 / 01 / 02 / 03	UM34	U	D	M	47 / 52 / 01
UM10	D	D	D	93 / 00 / 03 / 04	UM35	M	NP	M	72 / 24 / 04
UM11	D	D	D	93 / 01 / 04 / 02	UM36	M	NP	M	69 / 30 / 01
UM12	D	D	D	92 / 01 / 05 / 02	UM37	M	NP	M	57 / 42 / 01
UM13	D	D	D	88 / 01 / 04 / 07	UM38	M	M	M	58 / 41 / 01
UM14	D	F	D	98 / 01 / 01	UM39	M	M	M	54 / 46

Table 2.1. (Contineud)

Cultured cells					Isolated nuclei				
ID	Karyo- typing	FISH	FISH	D/M/T/4 (%)	ID	Karyo- typing	FISH	FISH	D/M/T/4 (%)
UM15	D	F	D	97 / 01 / 02	UM40	M	M	M	43 / 57
UM16	D	F	D	97 / 00 / 01 / 02	UM41	M	M	M	42 / 58
UM17	F	D	D	88 / 03 / 08 / 01	UM42	M	M	M	40 / 60
UM18	F	D	D	96 / 02 / 02	UM43	M	M	M	40 / 60
UM19	U	D	D	96 / 04	UM44	M	M	M	37 / 63
UM20	D	D	M	89 / 07 / 01 / 03	UM45	M	M	M	36 / 64
UM21	D	D	M	73 / 27	UM46	M	M	M	22 / 78
UM22	D	D	M	60 / 40	UM47	M	M	M	18 / 82
UM23	D	D	M	60 / 40	UM48	M	M	M	18 / 82
UM24	D	D	M	38 / 62	UM49	M	NP	M	06 / 94
UM25	D	D	M	36 / 64	UM50	T	NP	M	49 / 51

Interphase FISH on Paraffin-Embedded Tissue Sections

In situ hybridization was performed on paraffin sections of all 50 choroidal melanomas. Primary uveal melanomas typically do not contain significant amounts of stromal elements (Lin et al., 2005) so the detection of signals on histologic preparations is likely to originate from tumor cells. Furthermore, the nuclei of tumor cells are significantly larger than in fibroblasts or endothelial cells, thus ensuring that the signals counted in this study originated from tumor cells. However, heavy pigmentation in histologic sections made it difficult to evaluate the FISH preparations. Another problem is that the use of 4- μ m-thick sections of paraffin tissue, in which nuclei are cut through, results in different hybridization patterns and leads to difficulties in interpreting these sections in a diagnostic setting as described earlier (Hensen et al., 2004) (Figure 2.1). Still, in 7 tumors without heavy pigmentation, tumor heterogeneity for chromosome 3 was clearly observed and these tumors should therefore be considered as being partly disomy and partly monosomy for chromosome 3 (Figure 2.2, A through D). In 18 tumors, 2 hybridization signals were found in almost all cells, suggesting the presence of 2 copies of chromosome 3 in each cell; in 8 cases, 1 or no signals was seen in almost every cell, which implies the homogeneous presence of a monosomy 3. In the remaining 17 tumor sections, analysis was difficult because of necrosis and heavy pigmentation.

Figure 2.1. Schematic representation of nuclei with different patterns of hybridization signals in tissue sections. As tissue sections cut through nuclei at different levels, more hybridization signal patterns can be seen in tissue sections than when using isolated nuclei. When 2 hybridization signals are observed in a cell, the presence of 2 copies of chromosome 3 is assumed; if 1 or no signal is observed, this implies the presence of a cell with monosomy 3.

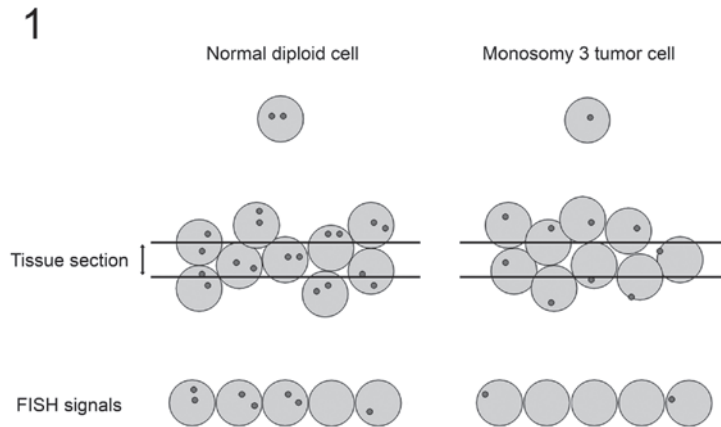
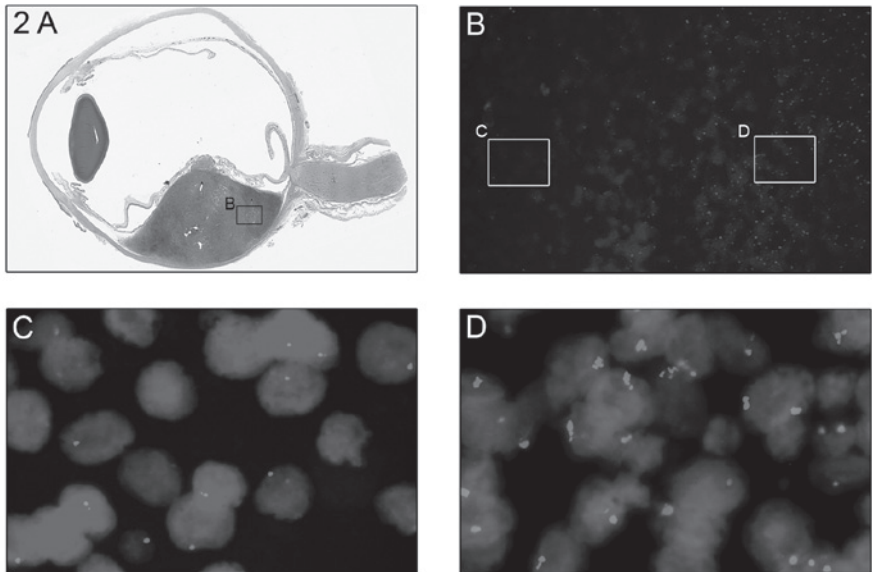


Figure 2.2. A, Hematoxylin-eosin staining of a uveal melanoma (whole mount). B, Area indicated in A that shows heterogeneity for chromosome 3 by fluorescence in situ hybridization (FISH) analysis on a paraffin section (original magnification x400). The red areas are positively stained pigment deposits. C, Typical hybridization pattern representative of disomy of chromosome 3 in the area indicated in B (original magnification x1000). D, Typical hybridization pattern representative of monosomy of chromosome 3 in the area indicated in B (original magnification x1000). A through D are derived from the same uveal melanoma.



Interphase FISH on Nuclei Isolated From Paraffin-Embedded Tissue

As interpretation of FISH signals on paraffin sections was difficult, we identified the number of copies of chromosome 3 on nuclei isolated from paraffin tissue. This technique was successful in all cases. Signals were bright and intense and easily recognizable (Figure 2.3). With a threshold value of 5% (as based on the normal controls), 19 uveal melanomas (38%) were categorized as disomic for chromosome 3 and 31 (62%) as having monosomy 3. Twelve of the tumors that showed monosomy 3 on isolated nuclei had not been recognized by the combination of karyotyping and FISH on cultured cells (Figure 2.4). In a minor percentage of nuclei, more than 2 signals were observed, which is the result of fragmentation of the centromere signal and does not reflect an aberration. A comparison of the data on isolated nuclei versus the chromosome analysis on cultured cells is provided in the Table 2.1. Although the analysis on cultured cells led to the identification of monosomy 3 in 38% of cases, FISH analysis on isolated nuclei identified 62% of uveal melanoma as having monosomy 3.

Figure 2.3. Example of nuclei isolated from paraffin tissue of a uveal melanoma after hybridization for the centromere of chromosome 3. Two nuclei show 2 signals, indicating disomy of chromosome 3. The nucleus at the bottom shows 1 signal, indicative for loss of 1 copy of chromosome 3 (original magnification x1000).

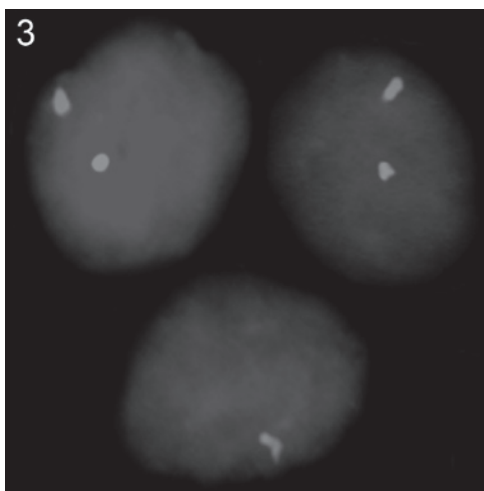
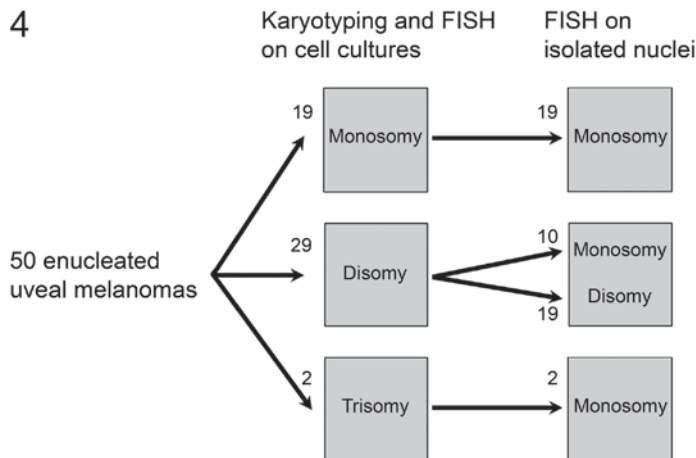


Figure 2.4. Fifty uveal melanomas were studied for the presence of numerical aberrations of chromosome 3. Combining karyotyping and fluorescence in situ hybridization (FISH) analysis on short-term uveal melanoma cell cultures, 19, 29, and 2 tumors were identified as having monosomy 3, disomy 3, and trisomy 3, respectively. Using FISH analysis on nuclei isolated from paraffin-embedded tissue, 31 tumors (62%) possessed monosomy 3.



Comment

Genetic imbalance for chromosome 3 represents a significant risk factor for metastatic disease and reduced survival in patients with primary uveal melanoma (Prescher et al., 1996). Clinically significant genetic imbalances can occur as a result of a single loss of chromosome 3 with single or multiple losses or gains of other chromosomes, such as of chromosome 1 (Kilic et al., 2005). Conversely, the absence of any genetic imbalance is associated with a favorable prognosis. Monosomy 3 is the most reliable cytogenetic marker identified to date. Tumors that contain 2 copies of chromosome 3 rarely metastasize, whereas 57% of patients with a primary uveal melanoma that showed loss of 1 chromosome 3 developed metastasis within 3 years, most commonly to the liver (Prescher et al., 1996). The ability to identify those individuals at high risk for metastases may allow these patients to be monitored closely for the development of liver metastases so they can be offered adjuvant chemotherapy or liver resection surgery at an early stage (Missotten and Keunen, 2004; Kodjikian et al., 2005). As showed by Hoglund et al, (Hoglund et al., 2004) the imbalance in the number of copies of chromosome 3 may represent a starting point of karyotypic evolution. Theoretically, this karyotypic evolution may represent the initial event of oncogenesis and lay the basis of tumor formation. On the other hand, loss of chromosome 3 may be a late clonal event, which will occur when the tumor has already grown out, thus leading to heterogeneity with regard to the number of copies of chromosome 3. Studying chromosome 3 in a clinical setting is important in determining the patient's prognosis. As we show here, detection of monosomy 3 is possible in uveal

melanoma cell cultures after enucleation, but concerns about normal cell overgrowth in long-term cell cultures are unmistakably realistic. The fastest manner to detect monosomy 3 with the smallest number of artifacts would be to study only the number of copies of chromosome 3 using FISH on fresh tissue. Our observations regarding FISH on paraffin sections indicate that some uveal melanomas show disomy 3 and monosomy 3 in different areas within the same tumor. This suggests that clonal expansion of cells with loss of 1 copy of chromosome 3 is probably a secondary event in uveal melanoma, which may subsequently be associated with dissemination.

This puts patients with tumors containing monosomy 3 cells at high risk for metastatic disease, even if monosomy 3 only occurs in part of the tumor. Identification of high-risk patients by using FNABs on tumors that will only be treated locally and will not undergo enucleation is of major importance but is, however, still under debate. Recently, Miden et al. (Miden et al, 2006) showed that FISH analysis on trans-scleral FNABs can yield sufficient material for molecular cytogenetic analysis and that FNAB is a reliable and efficient technique that may be valuable in predicting the prognosis in individuals with uveal melanoma. Fine-needle aspiration biopsies, however, will only detect changes in cells in the area from which the FNAB is taken and they do not provide information about the number of copies of chromosome 3 outside that region. In 1985, Folberg et al. (Folberg et al., 1985) compared the standard deviation of the nucleolar area measured from FNABs with the standard deviation of the nucleolar area measured from the matched enucleation specimen and concluded that FNABs do not yield the same prognostic information as larger enucleation specimens and that FNABs may not be representative of the tumor. Naus et al. (Naus et al., 2002) indicated that the application of FISH to FNABs is a reliable method for assaying genetic prognostic parameters such as chromosome 3 loss and that statistical analysis showed a very good agreement between the FISH results from the biopsies and those from the main tumor. However, FNABs were performed *ex vivo* under ideal circumstances on relatively large tumors and the article did not describe how many samples were obtained from 1 tumor. As we show here, uveal melanomas can be heterogeneous for the number of copies of chromosome 3. This implies that when using FNABs, one may miss the cells with monosomy 3 if they occur only in low numbers. A negative test does not exclude the presence of monosomy 3 somewhere in the tumor.

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References

- Bergman L, Seregard S, Nilsson B, et al. Uveal melanoma survival in Sweden from 1960 to 1998. *Invest Ophthalmol Vis Sci.* 2003;44:3282–3287.
- Collaborative Ocular Melanoma Study Group: Mortality in patients with small choroidal melanoma: COMS report No. 4: *Arch Ophthalmol.* 1997;115: 886–893.
- Collaborative Ocular Melanoma Study Group: Trends in size and treatment of recently diagnosed choroidal melanoma, 1987–1997: findings from patients examined at collaborative ocular melanoma study (COMS) centers: COMS report No. 20. *Arch Ophthalmol.* 2003;121:1156–1162.
- Dauwerse JG, Jumelet EA, Wessels JW, et al. Extensive cross-homology between the long and the short arm of chromosome 16 may explain leukemic inversions and translocations. *Blood.* 1992;79:1299–1304.
- Folberg R, Augsburger JJ, Gamel JW, Shields JA, Lang WR. Fine-needle aspirates of uveal melanomas and prognosis. *Am J Ophthalmol.* 1985;100:654–657.
- Haralambieva E, Kleiverda K, Mason DY, Schuurin E, Kluin PM. Detection of three common translocation breakpoints in non-Hodgkin's lymphomas by fluorescence in situ hybridization on routine paraffin-embedded tissue sections. *J Pathol.* 2002;198:163–170.
- Hensen EF, Jordanova ES, van Minderhout IJ, et al. Somatic loss of maternal chromosome 11 causes parent-of-origin-dependent inheritance in SDHD-linked paraganglioma and pheochromocytoma families. *Oncogene.* 2004;23:4076–4083.
- Hoglund M, Gisselsson D, Hansen GB, et al. Dissecting karyotypic patterns in malignant melanomas: temporal clustering of losses and gains in melanoma karyotypic evolution. *Int J Cancer.* 2004;108:57–65.
- Horsman DE, Sroka H, Rootman J, White VA. Monosomy 3 and isochromosome 8q in a uveal melanoma. *Cancer Genet Cytogenet.* 1990;45:249–253.
- Jordanova ES, Riemersma SA, Philippo K, et al. Hemizygous deletions in the HLA region account for loss of heterozygosity in the majority of diffuse large B-cell lymphomas of the testis and the central nervous system. *Genes Chromosomes Cancer.* 2002;35:38–48.
- Kilic E, Naus NC, van Gils W, et al. Concurrent loss of chromosome arm 1p and chromosome 3 predicts a decreased disease-free survival in uveal melanoma patients. *Invest Ophthalmol Vis Sci.* 2005;46:2253–2257.
- Kodjikian L, Grange JD, Baldo S, et al. Prognostic factors of liver metastases from uveal melanoma. *Graefes Arch Clin Exp Ophthalmol.* 2005;243:985–993.
- Lin AY, Maniotis AJ, Valyi-Nagy K, et al. Distinguishing fibrovascular septa from vasculogenic mimicry patterns. *Arch Pathol Lab Med.* 2005;129:884–892.
- Midena E, Bonaldi L, Parrozzani R, et al. In vivo detection of monosomy 3 in eyes with medium-sized uveal melanoma using transscleral fine needle aspiration biopsy. *Eur J Ophthalmol.* 2006;16:422–425.
- Missotten GS, Keunen JE. Screening for uveal melanoma metastasis: literature review. *Bull Soc Belge Ophtalmol.* 2004;13–22.
- Mooy CM, De Jong PT. Prognostic parameters in uveal melanoma: a review. *Surv Ophthalmol.* 1996;41:215–228.
- Naus NC, van Drunen E, de Klein A, et al. Characterization of complex chromosomal abnormalities in uveal melanoma by fluorescence in situ hybridization, spectral karyotyping, and comparative genomic hybridization. *Genes Chromosomes Cancer.* 2001;30:267–273.
- Naus NC, Verhoeven AC, van Drunen E, et al. Detection of genetic prognostic markers in uveal melanoma biopsies using fluorescence in situ hybridization. *Clin Cancer Res.* 2002;8:534–539.
- Prescher G, Bornfeld N, Hirsch H, et al. Prognostic implications of monosomy 3 in uveal melanoma. *Lancet.* 1996;347:1222–1225.
- Singh AD, Topham A. Incidence of uveal melanoma in the United States: 1973–1997. *Ophthalmology.* 2003;110:956–961.
- Sisley K, Rennie IG, Cottam DW, et al. Cytogenetic findings in six posterior uveal melanomas: involvement of chromosomes 3, 6, and 8. *Genes Chromosomes Cancer.* 1990;2:205–209.
- Vaandrager JW, Schuurin E, Raap T, et al. Interphase FISH detection of BCL2 rearrangement in follicular lymphoma using breakpoint-flanking probes. *Genes Chromosomes Cancer.* 2000;27:85–94.

Chapter 3

Epigenetic Inactivation of RASSF1a in Uveal Melanoma

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Abstract

PURPOSE. The RAS association domain family 1 (*RASSF1*) gene is a tumor-suppressor gene located on chromosome 3p21.3. The alternative transcript (*RASSF1a*) has been shown to be inactivated by hypermethylation in several human malignancies, including breast, prostate, and lung cancer, and in cutaneous melanoma. The purpose of this study was to evaluate the methylation status of *RASSF1a* in human uveal melanoma.

METHODS. The methylation status of the *RASSF1a* promoter region was analyzed using PCR in combination with melting curve analysis, sequencing, and restriction enzyme analysis. Eleven human uveal melanoma cell lines, normal melanocytes, 39 archival frozen tumor specimens, and a metastatic lesion of untreated primary uveal melanoma were studied. In addition, whether *RASSF1a* methylation correlates with patient survival and development of metastatic disease was investigated.

RESULTS. *RASSF1a* promoter methylation was detected in 10 of the 11 (91%) cell lines, in 19 of the 38 (50%) patients with primary uveal melanoma and in the metastatic lesion. A positive correlation was found between *RASSF1a* promoter methylation and development of metastatic disease ($P = 0.041$). A correlation with disease-free survival could not be established, but a positive trend was observed ($P = 0.063$).

CONCLUSIONS. These data show that *RASSF1a* methylation is a common epigenetic event in uveal melanoma development, potentially of clinical relevance. The presence of a methylated *RASSF1a* promoter region might therefore serve as a tumor marker and as a possible target for therapeutic intervention.

Introduction

Uveal melanoma is the most common primary intraocular neoplasm in adults. It accounts for 70% of all primary eye tumors and occurs at an annual incidence of 6 to 8 per million in white populations (Egan et al., 1988). Uveal melanoma develops without any obvious genetic or environmental predisposing factors (Hanahan and Weinberg, 2000). An effective starting point for the identification of genetic changes is to study the chromosomes involved in the progression and development of uveal melanoma. Cytogenetic studies have identified a number of chromosomal abnormalities in uveal melanoma, of which loss of chromosome 1p, gain of 6p and 8q, and loss of one copy of chromosome 3 (i.e., monosomy 3) commonly occur (Horsman et al., 1990, Prescher et al., 1990, Sisley et al., 1990, Naus et al., 2002, Kiliç et al., 2005). Monosomy 3 is highly correlated with decreased survival and metastatic disease (Prescher et al. 1996, White et al., 1998), suggesting the presence of a tumor suppressor gene (*TSG*) at this location. Despite this information, genetic analysis of this chromosome has not revealed specific *TSGs* as players in uveal melanoma pathogenesis. Alternatively, hypermethylation of promoter-associated CpG islands has recently emerged as an important epigenetic mechanism leading to the transcriptional silencing of *TSGs* in cancer development (Jones and Baylin, 2002), including the development of uveal melanoma (Van der Velden et al., 2002, Van der Velden et al., 2003, Van Dinten et al., 2005). Recent findings in other malignancies have identified the Ras association domain family 1 (*RASSF1*) gene, located on chromosome 3p21.3, as an important regulator of cell proliferation (Dammann et al., 2000, Hamilton et al., 2005, Choi et al., 2006). Hypermethylation of the CpG island in the promoter region of a major alternative transcript of this gene, *RASSF1a*, occurs frequently in various carcinomas, including those of the breast, prostate, and lung, and in cutaneous malignant melanoma (Spugnardi et al., 2003, Kang et al., 2004, Yeo et al., 2005, Fukasawa et al., 2006). In this study, we investigated *RASSF1a* gene hypermethylation in uveal melanoma. We demonstrated that *RASSF1a* methylation of its promoter CpG island in uveal melanoma cell lines and primary tumors is a common event. Furthermore, we showed a correlation between *RASSF1a* hypermethylation and the development of metastatic disease. Consequently, we postulated that the methylation of *RASSF1a* is a prognostic tumor marker for uveal melanoma and may serve as a potential target for therapeutic intervention.

Materials and methods

Cell Lines, Primary Uveal Melanoma Specimens, and the Metastatic Lesion

In total, 11 cell lines derived from primary uveal melanomas (92.1, OCM-1, OCM-3, OCM-8, Mel-202, Mel-270, Mel-285, Mel-290) and uveal melanoma metastases (OMM-1, OMM-1.3, OMM-1.5) and a culture of normal uveal melanocytes (Mel-1a) were analyzed for promoter hypermethylation. Cell lines Mel-270, OMM-1.3, and OMM-1.5 represent a progression model because they were derived from a primary uveal melanoma and two of its liver metastases, respectively. All melanoma cell lines were cultured in RPMI 1640 medium (Gibco, Paisley, Scotland) supplemented with 3 mM L-glutamine (Gibco), 2% penicillin/streptomycin, and 10% FBS (Hyclone, Logan, UT). The melanocyte cell line (Mel-1a) was grown in F12

medium (Gibco) (Zuidervaart et al., 2003). All cell cultures were incubated at 37°C in a humidified 5% CO₂ atmosphere. Archival frozen tumor specimens of primary uveal melanoma came from 39 patients who attended the Leiden University Medical Center between 1988 and 1996. The metastatic lesion (adrenal gland metastasis) was derived from the patient with a primary uveal melanoma (tumor 31), from which cell line 92.1 was derived (Waard-Siebinga et al., 1995, Blom et al., 1997). All tumors were primary lesions with diameters greater than 12 mm and prominences greater than 6 mm; patients had not received any treatment before enucleation. The validity of the diagnosis, uveal melanoma, was confirmed histologically in all patients, and clinical and survival data were listed for use in this study (Table 3.1). The research protocol followed the tenets of the Declaration of Helsinki (World Medical Association Declaration of Helsinki 1964; ethical principles for medical research involving human subjects).

Table 3.1. Primers used in PCR

RASSF1a Forward Primer:	3'-GAAGGAGGGAAGGAAGGGTAAGG-5'
RASSF1a Reverse Primer:	5'-GGAGTTTGAGTTATTGAGTTGCGG-3'

DNA Extraction and Sodium Bisulfite Modification

With the use of a column-based extraction kit (Qiagen Genomic tip 100/G; Qiagen Benelux BV, Venlo, The Netherlands), DNA was extracted from cell lines, frozen tumor material, and cultured uveal melanocytes according to the kit manufacturer's guidelines. Genomic DNA was modified with sodium bisulfite (EZ Methylation Gold Kit; Zymo Research Corporation, Orange, CA.). Enzymatically methylated human DNA (Chemicon Europe Ltd., Hampshire, UK) was used as a positive control in all experiments.

Methylation Analysis

Methylation status of the promoter region was determined by polymerase chain reaction with specific primers and by melting temperature analysis and was further validated with restriction digestion analysis. Primers were designed using bisulfite-converted DNA sequences (Beacon Designer Software version 5.0; Premier Biosoft International, Palo Alto, CA) and amplified a region of the *RASSF1a* gene promoter CpG island (NCBI accession *605082; bases 353–618). Primers are shown in Table 3.2. PCR was carried out in a final volume of 21 µL containing 10 µL mix (iQ SYBR Green Supermix; Bio-Rad Laboratories BV, Veenendaal, The Netherlands), 1 µL (10 pmol/µL) of each primer, 8 µL H₂O, and 1 µL bisulfite-converted DNA. PCR was initiated by hot start, followed by 40 cycles at 96°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, and with a final melting curve from 70°C to 97°C with an increase in temperature of 0.2°C every 10 seconds. The fluorescence of SYBR Green was measured once per cycle to monitor template amplification.

Table 3.2. Clinical and Survival Data

Tumor ID	Cell-type	Survival in months	Present status	Hypermethylated RASSF1a
UM 1	Spindle	198	Alive	Present
UM 2	Mixed	29	Dead, metastases	Present
UM 3	Mixed	95	Dead, metastases	Present
UM 4	Spindle	31	Dead, metastases	Present
UM 5	Spindle	50	Dead, metastases	Present
UM 6	Epithelioid	57	Dead, unknown	Not present
UM 7	Mixed	63	Dead, unknown	Not present
UM 8	Spindle	179	Alive	Not present
UM 9	Mixed	131	Alive	Not present
UM 10	Mixed	124	Alive	Not present
UM 11	Epithelioid	50	Dead, other cause	Not present
UM 12	Mixed	13	Dead, metastases	Present
UM 13	Epithelioid	128	Alive	Present
UM 14	Epithelioid	30	Dead, metastases	Not present
UM 15	Epithelio	21	Dead, unknown	Failed
UM 16	Mixed	42	Dead, metastases	Present
UM 17	Mixed	23	Dead, metastases	Present
UM 18	Epithelioid	115	Dead, unknown	Present
UM 19	Epithelioid	33	Dead, metastases	Present
UM 20	Spindle	12	Dead, metastases	Present
UM 21	Spindle	15	Dead, metastases	Not present
UM 22	Mixed	187	Alive	Present
UM 23	Epithelioid	167	Dead, metastases	Present
UM 24	Spindle	2	Lost to follow up	Not present
UM 25	Spindle	145	Alive	Not present
UM 26	Mixed	131	Alive	Present
UM 27	Mixed	13	Dead, other cause	Not present
UM 28	Mixed	29	Dead, other cause	Not present
UM 29	Spindle	122	Alive	Not present
UM 30	Mixed	23	Dead, unknown	Not present
UM 31	Epithelioid	33	Dead, metastases	Present
UM 32	Spindle	24	Dead, metastases	Not present
UM 33	Epithelioid	30	Dead, metastases	Not present
UM 34	Mixed	13	Dead, metastases	Present
UM 35	Spindle	101	Alive	Present
UM 36	Mixed	34	Dead, metastases	Present
UM 37	Epithelioid	36	Dead, unknown	Not present
UM 38	Mixed	24	Dead, unknown	Not present
UM 39	Spindle	94	Alive	Not present

Melting Temperature Analysis

PCR amplification of bisulfite-treated DNA and subsequent melting analysis (iQ5 Real-time PCR Detection System; Bio-Rad Laboratories BV) allowed detection of heterogeneous methylation (in which the content and distribution of methylated cytosines differed between different molecules in the same sample). DNA melting peaks were acquired by measuring the fluorescence of SYBR Green during a linear temperature transition from 70°C to 97°C at 0.2°C each 10 seconds with accompanying software (Bio-Rad Laboratories BV).

Restriction Digestion Analysis and Sequence Analysis

After amplification with specific primers for methylated DNA, the PCR-amplified fragments were digested using 4 U restriction enzyme RSA1 (Fermentas GmbH, St. Leon Rot, Germany) directly added to the PCR mixture (under conditions specified by the manufacturer). The *RASSF1a* amplicon of methylated DNA contains one RSA1 recognition site and is dependent on CT conversion and methylation of a CpG. The recognition site 5'-GT_{AC}-3' only appears when the first C in the GCAC sequence is converted to thymine, whereas the second must be methylated and remains C. Amplified products were analyzed by electrophoresis on a 2% agarose gel. DNA bands were excised from the gel, purified with a gel extraction kit (Nucleospin Extract II; Macherey-Nagel GmbH & Co., Düren, Germany), and sequenced on a DNA sequencing system (ABI PRISM 3700; Applied Biosystems, Foster City, CA).

Statistical Analysis

Survival analysis for *RASSF1a* promoter methylation was performed with Kaplan-Meier analysis and log rank test (SPSS version 12.0.1 for Windows; SPSS Inc., Chicago, IL). For comparison between the presence or absence of *RASSF1a* methylation and metastatic disease, the X2 test was performed.

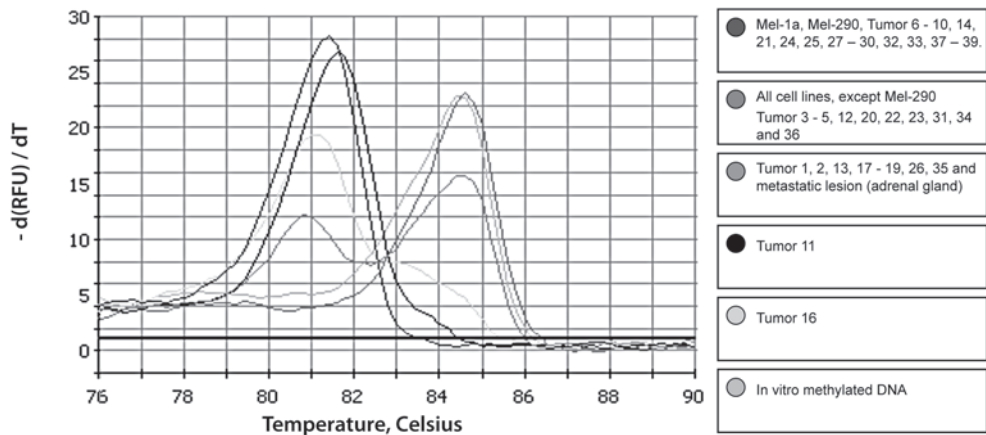
Results

Methylation Analysis in Cell Lines, Primary Tumor Tissue, and Metastasis

To evaluate whether *RASSF1a* methylation may play a role in uveal melanoma, we analyzed the methylation status of the *RASSF1a* promoter region in a panel of uveal melanoma cell lines and archival fresh frozen tumor tissue using melting temperature analysis, sequencing, and restriction enzyme analysis. In unmethylated DNA, all cytosines in a CpG dinucleotide are converted to thymines, but in methylated DNA such cytosines remain unchanged. Replacement of cytosines by thymines results in change of a CG base pair to a TA base pair in double-stranded DNA. Subsequently, the temperature at which DNA becomes single stranded is higher in methylated DNA. PCR performed on sodium bisulfite-converted DNA isolated from uveal melanoma cell lines showed a melting peak at 83.6° to 84.6°C in 10 of 11 cell lines (all except Mel-290), which coincided with the peak of the positive control (full methylation). In cell line Mel-290, a melting peak was observed at 81.2° to 81.4°C, similar to the normal cultured melanocytes, indicating an unmethylated promoter (Figure 3.1). This result is consistent with the fact

that the amplified RASSF1a amplicon contained 20 CpGs (primer design software predicts a melting temperature difference of 3.4°C).

Figure 3.1. Different melting peaks for the RASSF1a amplicon. Bisulfite treated DNA was amplified from cell lines, primary tumors, a metastatic lesion, and completely methylated DNA. Different melting peaks are shown that are also representative of corresponding samples. Melting peaks were obtained by plotting the negative derivative of fluorescence over temperature versus temperature ($-d(RFU)/dT$ vs. T).



In addition, 39 primary tumors and a metastatic lesion in patient 31 were analyzed in the same manner as the cell lines. One tumor sample failed repeatedly in the PCR and was excluded (sample 15). Of the remaining 38 primary tumor samples, 19 (50%) included a methylated RASSF1a promoter region. Nine of these samples and the metastatic lesion showed an additional melting peak at 81.2° to 81.4°C, indicating the presence of methylated and unmethylated DNA in the same sample and signaling the presence of heterogeneous cell populations (Figure 3.1).

Restriction Digestion Analysis and Sequence Analysis

Amplified PCR fragments were digested using the restriction enzyme *RSA1*. Incubation with *RSA1* and gel analysis of the fragments confirmed the methylation status as already observed with the melting curve analysis. Ten of 11 cell lines showed bands representative for methylated DN; again, hypermethylation was not observed in the melanocyte culture Mel-1a (normal control; Figure 3.2). Of the 38 primary uveal melanomas, 19 samples were found to be methylated by restriction enzyme analysis. These were in exact correspondence with those found to be methylated in melting curve analysis. As a final check, methylation of RASSF1a was confirmed by direct sequencing of the RASSF1a product in all samples (Figure 3.3 depicts an example of cell lines Mel-270 and Mel-290).

Figure 3.2. Restriction digestion analysis. Agarose gel electrophoresis showing *RASSF1a*-amplified DNA fragments of all uveal melanoma cell lines after digestion with restriction enzyme *RsaI*. The amplicon contains one *RsaI* recognition site and is dependent on CT conversion and CpG methylation. The recognition site GT_{AC} only appears when the first C in the GCAC sequence is converted to thymidine; the second must be methylated and remains a C. In vitro-methylated DNA was used as a positive control, and a normal uveal melanocyte culture was used as a normal control. With the exception of cell line Mel-290, all cell lines showed complete methylation of the *RASSF1a* promoter region.

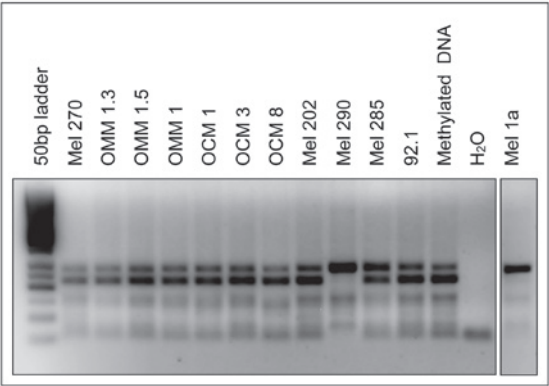
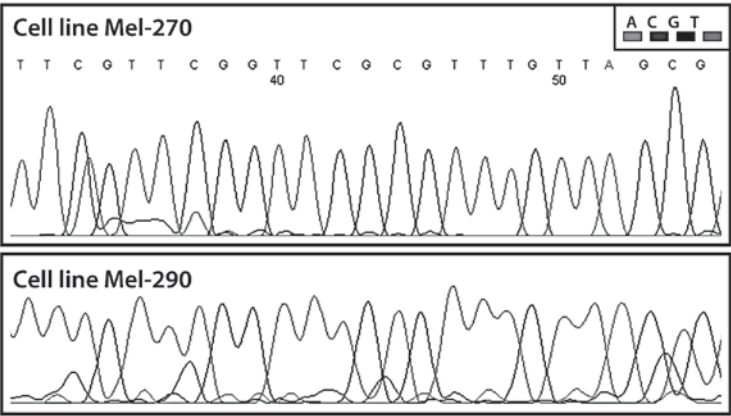


Figure 3.3. Sequence analysis of cell lines Mel270 and Mel-290. Sequence results from uveal melanoma cell line Mel-270 DNA (top) and cell line Mel-290 (bottom). The absence of cytosines in amplified bisulfite-converted DNA of cell line Mel-290 confirmed the presence of an unmethylated *RASSF1a* promoter region in this cell line, as found earlier by digestion analysis.

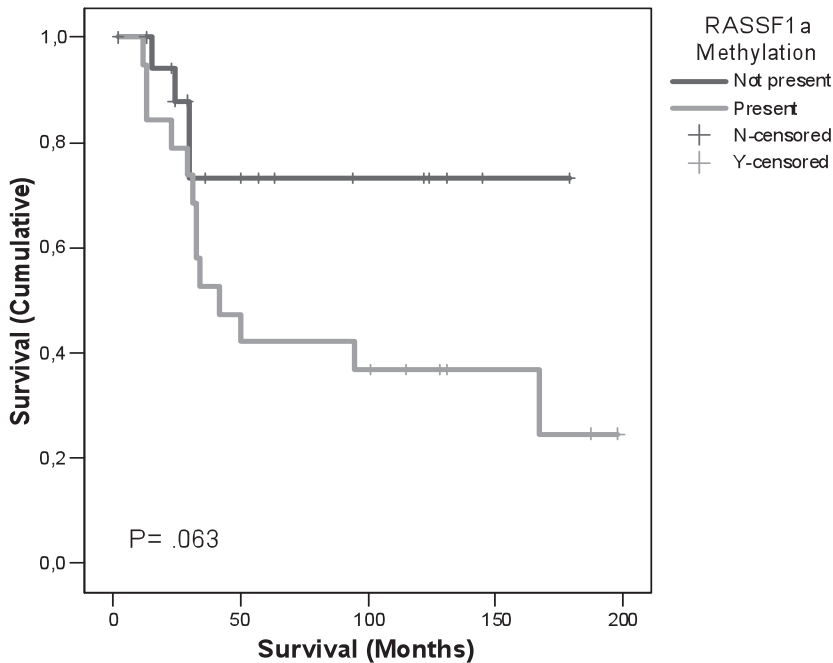


Survival Analysis

To assess whether the presence of hypermethylated *RASSF1a* was correlated with a clinical parameter, correlations between methylation status and melanoma-associated survival and development of metastatic disease were evaluated. Of the initial group of 39 patients, one was lost after 2 months of follow-

up. Mean survival of the remaining 38 patients was 68 months (range, 12–198 months). Development of metastatic disease correlated with the presence of a hypermethylated *RASSF1a* promoter region ($P = 0.041$). An association between disease free survival and the presence of a hypermethylated *RASSF1a* promoter could not be established, though a positive trend was observed ($P = 0.063$; log rank test), as shown in Figure 3.4.

Figure 3.4. Kaplan-Meier analysis and log rank test showed the difference in disease-free survival according the absence or presence of a hypermethylated *RASSF1a* promoter region in primary uveal melanoma tumor tissue ($P = 0.063$; log rank test). Although not significant, a positive trend was observed.



Discussion

Epigenetic modification of gene expression is important in tumor development (Das and Singal, 2004, Baylin and Ohm, 2006). Methylation of *TSG* is now commonly analyzed in tumors and even rivals mutation and deletion as the main mechanism in tumor development in certain tumors (Robertson, 2005). *RASSF1a* is an example of a potential *TSG* that has been studied in many tumors in which methylation correlates with reduced expression (Pfeifer and Dammann, 2005).

The frequent methylation of the *RASSF1a* gene in uveal melanoma cell lines (91%) and also in primary tumor tissue (50%) suggests that *RASSF1a* also plays a role in uveal melanoma pathogenesis. It has been suggested that the *RASSF1a* protein acts at the level of G1/S-phase cell cycle progression (Shivakumar et al., 2002). Loss of expression of *RASSF1a* as caused by hypermethylation could therefore reduce G1/S-

phase cell cycle control. In lung and breast tumor-derived epithelial cells, reintroduction of *RASSF1a* expression resulted in growth arrest that was correlated with reduced cyclin D1 protein accumulation, whereas iRNA-mediated inhibition of *RASSF1a* expression resulted in abnormal accumulation of native cyclin D1 (Shivakumar et al., 2002). Shivakumar et al. reported that *RASSF1A* functions as a negative regulator of cell proliferation through the inhibition of G1/S-phase progression.

Loss of one copy of chromosome 3 (monosomy 3) has been reported in approximately 50% of all uveal melanomas and is associated with metastatic behavior of this type of tumor. Until now, no specific *TSG* at chromosome 3 has been identified that is targeted by this deletion. Therefore, the location of the *RASSF1a* gene on chromosome 3p21.3 could be of utmost importance for uveal melanoma. Although methylation of *RASSF1a* may not be held wholly responsible for uveal melanoma development, it could be a contributing factor for uveal melanoma tumor formation and progression. *RASSF1a* methylation could be the second hit in a classic *TSG* inactivation in uveal melanoma with monosomy 3. Loss of one *RASSF1a* copy because of monosomy 3, in combination with hypermethylation of the other copy, could promote progression through the G1/S phase of the cell cycle, stimulating the formation of uveal melanoma tumor.

The high frequency of *RASSF1A* methylation in uveal melanoma cell lines compared with primary tumors has also been observed in cell lines derived from breast and ovarian cancer (Agathangelou et al., 2001). In addition, the methylation of p16INK4a in uveal melanoma was shown to be more common in uveal melanoma cell lines, in contrast to primary tumors (Van der Velden et al, 2001). This observation might indicate that tumors with *RASSF1A* methylation are more oncogenic and are likely to be established as cell lines and is consistent with the observation that, in contrast to the cell lines, one third of the positive tumors presented methylated and unmethylated DNA, suggesting that heterogeneity of the primary tumor is lost in the cell line cultures.

An obvious example of tumor heterogeneity is shown in Figure 3.1. Primary tumor sample 11 seems to be unmethylated, but the tiny shoulder at the position of methylated DNA suggests the presence of a minimal amount of methylated DNA. An attempt to validate this minor methylated DNA fraction through digestion analysis was inconclusive. Future analysis using more sensitive and advanced techniques should be conducted to clarify the possible presence of small quantities of methylated DNA in such samples.

Although *RASSF1a* methylation alone may not be responsible for uveal development, it could be a contributing factor for uveal melanoma tumor formation and progression. The three cell lines derived from one patient (OMM-1.3 and OMM-1.5 are from two different metastases, and Mel-270 is from the primary tumor) were all methylated. The metastatic lesion derived from the same patient bearing the *RASSF1a* methylated tumor 31 showed two melting peaks, indicating a mixture of methylated and unmethylated DNA probably as a result of contamination of the sample with normal tissue. Furthermore, *RASSF1a* promoter hypermethylation was found to be associated with the development of metastatic disease ($P = 0.041$), and the presence of methylation in the primary tumor and its metastasis indicates a role for methylated *RASSF1a* in tumor progression. A correlation with decreased survival could not be

established in this study, but a positive trend was observed ($P = 0.063$).

Based on these findings, we propose that the epigenetic regulation of the *RASSF1a* gene through promoter hypermethylation may play an important role in the development and progression of uveal melanoma, which once again adds to the proposed high frequency of methylation in uveal melanoma compared with normal tissue. We also propose that *RASSF1a* might serve as a tumor marker in uveal melanoma.

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References

- Agathanggelou A, Honorio S, Macartney DP, et al. Methylation associated inactivation of RASSF1A from region 3p21.3 in lung, breast and ovarian tumours. *Oncogene*. 2001;20:1509–1518.
- Baylin SB, Ohm JE. Epigenetic gene silencing in cancer—a mechanism for early oncogenic pathway addiction? *Nat Rev Cancer*. 2006;6:107–116.
- Blom DJ, Schurmans LR, Waard-Siebinga I, et al. HLA expression in a primary uveal melanoma, its cell line, and four of its metastases. *Br J Ophthalmol*. 1997;81:989–993.
- Choi YL, Kang SY, Choi JS, et al. Aberrant hypermethylation of RASSF1A promoter in ovarian borderline tumors and carcinomas. *Virchow Arch*. 2006;448:331–336.
- Das PM, Singal R. DNA methylation and cancer. *J Clin Oncol*. 2004;22:4632–4642.
- Dammann R, Li C, Yoon JH, et al. Epigenetic inactivation of a RAS association domain family protein from the lung tumour suppressor locus 3p21.3. *Nat Genet*. 2000;25:315–319.
- Egan KM, Seddon JM, Glynn RJ, Gragoudas ES, Albert DM. Epidemiologic aspects of uveal melanoma. *Surv Ophthalmol*. 1988;32: 239–251.
- Fukasawa M, Kimura M, Morita S, et al. Microarray analysis of promoter methylation in lung cancers. *J Hum Genet*. 2006;51: 368–374.
- Hamilton DW, Lusher ME, Lindsey JC, Ellison DW, Clifford SC. Epigenetic inactivation of the RASSF1A tumour suppressor gene in ependymoma. *Cancer Lett*. 2005;227:75–81.
- Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell*. 2000; 100:57–70.
- Horsman DE, Sroka H, Rootman J, White VA. Monosomy 3 and isochromosome 8q in a uveal melanoma. *Cancer Genet Cytogenet*. 1990;45:249–253.
- Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. *Nat Rev Genet*. 2002;3:415–428.
- Kang GH, Lee S, Lee HJ, Hwang KS. Aberrant CpG island hypermethylation of multiple genes in prostate cancer and prostatic intraepithelial neoplasia. *J Pathol*. 2004;202:233–240.
- Kilic E, Naus NC, van Gils W, et al. Concurrent loss of chromosome arm 1p and chromosome 3 predicts a decreased disease-free survival in uveal melanoma patients. *Invest Ophthalmol Vis Sci*. 2005;46:2253–2257.
- Naus NC, Verhoeven AC, van Drunen E, et al. Detection of genetic prognostic markers in uveal melanoma biopsies using fluorescence in situ hybridization. *Clin Cancer Res*. 2002;8:534–539.
- Pfeifer GP, Dammann R. Methylation of the tumor suppressor gene *RASSF1A* in human tumors. *Biochemistry (Mosc)*. 2005;70:576–583.
- Prescher G, Bornfeld N, Becher R. Nonrandom chromosomal abnormalities in primary uveal melanoma. *J Natl Cancer Inst*. 1990; 82:1765–1769.
- Prescher G, Bornfeld N, Hirche H, et al. Prognostic implications of monosomy 3 in uveal melanoma. *Lancet*. 1996;347:1222–1225.
- Robertson KD. DNA methylation and human disease. *Nat Rev Genet*. 2005;6:597–610.
- Shivakumar L, Minna J, Sakamaki T, Pestell R, White MA. The RASSF1A tumor suppressor blocks cell cycle progression and inhibits cyclin D1 accumulation. *Mol Cell Biol*. 2002;22:4309–4318.
- Spugnardi M, Tommasi S, Dammann R, Pfeifer GP, Hoon DS. Epigenetic inactivation of RAS association domain family protein 1 (RASSF1A) in malignant cutaneous melanoma. *Cancer Res*. 2003;63:1639–1643.
- Sisley K, Rennie IG, Cottam DW, et al. Cytogenetic findings in six posterior uveal melanomas: involvement of chromosomes 3, 6, and 8. *Genes Chromosomes Cancer*. 1990;2:205–209.
- van der Velden PA, Metzelaar-Blok JA, Bergman W, et al. Promoter hypermethylation: a common cause of reduced p16(INK4a) expression in uveal melanoma. *Cancer Res*. 2001;61:5303–5306.
- van der Velden PA, Zuidervaart W, Hurks MH, et al. Expression profiling reveals that methylation of TIMP3 is involved in uveal melanoma development. *Int J Cancer*. 2003;106:472–479.
- van Dinten LC, Pul N, van Nieuwpoort AF, et al. Uveal and cutaneous melanoma: shared expression characteristics of melanoma associated antigens. *Invest Ophthalmol Vis Sci*. 2005;46:24–30.

- Waard-Siebinga I, Blom DJ, Griffioen M, et al. Establishment and characterization of a uveal-melanoma cell line. *Int J Cancer*. 1995; 62:155–161.
- White VA, McNeil BK, Horsman DE. Acquired homozygosity (isodisomy) of chromosome 3 in uveal melanoma. *Cancer Genet Cytogenet*. 1998;102:40–45.
- Yeo W, Wong WL, Wong N, et al. High frequency of promoter hypermethylation of RASSF1A in tumorous and non-tumorous tissue of breast cancer. *Pathology*. 2005;37:125–130.
- Zuidervaat W, van der Velden PA, Hurks MH, et al. Gene expression profiling identifies tumour markers potentially playing a role in uveal melanoma development. *Br J Cancer*. 2003;89:1914–1919.

Chapter 4

Epigenetic Regulation Identifies *RASEF* as a Tumor-Suppressor Gene in Uveal Melanoma

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Abstract

PURPOSE. Recently, a segregation study in families with uveal and cutaneous melanoma identified 9q21 as a potential locus harboring a tumor-suppressor gene (*TSG*). One of the genes in this area, *RASEF*, was then analyzed as a candidate *TSG*, but lack of point mutations and copy number changes could not confirm this. In this study, the *RASEF* gene was investigated for potential mutations and gene silencing by promoter methylation in uveal melanoma.

METHODS. Eleven uveal melanoma cell lines and 35 primary uveal melanoma samples were screened for mutations in the *RASEF* gene by high-resolution melting-curve and digestion analysis. Expression of *RASEF* was determined by real-time RT-PCR in all cell lines and 16 primary uveal melanoma samples, and the methylation status of the promoter of the *RASEF* gene was analyzed and confirmed by direct sequencing.

RESULTS. Mutation screening revealed a known polymorphism (R262C; C→T) in exon 5 of the *RASEF* gene that displayed a normal frequency (54%). Of the primary uveal melanomas, 46% presented a heterozygous genotype, and 10 (91%) of 11 cell lines showed a homozygous genotype. Melting-curve analysis indicated loss of heterozygosity in at least two primary tumors. Low *RASEF* expression in the cell lines and primary tumors correlated with methylation of the *RASEF* promoter region. Homozygosity and methylation of the *RASEF* gene in primary tumors were associated with decreased survival ($P = 0.019$).

CONCLUSIONS. Homozygosity, in combination with methylation, appears to be the mechanism targeting *RASEF* in uveal melanoma, and allelic imbalance at this locus supports a *TSG* role for *RASEF*.

Introduction

Uveal melanoma is the most common primary intraocular neoplasm in adults, with an annual incidence of six to eight per million in Caucasian populations (Egan et al., 1988). In contrast to cutaneous melanoma, clustering of uveal melanoma in families is extremely rare (Van Hees et al., 1998; Kodjikian et al., 2003; Smith et al., 2007). Occurrence of both uveal melanoma and cutaneous melanoma in a single family has been observed (van Hees et al., 1998). Recently, Jönsson et al. (Jönsson et al., 2005) revealed a genetic component in three such families, in which members are affected by either uveal or cutaneous melanoma. Linkage analysis in these families identified a potential uveal melanoma susceptibility locus on chromosome 9, area q21. This locus has a long history in melanoma that started with detection of isochromosome 9q with cytogenetic analysis (Kopf et al., 1992; Albino et al., 1992). Loss of heterozygosity (LOH) of markers at 9q22 was subsequently frequently reported and was shown to be associated with proliferation and tumor progression (Boni et al., 1998; Kumar et al., 1999).

Recently, single nucleotide polymorphism (SNP) analysis has confirmed the LOH of this locus in melanoma, while genome-wide analysis in dizygotic twins for nevi numbers also showed linkage with this 9q region (Zhu et al., 2007; Stark et al., 2007). In addition, a gene slightly distal to *RASEF*, *RMI1*, has recently been shown to be a risk factor for cutaneous melanoma, whereas the locus for familial melanoma susceptibility is located on the short arm of chromosome 9 (Cannon-Albright et al., 1992; Boni et al., 1998; Broberg et al., 2007). Cutaneous melanomas are often characterized by loss of the cell-cycle regulator *p16* and/or activation of the RAS-RAF-ERK pathway (Cohen et al., 2002; Satyamoorthy et al., 2003). These hallmarks of melanoma are also recognized in uveal melanoma, although the underlying mechanisms differ (Weber et al., 2003; Calipel et al., 2006). Whereas in cutaneous melanoma, *p16* is commonly lost by chromosomal deletion of the *CDKN2A* gene, the preferential mechanism in uveal melanoma appears to be silencing of the *p16*-encoding *CDKN2A* promoter by methylation (van der Velden et al., 2001). Mutations in *BRAF*, *NRAS*, or *c-kit* lead to constitutive ERK activation in most cutaneous melanomas (van Elsas et al., 1996; Davies et al., 2002). However, mutations in *BRAF* have only rarely been reported in uveal melanoma, whereas activating *NRAS* and *c-kit* mutations have never been reported (Zuidervaart et al., 2005). Still, ERK activation is also present in uveal melanoma, and this knowledge leads to the question of what causes ERK activation in the absence of activating mutations in *BRAF*, *NRAS*, or *c-kit* (Weber et al., 2003; Zuidervaart et al., 2005; Calipel et al., 2006).

The *RASEF* (RAS and EF hand domain containing) gene is located on chromosome 9, area q21, and encodes a protein with calcium-binding EF-hand and Ras GTPase (Rab family) motifs (<http://www.genome.ucsc.edu/> provided in the public domain by the Genome Bioinformatics Group, University of Santa Cruz, CA); it is also known as *RAB45* or *FLJ31614.22* (Sweetser et al., 2005). Based on the functional domains in *RASEF*, the gene product may be engaged in the *RAS* pathway and in combination with evidence for linkage of the *RASEF* region with cutaneous and uveal melanoma, molecular analysis of this gene is warranted. In line with the analysis of cutaneous melanoma reported by Jönsson et al. (Jönsson et al., 2005) we therefore set out to analyze *RASEF* for mutations and for expression of the gene in uveal melanoma.

Materials and methods

Cell Lines and Primary Uveal Melanoma Specimens

In total, 11 cell lines derived from primary uveal melanomas (92.1; OCM-1, -3, and -8; and Mel-202, -270, -285, and -290) and uveal melanoma metastases (OMM-1, -2.3, and -2.5) were analyzed. All melanoma cell lines were cultured in RPMI 1640 medium (Invitrogen-Gibco, Paisley, Scotland, UK) supplemented with 3 mM L-glutamine (Invitrogen- Gibco), 2% penicillin streptomycin, and 10% FBS (Hyclone, Logan, UT). All cell cultures were incubated at 37°C in a humidified 5% CO₂ atmosphere. Archival frozen tumor specimens of primary uveal melanoma came from 35 patients who attended the Leiden University Medical Center between 1988 and 1996. All tumors were primary lesions with a tumor diameter greater than 12 mm, a prominence greater than 6 mm, and no treatment before enucleation. The validity of the diagnosis of uveal melanoma was confirmed histologically in all cases, and clinical and survival data were listed for use in the study (Table 4.1). The research protocol followed the tenets of the current version of the Declaration of Helsinki (World Medical Association Declaration of Helsinki 1964; Ethical Principles for Medical Research Involving Human Subjects).

Table 4.1. Tumor Characteristics and Survival Data of 35 Uveal Melanoma Patients Sorted by Methylation Status and *RASEF* Genotype

Tumor ID	Cell type	Survival (mo)	Present status	Hypermethylated <i>RASEF</i>	Genotype
UM1	Spindle	210	Alive	Not present	Hom C
UM2	Mixed	29	Dead, due to metastases	Not present	Het
UM3	Mixed	95	Dead, due to metastases	Not present	Hom C
UM4	Spindle	31	Dead, due to metastases	Not present	Hom T
UM5	Spindle	50	Dead, due to metastases	Present	Hom T
UM6	Epithelioid	57	Dead, due to metastases	Not present	Hom T
UM7	Mixed	63	Dead, other cause	Not present	Het
UM8	Spindle	191	Alive	Not present	Het
UM9	Mixed	131	Alive	Not present	Het
UM10	Mixed	136	Alive	Not present	Het
UM11	Mixed	13	Dead, due to metastases	Present	Hom T
UM12	Epithelioid	137	Dead, due to metastases	Not present	Hom T
UM13	Epithelioid	30	Dead, due to metastases	Present	Het*
UM14	Mixed	42	Dead, due to metastases	Not present	Het
UM15	Mixed	23	Dead, due to metastases	Present	Het
UM16	Epithelioid	115	Alive	Not present	Hom C
UM17	Epithelioid	33	Dead, due to metastases	Present	Het
UM18	Spindle	12	Dead, due to metastases	Present	Hom T
UM19	Spindle	15	Dead, due to metastases	Not present	Hom T

Table 4.1. (Continued)

Tumor ID	Cell type	Survival (mo)	Present status	Hypermethylated RASEF	Genotype
UM20	Mixed	187	Alive	Not present	Het
UM21	Epithelioid	167	Dead, due to metastases	Present	Het*
UM22	Spindle	2	Lost to follow up	Not present	Het
UM23	Spindle	152	Dead, unknown cause	Not present	Het
UM24	Mixed	143	Alive	Not present	Het
UM25	Mixed	29	Dead, other cause	Not present	Het
UM26	Spindle	122	Alive	Not present	Hom T
UM27	Mixed	23	Dead, due to metastases	Present	Hom C
UM28	Epithelioid	33	Dead, due to metastases	Present	Hom C
UM29	Spindle	24	Dead, due to metastases	Not present	Hom C
UM30	Spindle	113	Alive	Present	Hom C
UM31	Mixed	34	Dead, due to metastases	Not present	Hom T
UM32	Epithelioid	63	Dead, due to metastases	Not present	Hom T
UM33	Mixed	24	Dead, due to metastases	Not present	Hom C
UM34	Mixed	106	Alive	Not present	Het
UM35	Mixed	94	Alive	Present	Hom T

* Loss of heterozygosity / Allelic imbalance

DNA and RNA Extraction and Sodium-Bisulfite Modification

Using a column-based extraction kit (Genomic tip 100/G; Qiagen Benelux BV, Venlo, The Netherlands), DNA was extracted from the cell lines and frozen tumor material, according to the manufacturer's guidelines. RNA was also extracted with a column-based extraction kit (RNeasy mini kit; Qiagen Benelux) from tumors in which enough frozen material was available ($n=16$). RNA was converted to cDNA (iScript cDNA synthesis kit; Bio-Rad Laboratories BV, Veenendaal, the Netherlands), according to the manufacturer's guidelines. Genomic DNA was modified with sodium bisulfite (EZ Methylation Gold kit; Zymo Research Corp., Orange, CA). Enzymatically methylated human DNA (Chemicon Europe Ltd., Hampshire, UK) was used as the positive control in all experiments. DNA and RNA concentrations were determined by spectrophotometer (model ND-1000; NanoDrop Technologies Inc., Wilmington, DE).

Mutation Screening and Genotyping

A 96-well light scanner (Idaho Technologies Inc., Salt Lake City, UT) for high-resolution melting-curve analysis was used to scan all amplicons of the *RASEF* gene. The primers are shown in Table 4.2. DNA samples were amplified with a double-stranded DNA-binding dye (LC Green Plus; Idaho Technologies). Melting curves were analyzed in plots showing differences in fluorescence. The shift and curve shapes of melting profiles were used to distinguish between samples from control subjects and patients. PCR

reaction with the green dye contained PCR buffer (Invitrogen, Breda, The Netherlands), 1.5 mM MgCl₂, 40 μM dNTPs, 1:10 diluted green dye, 0.4 μM of forward and reverse primers, and 1 unit *Taq* polymerase per 10-μL reaction (Fast Start; Roche Diagnostics BV, Almere, The Netherlands). PCR consisted of an initial denaturation at 94°C for 6 minutes followed by 40 cycles consisting of 15 seconds at 96°C, 30 seconds at 58°C, and 60 seconds at 72°C, and the PCR ended with a 1-minute denaturation at 94°C. After amplification, the amplified fragments (exon 5) were digested using 4 units of the restriction enzyme *Bst*U1 (New England Biolabs, Beverly, MA) directly added to the PCR mixture. Analysis was performed by overnight digestion of the amplified fragments at 60°C. The *Bst*U1 enzyme recognizes and cleaves the 5'-CG[^]CG-3' sequence. PCR products were separated on a 2% agarose gel in 1x TBE (0.09 M Tris-borate, 0.002 M EDTA; pH 8.2).

RASEF Expression

The expression of *RASEF* in 16 tumors for which RNA was available was analyzed with real-time RT-PCR and specific primers, which are shown in Table 4.2. PCR was performed as described earlier (Maat et al., 2007).

Table 4.2. RASEF Primers Used for Mutation Detection, Expression Analysis, and Methylation Analysis

Primer		Sequence
Exon 1	Forward	3'-GGCAAGCAGCGGTGGACTC-5'
	Reverse	5'-GTAGGTGAAGGAAGACAAGCAACTC-3'
Exon 2	Forward	3'-TCTTCCCTTCCTTCCGTTTCATTCTG-5'
	Reverse	5'-GTCCACCTATATCATAGTGTGACAATGC-3'
Exon 3	Forward	3'-TTCTCTTCATCTGTAATATATAGGGCTTAACG-5'
	Reverse	5'-CCCTCTCCGTAGAAACCACCTC-3'
Exon 4	Forward	3'-TCACCTTCCCTGTGTAGGAGAAC-5'
	Reverse	5'-CTGAGATGCTGAGGCTGTTCC-3'
Exon 5	Forward	3'-CAAAGCAATTCAAAGTGAGTTTGTAAAGC-5'
	Reverse	5'-TGAGGATGTGGTCTAACAGGAAGTG-3'
Exon 6	Forward	3'-GTGTGGGAGGGTGACAGGAC-5'
	Reverse	5'-AAATCATTAGAAAGTAAAGAAGATATTAGCAAAG-3'
Exon 7	Forward	3'-AAAGGGTCTGGGAGGGTAGG-5'
	Reverse	5'-AAACAAGTGAAATGTAATGTAATGAGC-3'
Exon 8	Forward	3'-CCCAATGATACTTTCCTGTCTCTCTTC-5'
	Reverse	5'-ACTTACTTGAGGCTCTCCTTTAAGAAATTAC-3'
Exon 9	Forward	3'-TAGTTACATTAGAAGTTTGAGTAGTGTGC-5'
	Reverse	5'-TTAACATACCTGTCATAGCCTAGAGG-3'
Exon 10	Forward	3'-AGCCCTCAGGTAAATTGGTCTTCC-5'
	Reverse	5'-TGACAGATAGAAGGCAATAAGGTGAC,-3'

Table 4.2. (Continued)

Primer		Sequence
Exon 11 - 12	Forward	3'-TGACATAAGGGATGAAGAGACATTTGG-5'
	Reverse	5'-TTATCAACCGAAATACGAGCCATACC-3'
Exon 13	Forward	3'-CAATGGAATTATTTACATCGTGCTCTC-5'
	Reverse	5'-TTTGAGTATGAAGAATCAAGTGG-3'
Exon 14	Forward	3'-GGCAACACAACTGACTGATGATG-5'
	Reverse	5'-TTTCTGTTTCTCCATTATGATTCTTACCTC-3'
Exon 15	Forward	3'-TGTTGCTGTTGTTCTGTGGTCATC-5'
	Reverse	5'-ACCGACTTCAAAGCCATTAAACCC-3'
Exon 16	Forward	3'-AAGGGCTTCATTAAATTGTGTGATTTC-5'
	Reverse	5'-CCACCATGACTGACAGATAAGAGAG-3'
Exon 17	Forward	3'-TATGAAGATTAAGTCAAGACCTATAAAGC-5'
	Reverse	5'-GACTTTGTGGGTAACCTAATTCAGC-3'
RASEF QPCR	Forward	3'-ATCAGACTTCAAAGCACAGAAATGG-5'
	Reverse	5'-TTCTCTTCCAACCTCACTCACTG-3'
RASEF Bisulfite	Forward	3'-GGGATGGAGGCGGATGGG-5'
	Reverse	5'-CCGCAACTCCGTACACAATACC-3'
RASEF PAP	Forward	3'-GGACGGAGAGGAGTTGGTTCGGTTG-ddC-5'
	Reverse	5'-CCGCAACTCCGTACACAATACCCGAAA-ddC-3'

Methylation Analysis

We applied bisulfite modification of tumor DNA in combination with PCR, as this introduces sequence differences between methylated and unmethylated DNA that can be analyzed with several methods. The sequence differences were initially determined with melting-temperature analysis, as this method provides both quantitative and qualitative measures of methylation (Figure 4.3). The methylation status of the *RASEF* promoter region was determined by polymerase chain reaction with specific primers and by melting-temperature analysis and was further validated with a restriction digestion analysis. Primers were designed on computer (Beacon Designer Software ver. 5.0; Premier Biosoft International, Palo Alto, CA) using bisulfite converted DNA sequences and amplified a CpG island in the *RASEF* gene promoter (Consensus CDS [Coding Sequence] accession number 6662.1; Gene ID 158158/ <http://www.ncbi.nlm.nih.gov/> a genome database hosted by the National Center for Biotechnology Information, Bethesda, MD). The primers are shown in Table 4.2. PCR was performed exactly as described earlier (Maat et al., 2007).

Melting-Temperature Analysis

A melting-temperature analysis was performed as described earlier (Maat et al., 2007)

Restriction Digestion Analysis and Sequence Analysis

After amplification with specific primers for bisulfite-converted DNA, the PCR-amplified fragments were digested with 4 units of restriction enzyme *HinfI* (Fermentas GmbH, St. Leon Rot, Germany) directly added to the PCR mixture (under conditions specified by the manufacturer). The *HinfI* enzyme recognizes and cleaves the 5'-G^AANTC-3' sequence. This sequence is not present in unmodified DNA and in modified unmethylated DNA. The *RASEF* amplicon of methylated DNA contains one *HinfI* recognition site and is dependent on both CT conversion and methylation of a CpG. The recognition site 5'- G^AANTC-3' appears only when the first C in the 5'-GANCC-3' sequence is converted into thymine, whereas the second must be methylated and remains a cytosine. PCR products were separated on a 2% agarose gel in 1x TBE (0.09 M Tris-borate, 0.002 M EDTA; pH 8.2). DNA bands were excised from the gel, purified using a gel extraction kit (Nucleospin Extract II; Macherey-Nagel, GmbH, Düren, Germany) and sequenced (Prism 3700 DNA sequencing system; Applied Biosystems, Foster City, CA).

Pyrophosphorolysis-Activated Polymerization

In the pyrophosphorolysis-activated polymerization (PAP) reaction, primers are used that contain a dideoxy-nucleotide (ddNTP) at their 3' terminus and hence will not be extended. A polymerase with Pyrophosphorolysis activity can remove the dideoxy-cytosine and thereby activate polymerization. Since this pyrophosphorolysis activity is dependent on double-stranded DNA, only primers that perfectly match the template will be activated. The specificity of Pyrophosphorolysis allows us to amplify specifically the minute amounts of methylated *RASEF* DNA in the background of unmethylated DNA. The PAP products can be further validated with sequence analysis for internal CpGs. The primers are shown in Table 4.2. The amplification was performed in a final volume of 25 μ L containing 5 μ L 5x PAP buffer (prepared as described by Liu and Sommer), 0.3 μ L (10 picomoles/ μ L) of each primer, 0.5 μ L *Taq* polymerase (Klen*Taq*; DNA Polymerase Technology, Inc., St. Louis, MO), 17.9 μ L H₂O, and 1 μ L DNA sample (Liu and Sommer, 2004). Amplification was initiated by hot start, followed by 40 cycles at 94°C for 15 seconds, 60°C for 40 seconds, 64°C for 40 seconds (T-anneal); 68°C for 40 seconds (pyrophosphorolysis activity); and 72°C for 40 seconds (elongation), and using a final melting curve from 70°C to 97°C with an increase in temperature of 0.2°C every 10 seconds. PCR products were separated on a 2% agarose gel in 1x TBE (0.09 M Tris-borate, 0.002 M EDTA; pH 8.2).

Statistical Analysis

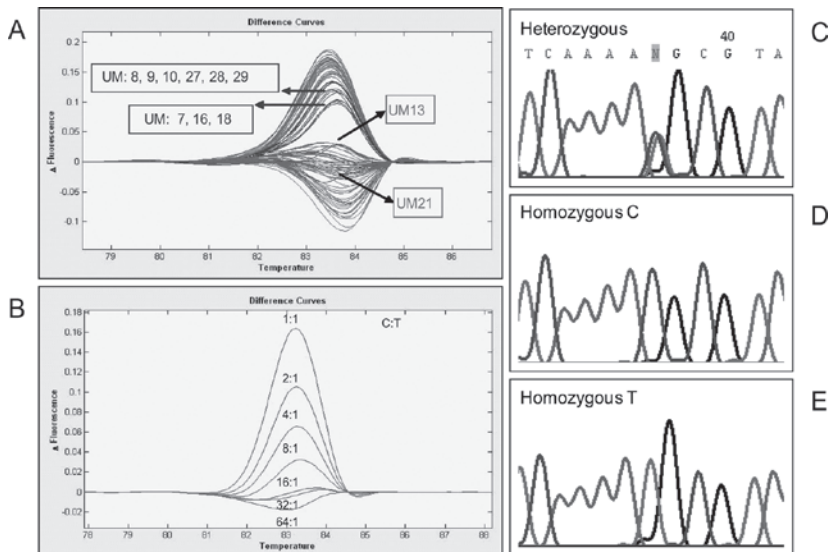
Survival analysis for *RASEF* promoter methylation was performed using a Kaplan Meier analysis and log rank test (SPSS ver. 14.0 for Windows; SPSS Inc., Chicago, IL). For a comparison between the presence and absence of *RASEF* methylation and metastatic disease and tumor characteristics, the χ^2 test and analysis of variance were performed.

Results

Mutation Screening in Uveal Melanoma

To analyze *RASEF* as a tumor-suppressor gene (TSG) candidate, we first investigated the gene for mutations. Mutation screening was performed in two steps: first, the 17 exons were prescreened by high-resolution melting-curve analysis. Though melting-curve analysis showed few variations, we nevertheless generated sequences for 2 tumor samples of each exon both sequenced with the forward and the backward PCR primer. We detected a sequence variation, which was a known polymorphism in exon 5 of *RASEF* encoding a R262C (C→T; Arginine →Cysteine) substitution (Figure 4.1). This SNP occurs frequently in the population, with reported frequencies between 50% and 58% of the Caucasian population (Sweetser et al., 2005). In 10 of the 11 cell lines, a homozygous genotype of the T allele was observed. The primary uveal melanomas ($n = 35$) displayed a normal frequency of the SNP (54%), with 16 uveal melanomas presenting a heterozygous genotype (Table 4.1). However, both melting curve analysis and restriction enzyme analysis revealed imbalance of the alleles in two samples. Whereas in gel analysis of *Bst*UI digested *RASEF*, exon 5 PCR indicated the presence of the C-allele, melting-curve analysis indicated that the relative concentration of the C-allele was at least 10-fold lower than the T-allele in UM13 and -21 (Figure 4.1).

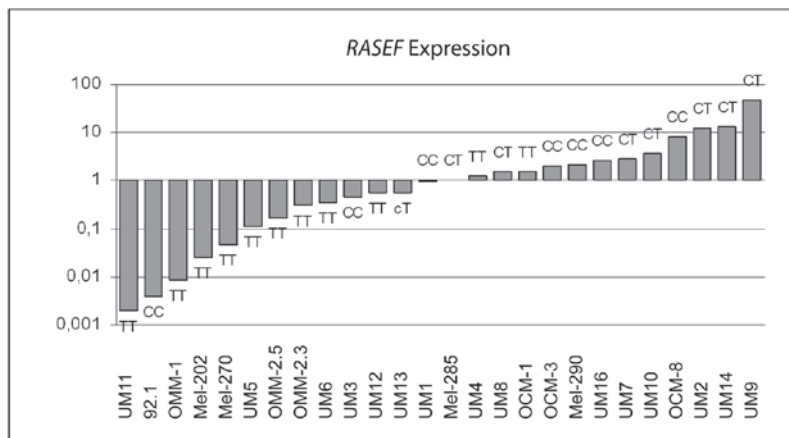
Figure 4.1. (A) Melting-curve analysis of the *RASEF* R262C polymorphism in control and primary uveal melanoma and cell lines. Red: heterozygous control and tumor samples; blue: heterozygous primary uveal melanoma samples with a lowered difference plot that is not seen in control samples. (B) A calibration curve was created with dilutions of the T allele in a constant background of the C allele. Based on this curve it was estimated that the relative abundance of the alleles in the primary uveal melanoma samples 13 and 21 was decreased at least 10-fold. (C–E) *RASEF* exon 5 sequence analysis of primary uveal melanomas shows the R262C polymorphism at position 37. The cytosine from the consensus sequence is substituted for a thymine.



Expression analysis

The allelic imbalance observed in the primary uveal melanoma was followed up by *RASEF* RT-PCR expression analysis. In the cell lines, two groups were distinguishable, based on expression levels. In 6 of 11 uveal melanoma cell lines (92.1; Mel 202 and -270; and OMM-1, -2.3, and -2.5) an approximately 30- to 100-fold reduced expression of *RASEF* was displayed compared with the other cell lines (OCM1, -3, and -8; Mel285 and -290; Figure 4.2). Cell lines Mel270 and OMM-2.3 and -2.5 are derived from the same patient and fell into the same group. Among the uveal melanomas cell lines with low *RASEF* expression, a homozygous (TT) genotype prevailed. When primary tumors were analyzed, variable levels of *RASEF* expression were also observed, but clustering into two groups was not as marked, and the absolute expression levels differed even more. One sample failed in the expression analysis (UM15). Correlating the expression levels with the genotypes of the tumors revealed that the homozygous tumors tended to present a lower *RASEF* expression ($P = 0.015$), as was the case in one tested uveal melanoma presenting allelic imbalance (UM13).

Figure 4.2. Expression analysis for *RASEF* in cell lines and primary uveal melanoma (UM), measured with real-time RT-PCR. Expression was normalized with the control gene *RPS11*. The change (x-fold) of expression is calculated compared with the median expression level. The *RASEF* genotypes of the samples are indicated in the graph. UM13 displays loss of the C allele indicated by a lowercase c. UM15 failed in the expression analysis.

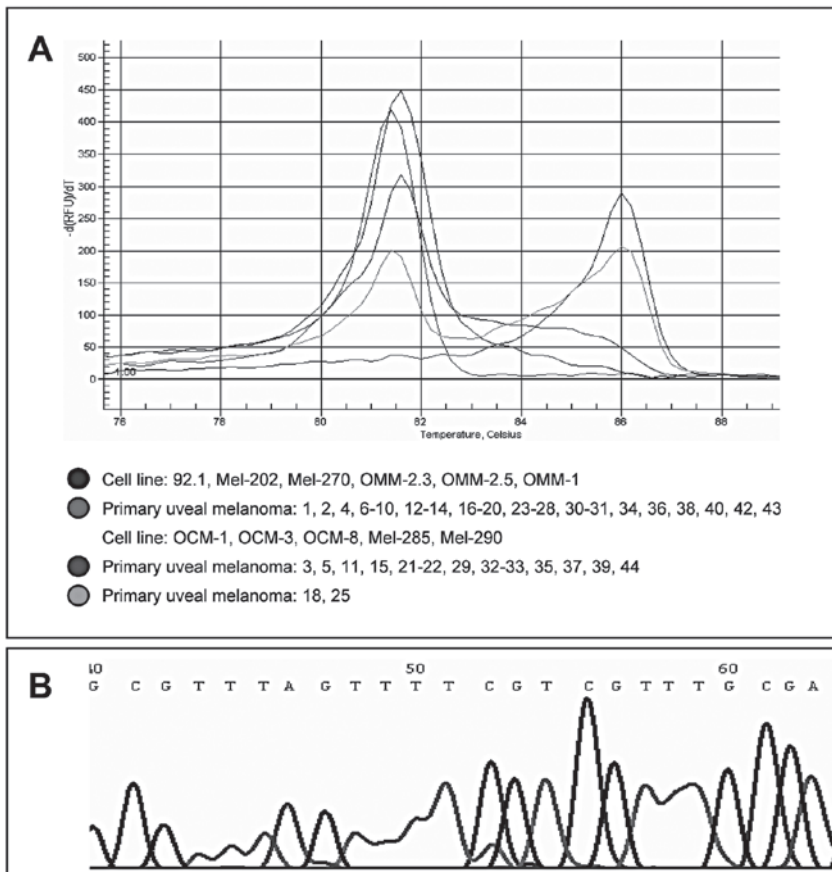


Methylation Analysis

Because we did not detect mutations that could explain the low *RASEF* expression in the primary uveal melanomas and the cell lines, we considered epigenetic regulation as the possible mechanism of downregulation. All five *RASEF*-expressing cell lines contained an unmethylated promoter while hypermethylation of all CpGs within the amplicon was present in all six cell lines that lacked *RASEF* expression. The analysis of methylation with melting temperature was confirmed by sequence analysis (Figure 4.3). In primary uveal melanomas, methylation was much more heterogeneous and never

reached the level of methylation observed in the cell lines. Uveal melanoma samples 15 (failed in expression analysis) and 21 displayed the highest methylation but also contained an equimolar level of unmethylated *RASEF*. In the other uveal melanoma samples with methylated *RASEF*, a minor fraction of the CpGs was methylated. Still, there was a correlation between methylation and expression of *RASEF* in the primary tumor samples, although not as obvious as in the cell lines.

Figure 4.3. (A) Melting-temperature analysis of amplified *RASEF* product reveals the methylation of primary uveal melanoma samples and cell lines. Blue: methylated samples; green and purple: samples with a mixed methylation pattern; red: unmethylated samples. (B) Methylation in the promoter region of the *RASEF* gene in primary uveal melanoma sample 25, as shown by sequence analysis after PAP. After bisulfite treatment and PCR, unmethylated cytosines converted into thymidine. Methylated cytosines remained unchanged.



To validate methylation in primary tumors, we used restriction-enzyme analysis. By *HinfI* digestion, we were able to confirm *RASEF* methylation in primary uveal melanoma (data not shown). Next, we set out to isolate the methylated fraction and applied PAP. By applying PAP, we were able to show completely

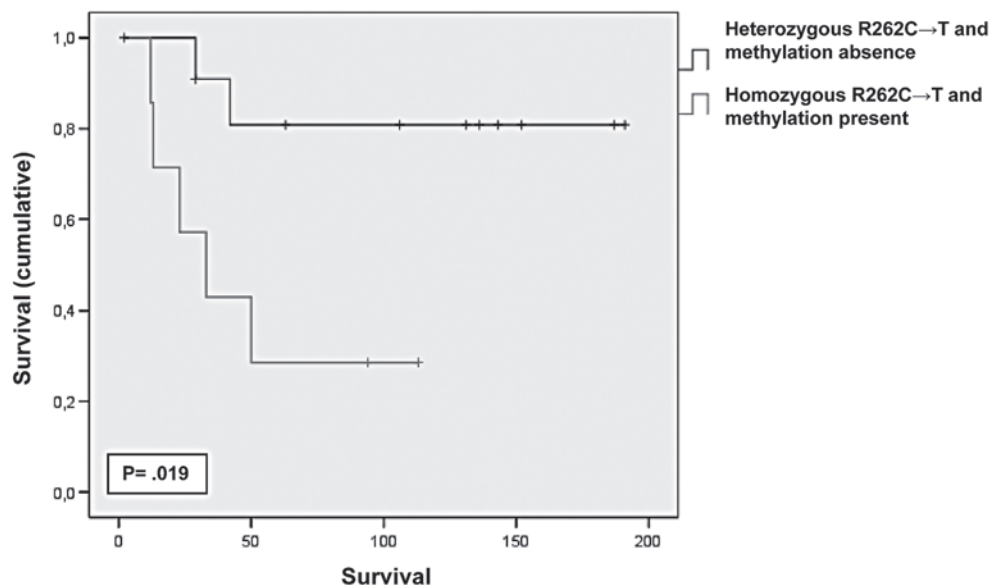
methylated alleles and thereby validate melting temperature analysis in five tumors that had shown a methylated fraction in the background of unmethylated DNA. In one sample, a methylated allele was detected in a tumor that had shown a normal curve with melting-temperature analysis, suggesting a very low level of the methylated allele (Figure 4.3).

Survival

The mean follow-up of the 35 patients was 78 months (2–210 months), and 20 patients had died of tumor-related metastasis at the time of analysis. Two patients had died of a metastasis from another primary tumor (UM7 and UM25), one patient was lost to follow-up (UM22) after 2 months, and two patients had died of unknown causes. The presence of methylation within the *RASEF* promoter region correlated with death due to metastatic disease ($P = 0.024$; log rank test). The genotype of the 35 tumors did not correlate to cell type, methylation status, or the development of metastatic disease ($P = 0.441$; Pearson χ^2).

Although the genotype itself was not associated with metastatic death, patients with a homozygous genotype and methylation of the *RASEF* gene ($n = 7$) had a significantly higher risk of development of metastasis than did patients with a heterozygous genotype and no methylation (survival 51 +/- 15.5 vs. 161 +/- 19.0 months; $P = 0.019$).

Figure 4.4. Kaplan-Meier analysis and log rank test showed the difference in survival according to genotype and presence or absence of methylation of the promoter region of the *RASEF* gene ($P = 0.019$).

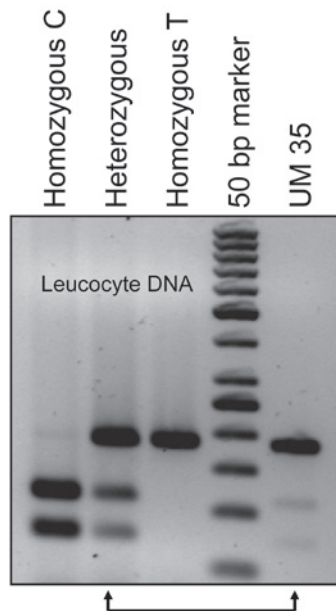


Discussion

Linkage analysis in uveal and cutaneous melanoma families identified the 9q21 region as a locus for a potential TSG involved in the development of melanoma. In addition, LOH analysis in two uveal melanomas from members of the families in which linkage was identified indicated 9q21 to be the possible region for a TSG (Jönsson et al., 2005). The 9q21 region harbors the *RASEF* gene, which is potentially involved in the *RAS* pathway prominent in the development of melanoma (Padua et al., 1984; Rimoldi et al., 2003). As patients with melanoma from the family just mentioned had been analyzed for *RASEF* mutations, we set out to analyze sporadic uveal melanoma and uveal melanoma cell lines. In line with the findings of Jönsson et al., (Jönsson et al., 2005) we did not detect any mutations in the *RASEF* gene other than a known SNP (Jönsson et al., 2005; Sweetser et al., 2005). Using this SNP, we detected allelic imbalance in some of the tumors that were heterozygous for this marker (UM13 and -21). Because the imbalances were not complete, we suspect tumor heterogeneity in the primary tumors in contrast to the cell lines, all of which, with one exception, displayed a homozygous genotype. Gene expression analysis revealed that 5 of 11 uveal melanoma cell lines had high *RASEF* expression, whereas the others hardly showed expression. As almost all the low expression cell lines displayed the homozygous T-allele, there appears to be an association between expression and genotype. This apparent association, however, could also be based on the small number of cell lines that were tested and the fact that three cell lines were derived from the same patient (Mel 270, OMM 2.3, and OMM 2.5). In the primary tumors, expression varied widely and often exceeded the expression seen in the cell lines. Among the uveal melanomas with low *RASEF* expression a homozygous genotype prevailed, but this fact does not favor a specific allele. This finding may indicate that there is no risk factor linked to either allele and that the low expression is more likely due to a somatic alteration. As we had not observed any mutations in the cell lines, we subsequently considered epigenetic modifications as the cause of low *RASEF* expression. Indeed, all cell lines that did not express *RASEF* contained a methylated promoter, whereas all cell lines with expression lacked this methylation, confirming our hypothesis. Hereafter, we performed demethylation experiments with 5-azacytidine, which revealed a highly induced expression in a cell line with methylated *RASEF*. Demethylation of an unmethylated cell line resulted in the opposite effect. The demethylating agent is highly toxic and may explain downregulation of *RASEF* expression in the unmethylated cell line. Toxicity of 5-azacytidine and demethylation of all the other genes during treatment are the reasons that we reserve functional analysis using genetically modified cell lines for follow-up research. The primary uveal melanomas displayed heterogeneity for *RASEF* methylation but never reached levels above ~ 50% methylation, and most commonly only a part of the CpGs present in the promoter region was methylated. Furthermore, methylation not only coincided with low expression but also with a homozygous genotype, which suggests a combination of methylation and LOH being the mechanism of loss of expression. The additional effect of LOH seems to be associated with the aggressiveness of the tumor, because homozygous tumors with a methylated *RASEF* promoter region tended to have a decreased survival compared with heterozygous tumors without methylation ($P = 0.019$; Figure 4.4). To confirm the suggested mechanism, we compared the *RASEF* homozygous

genotype of four tumors with the genotype of their peripheral blood leukocytes, also obtained at time of enucleation. One person, in whom the tumor showed a nearly complete homozygous T allele genotype, revealed a heterozygous genotype in leukocyte DNA, confirming the mechanism of LOH in tumor tissue (Figure 4.5). Of interest, in the tumor tissue of this patient, the *RASEF* gene was methylated, furthermore confirming our conclusion.

Figure 4.5. Digestion analysis of DNA from a patient in whom the tumor (UM35, right) showed a nearly complete homozygous T-allele genotype revealed a heterozygous genotype in leukocyte DNA (left), confirming the mechanism of LOH in tumor tissue.



We conclude that homozygosity in combination with methylation is the mechanism that targets *RASEF* in uveal melanoma, appointing *RASEF* as a bona fide tumor suppressor that is epigenetically silenced in uveal melanoma. Allelic imbalance at this locus supports a tumor-suppressor role for *RASEF*; however, analysis of *RASEF* in proliferation, survival, and migration of uveal melanoma is needed to confirm this.

Acknowledgements

The authors thank Bruce R. Ksander (Schepens Eye Institute, Harvard Medical School, Boston, MA) for cell lines Mel-202, -270, -285, and -290, and OMM-2.3 and -2.5, June Kan-Mitchell (Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, MI) for cell lines OCM-1, -3, and -8, and Rolf H.A.M. Vossen (Department of Human and Clinical Genetics, LUMC, Leiden, The Netherlands) for technical assistance.

References

- Albino AP, Sozzi G, Nanus DM, Jhanwar SC, Houghton AN. Malignant transformation of human melanocytes: induction of a complete melanoma phenotype and genotype. *Oncogene*. 1992;7:2315–2321.
- Boni R, Vortmeyer AO, Burg G, Hofbauer G, Zhuang Z. The PTEN tumour suppressor gene and malignant melanoma. *Melanoma Res*. 1998;8:300–302.
- Broberg K, Hoglund M, Gustafsson C, et al. Genetic variant of the human homologous recombination-associated gene RMI1 (S455N) impacts the risk of AML/MDS and malignant melanoma. *Cancer Lett*. 2007;258:38–44.
- Calipel A, Mouriaux F, Glotin AL, Malecize F, Faussat AM, Mascarelli F. Extracellular signal-regulated kinase-dependent proliferation is mediated through the protein kinase A/B-Raf pathway in human uveal melanoma cells. *J Biol Chem*. 2006;281:9238–9250.
- Cannon-Albright LA, Goldgar DE, Meyer LJ, et al. Assignment of a locus for familial melanoma, MLM, to chromosome 9p13–p22. *Science*. 1992;258:1148–1152.
- Cohen C, Zavala-Pompa A, Sequeira JH, et al. Mitogen-activated protein kinase activation is an early event in melanoma progression. *Clin Cancer Res*. 2002;8:3728–3733.
- Davies H, Bignell GR, Cox C, et al. Mutations of the BRAF gene in human cancer. *Nature*. 2002;417:949–954.
- Egan KM, Seddon JM, Glynn RJ, Gragoudas ES, Albert DM. Epidemiologic aspects of uveal melanoma. *Surv Ophthalmol*. 1988;32: 239–251.
- Joˆnsson G, Bendahl PO, Sandberg T, et al. Mapping of a novel ocular and cutaneous malignant melanoma susceptibility locus to chromosome 9q21.32. *J Natl Cancer Inst*. 2005;97:1377–1382.
- Kodjikian L, Nguyen K, Lumbroso L, et al. Familial uveal melanoma: a report on two families and a review of the literature. *Acta Ophthalmol Scand*. 2003;81:389–395.
- Kopf I, Stierner U, Islam Q, Delle U, Kindblom LG, Martinsson T. Characterization of four melanoma cell lines with electron microscopy, immunocytochemistry, cytogenetics, flow cytometry, and southern analysis. *Cancer Genet Cytogenet*. 1992;62:111–123.
- Kumar R, Smeds J, Lundh RB, Hemminki K. Loss of heterozygosity at chromosome 9p21 (INK4–p14ARF locus): homozygous deletions and mutations in the p16 and p14ARF genes in sporadic primary melanomas. *Melanoma Res*. 1999;9:138–147.
- Liu Q, Sommer SS. Pyrophosphorolysis by Type II DNA polymerases: implications for pyrophosphorolysis-activated polymerization. *Anal Biochem*. 2004;324:22–28.
- Liu Q, Sommer SS. PAP: detection of ultra rare mutations depends on P* oligonucleotides: “sleeping beauties” awakened by the kiss of pyrophosphorolysis. *Hum Mutat*. 2004;23:426–436.
- Maat W, van der Velden PA, Out-Luiting C, et al. Epigenetic inactivation of RASSF1a in uveal melanoma. *Invest Ophthalmol Vis Sci*. 2007;48:486–490.
- Padua RA, Barras N, Currie GA. A novel transforming gene in a human malignant melanoma cell line. *Nature*. 1984;311:671–673.
- Rimoldi D, Salvi S, Lienard D, et al. Lack of BRAF mutations in uveal melanoma. *Cancer Res*. 2003;63:5712–5715.
- Satyamoorthy K, Li G, Gerrero MR, et al. Constitutive mitogenactivated protein kinase activation in melanoma is mediated by both BRAF mutations and autocrine growth factor stimulation. *Cancer Res*. 2003;63:756–759.
- Smith JH, Padnick-Silver L, Newlin A, Rhodes K, Rubinstein WS. Genetic study of familial uveal melanoma: association of uveal and cutaneous melanoma with cutaneous and ocular nevi. *Ophthalmology*. 2007;114:774–779.
- Stark M, Hayward N. Genome-wide loss of heterozygosity and copy number analysis in melanoma using high-density single-nucleotide polymorphism arrays. *Cancer Res*. 2007;67:2632–2642.
- Sweetser DA, Peniket AJ, Haaland C, et al. Delineation of the minimal commonly deleted segment and identification of candidate tumor-suppressor genes in del(9q) acute myeloid leukemia. *Genes Chromosomes Cancer*. 2005;44:279–291.
- van der Velden PA, Metzelaar-Blok JA, Bergman W, et al. Promoter hypermethylation: a common cause of reduced p16(INK4a) expression in uveal melanoma. *Cancer Res*. 2001;61:5303–5306.
- van Elsland A, Zerp SF, van der Flier S, et al. Relevance of ultraviolet-induced N-ras oncogene point mutations in development of primary human cutaneous melanoma. *Am J Pathol*. 1996;149:883–893.

- van Hees CL, Jager MJ, Bleeker JC, Kemme H, Bergman W. Occurrence of cutaneous and uveal melanoma in patients with uveal melanoma and their first degree relatives. *Melanoma Res.* 1998;8: 175–180.
- Weber A, Hengge UR, Urbanik D, et al. Absence of mutations of the BRAF gene and constitutive activation of extracellular-regulated kinase in malignant melanomas of the uvea. *Lab Invest.* 2003;83:1771–1776.
- Zhu G, Montgomery GW, James MR, et al. A genome-wide scan for naevus count: linkage to CDKN2A and to other chromosome regions. *Eur J Hum Genet.* 2007;15:94–102.
- Zuidervaat W, van Nieuwpoort F, Stark M, et al. Activation of the MAPK pathway is a common event in uveal melanomas although it rarely occurs through mutation of BRAF or RAS. *Br J Cancer.* 2005;92:2032–2038.

Chapter 5

Pyrophosphorolysis Detects *B-RAF* Mutations in Primary Uveal Melanoma

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Abstract

PURPOSE. Mutations in the genes that control cell proliferation in cutaneous melanoma are generally uncommon in uveal melanoma. Despite the absence of known activating mutations, the RAF-MEK-ERK, or mitogen-activated protein kinase (MAPK), pathway is usually activated in uveal melanoma. An assay with increased potential to identify mutations is now available, and this study was therefore conducted to reanalyze uveal melanoma cell lines and primary tumors for this mutation.

METHODS. Eleven uveal melanoma cell lines and 45 primary uveal melanomas were analyzed for mutations in exon 15 of the *B-RAF* gene by using pyrophosphorolysis-activated polymerization (PAP). Mutations were validated by sequencing of the PAP product.

RESULTS. *B-RAF* mutations were detected in cell lines OCM-1 and -3 (V600E) and in six primary uveal melanomas. The V600K mutation was detected in one primary uveal melanoma, for which the V600E assay turned out to be sensitive as well. Direct sequencing of the exon 15 PCR product did not reveal the mutations found with the PAP-assay, indicating a low frequency of the mutant allele in primary samples.

CONCLUSIONS. Because of the very sensitive PAP technology, *B-RAF* mutations were found in cell lines and primary uveal melanomas, which suggests that they may occasionally play a role in the activation of the MAPK pathway in uveal melanoma and indicates a higher prevalence of *B-RAF* mutations in uveal melanoma than was reported earlier. However, the relative scarcity of the *B-RAF* mutation excludes an elemental role for this mutation in uveal melanoma.

Introduction

Uveal melanoma is a rare neoplasm arising from melanocytes in the eye, with an incidence rate of approximately six to eight new cases per million per year among Caucasians (Egan et al., 1988; Singh and Topham, 2003). Approximately 50% of patients who have a medium to large uveal melanoma will develop metastatic disease, mostly through hematogenic spread to the liver. Since there is hardly any effective treatment for such metastases, they are usually fatal within a year of onset of symptoms.

Compared with cutaneous melanoma, little is known about the molecular pathogenesis of uveal melanoma, and the role of different tumor pathways is less defined. Cutaneous melanoma shares the same embryonic origin and similar histologic features, but the mutations that activate the major oncogenic pathway in cutaneous melanoma, have not been found in uveal melanoma.

The RAF-MEK-ERK or mitogen-activated protein kinase (MAPK) pathway is of great importance in the development of many types of cancer, as well as in melanocytic neoplasia. In cutaneous melanocytes, activation of this pathway has been shown to occur by a variety of mechanisms, including endocrine and autocrine growth factor stimulation and mutation of the *RAS* and *RAF* genes. Activation of the MAPK pathway has also been reported in uveal melanoma, although it only rarely occurs through mutations in *B-RAF* or *RAS* (Zuidervaart et al., 2005). 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 in this exon (Brose et al., 2002; Davies et al., 2002; Pollock et al., 2003; Satyamoorthy et al., 2003; Uribe 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 (the National Center for Biotechnology Information [NCBI; Bethesda, MD] GenBank re-named the V599E mutation based on newly available sequence data; accession number NM_004333.2; hereafter referred to as *B-RAF* V600E), leading to a constitutive activation of proliferation signaling (Zhang and Guan, 2000; Wellbrock et al., 2004).

In contrast to these findings, *B-RAF* mutations have been reported only rarely in uveal melanoma (Table 5.1). In 2003, Calipel et al. (Calipel et al., 2003) analyzed four primary uveal melanoma cell lines for mutations in the *B-RAF* gene and reported the presence of the V600E mutation in uveal melanoma cell lines OCM-1 and TP-31, of which the mutation in OCM-1 was confirmed in two studies by other groups (Calipel et al., 2003; Kilic et al., 2004; Zuidervaart et al., 2005). The same group also reported *B-RAF* mutations in cell lines MKT-BR and SP6.5 (Kilic et al., 2004). To our knowledge, only one *B-RAF* mutation (V600E) has been described in a primary uveal melanoma, (Malaponte et al., 2006) whereas several studies reported a lack of *B-RAF* mutations in cell lines and primary tumors (Cohen et al., 2003; Cruz et al., 2003; Edmunds et al., 2003; Rimoldi et al., 2003; Weber et al., 2003; Kilic et al., 2004; Zuidervaart et al., 2005). A possible explanation for the apparent lack of *B-RAF* mutations is that uveal melanomas are genetically heterogeneous, and therefore mutations are not present in each cell, similar to our findings

in previous studies on the heterogeneous distribution of monosomy of chromosome 3 and methylation of *RASSF1a* (Maat et al., 2007a; Maat et al., 2007b). To detect mutations in a background of normal DNA, we used pyrophosphorolysis-activated polymerization (PAP) (Liu and Sommer, 2004) to test whether *B-RAF* mutations are indeed present in uveal melanoma and to test tumor heterogeneity. We set out to screen exon 15 of the *B-RAF* gene in uveal melanoma cell lines and primary tumors with PAP and showed that with PAP it is possible to amplify specifically minute amounts of mutant DNA in a background of wild-type DNA, and that, consequently, it has a high sensitivity for mutations present in just a small number of tumor cells.

Table 5.1. Summary of Published *B-RAF* Mutation Studies in Uveal Melanoma Cell Lines

Study	<i>B-RAF</i> mutation frequency *	Cell line with <i>B-RAF</i> mutation
Calipel et al. (2003)	2/4	OCM-1, TP-31
Kiliç et al. (2004)	1/11	OCM-1
Zuidervaart et al. (2005)	1/10	OCM-1
Calipel et al. (2006)	3/3	OCM-1, MKT-BR, SP6.5
This study	2/11	OCM-1, OCM-3
* All V600E mutations		

Materials and methods

Cell Lines and Primary Uveal Melanoma Specimens

In total, 11 cell lines derived from primary uveal melanomas (92.1, OCM-1, -3 and -8; Mel-202, -270, -285, -290) and uveal melanoma metastases (OMM-1, -2.3, and -2.5) were analyzed for *B-RAF* mutations. OMM-2.3 and -2.5 were derived from separate tumor nodules in the liver of the same patient from whom cell line Mel-270 was obtained and thus represent a progression model (Chen et al., 1997). All melanoma cell lines were cultured in RPMI 1640 medium (Invitrogen-Gibco, Paisley, Scotland, UK) supplemented with 3 mM L-glutamine (Gibco), 2% penicillin streptomycin and 10% FBS (Hyclone, Logan, UT). All cell cultures were incubated at 37°C in a humidified 5% CO₂ atmosphere. Archival frozen tumor specimens of primary uveal melanoma came from 37 consenting patients who attended the Leiden University Medical Center and from eight patients attending the Erasmus Medical Center (Rotterdam, The Netherlands). All tumors were primary lesions with a tumor diameter greater than 12 mm and a prominence greater than 6 mm, and they had not been treated before enucleation. The validity of the diagnosis of uveal melanoma was confirmed histologically in all cases. The research protocol followed the tenets of the current principles of the Declaration of Helsinki (World Medical Association Declaration of Helsinki 1964; ethical principles for medical research involving human subjects).

DNA Extraction

A column-based extraction kit (Genomic tip 100/G; Qiagen Benelux BV, Venlo, The Netherlands) was used to extract DNA from the cell lines and frozen tumor material according to the kit manufacturer's guidelines. A V600E-positive colon carcinoma cell line (HT29) was used as the control (Davies et al., 2002). DNA concentrations were determined with a spectrophotometer (model ND-1000; NanoDrop Technologies Inc., Wilmington, DE).

Table 5.2. Primers

Primer	Sequence
PAP - B-RAF - Forward	5'-GTTTCTCTTACTTAC TACACCTCAGATATATTCCTCATG-3'
PAP - B-RAF - Reverse	5'-CTGTCAAACCTGATGGGACCCACTCCATCGAGATTTC-dd*T-3'
Sequence – B-RAF exon 15 - Forward	5'-AACTCTTCATAATGCTTGCTCTGTAGG-3'
Sequence – B-RAF exon 15 - Reverse	5'-GCCTCAATTCTTACCATCCACAAAATG-3'
* dd: dideoxy-nucleotide (ddNTP)	

PAP and Sequence Analysis

In the PAP reaction, primers are used that contain a dideoxy-nucleotide (ddNTP) at their 3' terminus and hence cannot be extended. Primers are shown in Table 5.2. A polymerase with pyrophosphorolysis activity can remove the dideoxy-nucleotide and thereby activate polymerization. The mutant base is resynthesized based on the original template sequence. Since this pyrophosphorolysis activity is dependent on double-stranded DNA, only primers that perfectly match the template will be activated (Figure 5.1). Because the PAP products are based on resynthesis, mutations can be validated by sequence analysis. Amplification was performed on a standard thermal cycler (MJ Research, Watertown, MA) in a final volume of 25 μ L containing 5 μ L 5x PAP buffer (prepared as described by Liu and Sommer (Liu and Sommer, 2004)), 0.3 μ L (10 picomoles/ μ L) of each primer (Eurogentec Nederland BV, Maastricht, The Netherlands), 0.75 μ L KlenTaq-S (ScienTech, St. Louis, MO), 16.65 μ L H₂O, and 2 μ L DNA sample. Amplification was initiated by hot start, followed by 50 cycles at 94°C for 15 seconds, 60°C for 40 seconds, 64°C for 40 seconds, 68°C for 40 seconds, and 72°C for 40 seconds. The product was electrophoresed through a standard 2% agarose gel in 1x TBE (0.09 M Tris-borate, 0.002 M EDTA; pH 8.2). The gel was stained with ethidium bromide for UV photography by a charge-coupled device camera (G:BOX Chemi; Syngene Europe, Cambridge, UK). After gel electrophoresis, DNA bands were excised from the gel, purified with a gel extraction kit (Nucleospin Extract II; Macherey-Nagel GmbH & Co., Düren, Germany) and sequenced on a DNA sequencing system (Prism 3700 Applied Biosystems [ABI], Foster City, CA). Direct sequencing with specific primers (Invitrogen, Breda, The Netherlands) (Table 5.2) was used for screening for the B-RAF mutation in all cell lines and primary tumors.

Figure 5.1. (A) *B-RAF* exon 15 sequence and primers used in PAP experiments. (B) Situation in which the primer with a dideoxy nucleotide (dd*T) at its 3' end does not match with the normal template and hence will not be removed by the phosphorolytic activity of *KlenTaq* and prevent amplification. (C) Situation in which the primer with dd*T-3' perfectly matches with the mutant template. The phosphorolytic activity of *KlenTaq* removes the dideoxy nucleotide and the primer initiates polymerization. The mutant base is resynthesized based on the template and thereby provides a means of validation by sequencing of the PAP product.

A *B-RAF* Exon 15 Sequence

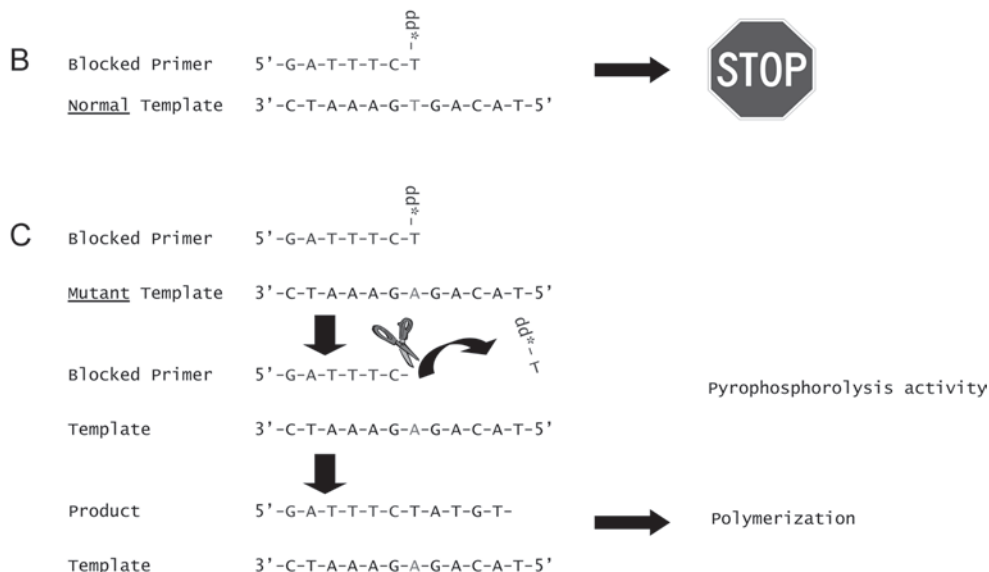
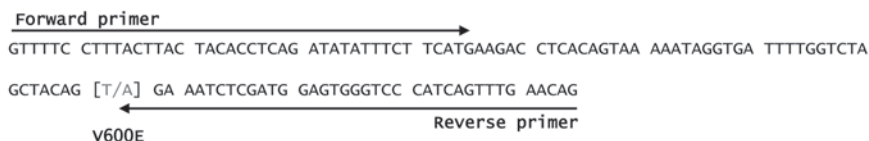
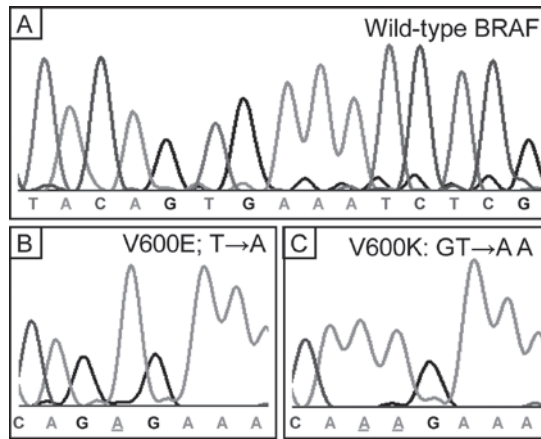


Figure 5.2. Sequence analysis of the PAP products showing the sequence of wild-type *B-RAF* exon 15 (A), the V600E mutation (B), and the V600K mutation (C) in primary uveal melanoma DNA samples.



Results

Numerous *B-RAF* mutations have been detected in primary cutaneous melanomas and their cell lines (Brose et al., 2002; Davies et al., 2002; Pollock et al., 2002). In contrast, an extremely low frequency of *B-RAF* mutations in uveal melanoma cell lines and primary tumors has been reported, whereas in vitro studies suggest that the *B-RAF* pathway plays a role in uveal melanoma cell growth (Calipel et al., 2003; Tsao et al., 2004; Zuidervaart et al., 2005; Malaponte et al., 2006). Applying PAP on 11 uveal melanoma cell lines and 45 primary uveal melanomas, we detected several *B-RAF* mutations. In cell line OCM-1, the V600E mutation was detected, confirming results from previous studies (Table 5.1). Of interest, cell line OCM-3 seems to harbor the same V600E mutation. Both cell lines were derived from the same laboratory, and their melanocytic origin has been demonstrated (Diebold et al., 1997). Several investigators have questioned the choroidal origin of cell line OCM-1 based on the presence of the *B-RAF* V600E mutation, which is definitely related to cutaneous melanoma (Kan-Mitchell et al., 1989; Luyten et al., 1996; Kilic et al., 2004). Cell lines OCM-1 and -3 showed different HLA-types and confirmed that they were indeed derived from different patients and were not interchanged (data not shown). The presence of the mutation found in OCM-3 has not been reported before, as far as we know, even though this cell line was analyzed in our own laboratory before by conventional sequencing (Zuidervaart et al., 2005).

The fact that direct sequencing reveals a homozygous genotype for the *BRAF* mutation indicates that all cells contain the V600E mutation. In primary uveal melanomas, the V600E mutation in the *B-RAF* gene, which consists of a T → A transversion at position 1799 and results in a valine-to-glutamic acid substitution, was detected in 6 (13%) of 45 primary tumors. The other mutation uncovered in our study was the rarely reported V600K mutation in one primary uveal melanoma sample. This mutation

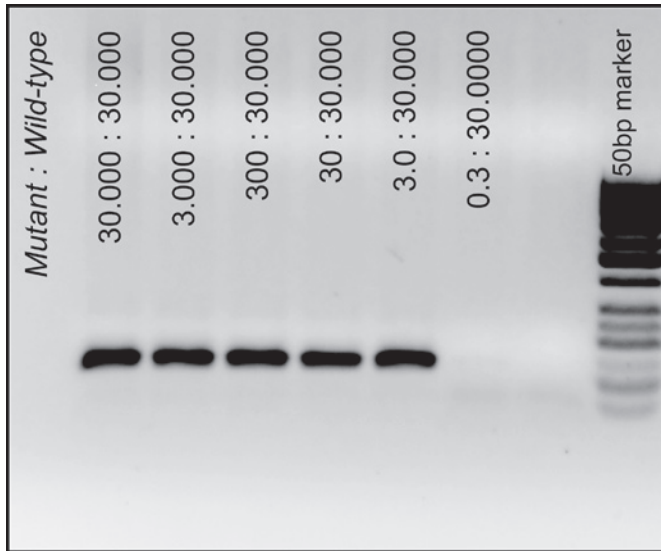
consists of a GT → AA substitution at position 1798-1799 and is also located in the serine/threonine kinase domain of *B-RAF* (Pollock et al., 2003). Results were confirmed by sequencing of the PAP product after purification from the electrophoresis gel (Figure 5.2). Our primers were not designed to detect occurrences of V600D or V600R mutations. We did not detect any differences in tumor size, location, cell type or patient survival between tumors with and without the detected V600E mutation.

Discussion

That in this study we detected *B-RAF* mutations, whereas other studies, including our own (Zuidervaart et al., 2005), reported the absence of mutations may be explained by the techniques used. PCR in combination with direct sequencing or ligase-detection reaction and mutation assay (Mutector; Biomol, Hamburg, Germany) were the techniques used to detect mutations in previous studies (Calipel et al., 2003; Cruz et al., 2003; Zuidervaart et al., 2005; Goldenberg-Cohen et al., 2005; Turner et al., 2005). However, these techniques are less sensitive than PAP, especially in samples with a low abundance of mutations in the presence of excess amounts of wild-type DNA in the tumor (Liu and Sommer, 2004a; Liu and Sommer, 2004b). Whereas conventional techniques used to detect mutations theoretically have a predicted sensitivity varying between $1:10^1$ to $1:10^5$ PAP has a predicted sensitivity of $1:10^9$ making it suitable for the detection of sporadic mutations (Parsons et al., 1997; Liu and Sommer, 2004a). Limited by the input of genomic DNA, the practical sensitivity of the essays is lower. For the PAP assay, the practical sensitivity is at least $1:10^4$ (Figure 5.3). Also in our study, direct sequencing of exon 15 PCR products did not reveal the mutations found with the PAP-assay suggesting a minor frequency for the mutant allele, apart from the V600E mutation in cell lines OCM-1 and -3, which could be detected by direct sequencing. Although the reverse primer in our PAP-assay is blocked at the 3-prime end, which first must be removed to start polymerization, the forward primer will start DNA polymerization each cycle, independent of the *B-RAF* genotype.

Because of the intrinsic error rate of the forward polymerase reaction, theoretically, an adenine can be misincorporated at position 1799. This erroneously synthesized copy can subsequently serve as a template for the blocked primer and falsely start a PCR reaction. However, the control assays that we performed indicate that the positive PAP-assays with primary tumors are not likely to be explained by polymerase artifacts. The assays with normal DNA that we always include in our experiments never resulted in a positive PAP assay and thereby suggest that this error rate is limited. Cross contamination as cause of positive PAP assays is prevented by using separate rooms before and after PCR. The negative controls furthermore indicate that this is not the explanation for the positive tumors, and the latter specifically applies to the V600K mutation that we have never detected before. Moreover, dilution experiments with OCM-1 and wild-type *B-RAF* genomic DNA illustrates the sensitivity of the PAP assay (Figure 5.3). Under experimental conditions a few mutant copies can be detected in the presence of tens of thousands of wild-type copies and supports the hypothesis that uveal melanoma display

Figure 5.3. The PAP dilution experiment shows the high specificity and sensitivity of this assay. The *B-RAF* mutation is still recognizable in the presence of tens of thousands of wild-type templates.



heterogeneity for *B-RAF* mutations. Unfortunately, it is not possible to quantify the number of *B-RAF* mutants in a tumor sample with a real time approach because PAP is inhibited by fluorescent dyes and the polymerase lacks the 5'→ 3' exonuclease activity necessary for the *TaqMan* approach (ABI). That to date only PAP is able to detect *B-RAF* mutations in primary uveal melanoma may indicate that cells with mutations are very rare in these tumors and may imply that mutations in *B-RAF* are not likely to drive uveal melanoma development and also adds further proof for the proposed heterogeneity in uveal melanoma (Maat et al., 2007a; Maat et al., 2007b). The role of these sporadic mutations remains unclear. It may be that the observed *B-RAF* mutations represent a sign of tumor progression or evolution or appear as spontaneous mutations within the developing tumor (Albertson et al., 2003; Duesberg et al., 2005). Mutations are found in exons 11 and 15, but only mutations in the activation domain of *B-RAF* such as the V600E are thought to have a selective advantage (Thomas et al., 2007). Of interest, the V600E mutation accounts for 92% of the *B-RAF* mutations detected in cutaneous melanoma samples (Davies et al., 2002). However, Pollock (Pollock et al., 2003) reported the presence of *B-RAF* mutations in 82% of cutaneous nevi, demonstrating that *B-RAF* activation alone is insufficient for the development of cutaneous melanoma, highlighting the requirement for additional molecular changes.

In this study, PAP detected *B-RAF* mutations in uveal melanoma cell lines, as well as in primary tumor samples. The PAP assay is sensitive enough to detect a single mutant sequence in excess of wild-type DNA sequences. Based on this assay, we conclude that *B-RAF* mutations occur in uveal melanoma, although the clinical relevance of such mutations in a minor percentage of cells has to be determined.

Our data reveal that *B-RAF* mutation frequency in uveal melanoma is higher than earlier anticipated and add to the rarely reported *B-RAF* mutations in uveal melanoma. However, the relative scarcity of the *B-RAF* mutation excludes an elemental role for this mutation in uveal melanoma.

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References

- Albertson DG, Collins C, McCormick F, Gray JW. Chromosome aberrations in solid tumors. *Nat Genet.* 2003;34:369–376.
- Brose MS, Volpe P, Feldman M, et al. BRAF and RAS mutations in human lung cancer and melanoma. *Cancer Res.* 2002;62:6997–7000.
- Calipel A, Lefevre G, Pouppnot C, et al. Mutation of B-Raf in Human Choroidal Melanoma Cells Mediates Cell Proliferation and Transformation through the MEK/ERK Pathway. *Journal of Biological Chemistry.* 2003;278:42409–42418.
- Calipel A, Mouriaux F, Glotin AL, et al. Extracellular signal-regulated kinase-dependent proliferation is mediated through the pro-tein kinase A/B-Raf pathway in human uveal melanoma cells. *J Biol Chem.* 2006;281:9238–9250.
- Cohen Y, Goldenberg-Cohen N, Parrella P, et al. Lack of BRAF mutation in primary uveal melanoma. *Invest Ophthalmol Vis Sci.* 2003;44:2876–2878.
- Chen PW, Murray TG, Uno T, et al. Expression of MAGE genes in ocular melanoma during progression from primary to metastatic disease. *Clin Exp Metastasis.* 1997;15:509–518.
- Cruz F III, Rubin BP, Wilson D, et al. Absence of BRAF and NRAS mutations in uveal melanoma. *Cancer Res.* 2003;63:5761–5766.
- Davies H, Bignell GR, Cox C, et al. Mutations of the BRAF gene in human cancer. *Nature.* 2002;417:949–954.
- Diebold Y, Blanco G, Saornil MA, Fernandez N, Lazaro MC. Morphologic and immunocytochemical characterization of four human uveal cell lines (melanoma- and melanocytes-derived). *Curr Eye Res.* 1997;16:487–495.
- Duesberg P, Li R, Fabarius A, Hehlmann R. The chromosomal basis of cancer. *Cell Oncol.* 2005;27:293–318.
- Edmunds SC, Cree IA, Di Nicolantonio F, et al. Absence of BRAF gene mutations in uveal melanomas in contrast to cutaneous melanomas. *Br J Cancer.* 2003;88:1403–1405.
- Egan KM, Seddon JM, Glynn RJ, Gragoudas ES, Albert DM. Epidemiologic aspects of uveal melanoma. *Surv Ophthalmol.* 1988;32: 239–251.
- Goldenberg-Cohen N, Cohen Y, Rosenbaum E, et al. T1799A BRAF mutations in conjunctival melanocytic lesions. *Invest Ophthalmol Vis Sci.* 2005;46:3027–3030.
- Kan-Mitchell J, Mitchell MS, Rao N, Liggett PE. Characterization of uveal melanoma cell lines that grow as xenografts in rabbit eyes. *Invest Ophthalmol Vis Sci.* 1989;30:829–834.
- Kilic E, Bruggenwirth HT, Verbiest MM, et al. The RAS-BRAF kinase pathway is not involved in uveal melanoma. *Melanoma Res.* 2004; 14:203–205.
- Liu Q, Sommer SS. PAP: detection of ultra rare mutations depends on P* oligonucleotides: “sleeping beauties” awakened by the kiss of pyrophosphorolysis. *Hum Mutat.* 2004a;23:426–436.
- Liu Q, Sommer SS. Pyrophosphorolysis by Type II DNA polymerases: implications for pyrophosphorolysis-activated polymerization. *Anal Biochem.* 2004b;324:22–28.
- Luyten GP, Naus NC, Mooy CM, et al. Establishment and characterization of primary and metastatic uveal melanoma cell lines. *Int J Cancer.* 1996;66:380–387.
- Maat W, Jordanova ES, Zelderen-Bhola SL, et al. The heterogeneous distribution of monosomy 3 in uveal melanomas: implications for prognostication based on fine-needle aspiration biopsies. *Arch Pathol Lab Med.* 2007a;131:91–96.
- Maat W, van der Velden PA, Out-Luiting C, et al. Epigenetic inactivation of RASSF1a in uveal melanoma. *Invest Ophthalmol Vis Sci.* 2007b;48:486–490.
- Malaponte G, Libra M, Gangemi P, et al. Detection of BRAF gene mutation in primary choroidal melanoma tissue. *Cancer Biol Ther.* 2006;5:225–227.
- Parsons BL, Heflich RH. Genotypic selection methods for the direct analysis of point mutations. *Mutat Res.* 1997;387:97–121.
- Pollock PM, Meltzer PS. A genome-based strategy uncovers frequent BRAF mutations in melanoma. *Cancer Cell.* 2002;2:5–7.
- Pollock PM, Harper UL, Hansen KS, et al. High frequency of BRAF mutations in nevi. *Nat Genet.* 2003;33:19–20.
- Rimoldi D, Salvi S, Lienard D, et al. Lack of BRAF mutations in uveal melanoma. *Cancer Res.* 2003;63:5712–5715.

- Satyamoorthy K, Li G, Gerrero MR, et al. Constitutive mitogenactivated protein kinase activation in melanoma is mediated by both BRAF mutations and autocrine growth factor stimulation. *Cancer Res.* 2003;63:756–759.
- Singh AD, Topham A. Incidence of uveal melanoma in the United States: 1973–1997. *Ophthalmology.* 2003;110:956–961.
- Thomas NE, Edmiston SN, Alexander A, et al. Number of nevi and early-life ambient UV exposure are associated with BRAF-mutant melanoma. *Cancer Epidemiol Biomarkers Prev.* 2007;16:991–997.
- Turner DJ, Zirvi MA, Barany F, Elenitsas R, Seykora J. Detection of the BRAF V600E mutation in melanocytic lesions using the ligase detection reaction. *J Cutan Pathol.* 2005;32:334–339.
- Tsao H, Goel V, Wu H, Yang G, Haluska FG. Genetic interaction between NRAS and BRAF mutations and PTEN/MMAC1 inactivation in melanoma. *J Invest Dermatol.* 2004;122:337–341.
- Uribe P, Wistuba II, Gonzalez S. BRAF mutation: a frequent event in benign, atypical, and malignant melanocytic lesions of the skin. *Am J Dermatopathol.* 2003;25:365–370.
- Waard-Siebinga I, Blom DJ, Griffioen M, et al. Establishment and characterization of an uveal-melanoma cell line. *Int J Cancer.* 1995;62:155–161.
- Weber A, Hengge UR, Urbanik D, et al. Absence of mutations of the BRAF gene and constitutive activation of extracellular-regulated kinase in malignant melanomas of the uvea. *Lab Invest.* 2003;83:1771–1776.
- Wellbrock C, Karasarides M, Marais R. The RAF proteins take centre stage. *Nat Rev Mol Cell Biol.* 2004;5:875–885.
- Zhang BH, Guan KL. Activation of B-Raf kinase requires phosphorylation of the conserved residues Thr598 and Ser601. *EMBO J.* 2000;19:5429–5439.
- Zuidervaart W, van Nieuwpoort F, Stark M, et al. Activation of the MAPK pathway is a common event in uveal melanomas although it rarely occurs through mutation of BRAF or RAS. *Br J Cancer.* 2005;92:2032–2038.

Chapter 6

Episodic Src Activation in Uveal Melanoma Revealed by Kinase Activity Profiling

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Abstract

BACKGROUND. The RAS-RAF-MEK-ERK pathway is involved in the balance between melanocyte proliferation and differentiation. The same pathway is constitutively activated in cutaneous and uveal melanoma (UM) 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 UM.

METHODS. We set out to explore the RAS-RAF-MEK-ERK pathway and used mitogen-activated protein kinase profiling and tyrosine kinase arrays.

RESULTS. We identified Src as a kinase that is associated with ERK1/2 activation in UM. However, low Src levels and reduced ERK1/2 activation in metastatic cell lines suggest that proliferation in metastases can become independent of Src and RAS-RAF-MEK-ERK signaling. Inhibition of Src led to the growth reduction of primary UM cultures and cell lines, whereas metastatic cell line growth was only slightly reduced.

CONCLUSIONS. We identified Src as an important kinase and a potential target for treatment in primary UM. Metastasis cell lines seemed largely resistant to Src inhibition and indicate that in metastases treatment, a different approach may be required.

Introduction

Uveal melanoma (UM) is a rare neoplasm that arises from melanocytes in the eyes. It usually affects people in their sixties with an incidence rate of 6–8 new cases per million per year among Caucasians (Egan et al., 1988; Singh and Topham, 2003). Little is known about the molecular pathogenesis of UM compared with cutaneous melanoma (CM). Cutaneous melanoma and UM share the same embryonic origin and similar histological features, but mutations that regulate proliferation and cause a loss of cell-cycle control in CM can hardly be found in UM. Whereas p16-regulated cell cycle control is targeted by the deletion of chromosome 9p or by the mutation of *CDKN2A* in CM, most of the UM cell lines possess a wild-type p16-encoding gene that is, however, not expressed because of the epigenetic modification of the *CDKN2A* gene (van der Velden et al., 2001). The same may be true for the activation of the RAS-RAF-MEK-ERK or the classical mitogen-activated protein kinase (MAPK) pathway. The MAPK activation is crucial for the development of melanocytic neoplasia, and a constitutive activation of this pathway has been associated with many different types of cancer (Goding, 2000; Reddy et al., 2003). In CM, the activation of the MAPK pathway has been shown to occur by a variety of mechanisms, including autocrine growth factor stimulation and mutation of the *NRAS* (20% of cases) and *BRAF* (60% of cases) genes (van Elsas et al., 1995; Davies et al., 2002; Satyamoorthy et al., 2003). The *BRAF* mutations have only rarely been reported in UM and activating mutations in *NRAS*, which are found in 25% of all cancers, have never been reported (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). However, we and others have found that UM is heterogeneous and that, with more sensitive techniques, the percentage of mutant *BRAF*-positive UM may be higher (Janssen et al., 2008; Maat et al., 2008). 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 UM (Rimoldi et al., 2003; Weber et al., 2003; Zuidervaart et al., 2005). 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 UM 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 UM growth and suggests that an upstream factor is involved in autonomous UM proliferation. Recently, *c-Kit* was shown to be upregulated in UM and involved in an autocrine loop that also involved the RAS-RAF-MEK-ERK pathway (Lefevre et al., 2004). An incomplete response to *c-Kit* inhibition indicates that additional factors are involved (Hofmann et al., 2009). In addition, the *GNAQ* gene was shown to be mutated in almost half of UM (Van Raamsdonk et al., 2008). *GNAQ* is part of the G-protein heterotrimer and represents the GTP-binding part that couples GPCR signaling to MAPK activation, which marks it as a potential therapeutic target.

However, targeting downstream signaling molecules may be just as effective as they may be shared with other mutant pathways. Tyrosine kinase activity profiling in UM was used to explore the involved kinases. On the basis of a UM cell line and two related metastasis cell lines, which revealed reduced ERK1/2 activation in metastases, we were able to identify Src as a crucial upstream tyrosine kinase for ERK1/2 activation in primary UM. Unfortunately, metastasis cell lines seemed less dependent on Src and may indicate that metastasis may require an alternative approach for intervention.

Materials and methods

Cell lines and tumor material

A total of 11 cell lines derived from primary UM (92.1; OCM-1, -3 and -8; Mel-202, -270, -285, -290) and UM metastases (OMM-1, -2.3 and -2.5) were analysed for kinase activity (Kan-Mitchell et al., 1989; Waard-Siebinga et al., 1995; Luyten et al., 1996; Chen et al., 1997). UM cell lines were cultured in RPMI 1640 medium (Gibco, Paisley, Scotland) supplemented with 3mM L-glutamine (Gibco), 2% penicillin/streptomycin and 10% FBS (Hyclone, Logan, UT, USA). Primary UM was cultured in Amniochrome Pro Medium (Lonza Group Ltd, Basel, Switzerland). All cell cultures were incubated at 37°C in a humidified 5% CO₂ atmosphere. Cell lysates were obtained by lysing cells in M-PER Mammalian Protein Extract Reagent (Pierce, Rockford, IL, USA), supplemented with 1% Halt Protease Inhibitor Cocktail, EDTA-free (Pierce) and 1% Halt Phosphatase Inhibitor Cocktail (Pierce). Protein concentrations were measured by using the BCA Protein Assay kit (Pierce). Cell lysates were also acquired from three fresh primary UM samples obtained by enucleation and from three liver metastases of three different patients, in whom the diagnosis was confirmed.

Phospho-MAPK array

The Human Phospho-MAPK Array (R&D Systems, Abingdon, UK) was used to simultaneously detect the relative levels of nine MAP kinases and nine other serine/threonine kinases in cell lines, in primary UM and in liver metastasis. In this array, capture and control antibodies were spotted in duplicate on nitrocellulose membranes. Experiments were carried out according to the manufacturer's guidelines. In short, cell lysates were diluted and incubated with the array. After binding of both phosphorylated and unphosphorylated kinases, unbound material was washed away. A cocktail of phospho-site-specific biotinylated antibodies was used to detect phosphorylated proteins through streptavidin horseradish peroxidase and chemiluminescence. X-ray films of the blots were scanned and analysed using G-boxHR (Syngene, Frederick, MD, USA). Control spots with mouse, goat and rabbit antibodies were used for background correction.

PamGene tyrosine kinase array

Experiments were carried out using a 4-array semi-automated system (PamStation 4, PamGene, 's-Hertogenbosch, The Netherlands) designed for processing PamChip-4 arrays. The PamChip Tyrosine Kinase Array (PamGene) contains 144 phospho-peptides, immobilized on a porous microarray surface through the peptide N terminus, representing tyrosine kinase substrates. Each array was blocked with 0.2% bovine serum albumin (BSA), fraction V (Calbiochem Immunochemicals, Merck KGaA, Darmstadt, Germany) by pumping it through the porous microarray for 30 cycles of 30 s. Thereafter, each array was washed thrice for 8 s with 1x ABL Protein Tyrosine Kinase Reaction Buffer solution (New England Biolabs, Ipswich, MA, USA). Next, incubation was carried out at 30°C with the reaction mix, containing 5 µg cell lysate, 4 µl 100x BSA (New England Biolabs), 0.4 µl 10mM ATP (Sigma-Aldrich, Zwijndrecht, The Netherlands) and 0.5 µl 1mg ml⁻¹ monoclonal anti-phosphotyrosine FITC conjugate (clone Py20,

Exalpa Biologicals, Maynard, MA, USA), adjusted to 40 μ l with distilled H₂O. The sample was pulsed back and forth through the porous material for 45 cycles, which is coupled to the base of a well to maximize reaction kinetics and to reduce analysis time. At every fifth pump cycle, a 16-bit TIFF image was taken with a built-in CCD camera (PamGene). Blocking experiments were carried out with Src family-selective tyrosine kinase inhibitors, PP1, PP2 (Biomol International, LP, of Plymouth Meeting, PA, USA) and PP3 (the inactive analogue, Calbiochem), at an end concentration of 10 μ M in line with a large body of literature. Each particular inhibitor was mixed with lysates of cell lines and tissue, together with the reaction mix just before incubation on the array. Acquired data from the PamStation 4 were captured with the supplied software package BioNavigator (Version 0.3.1; PamGene). For the purpose of finding differentially phosphorylated substrates, data were imported in the LIMMA package (Bioconductor.org) and we applied the empirical Bayes method (Smyth, 2004). Background subtracted data were normalized for differences between experiments, and substrates and P-values of 0.05 or less were corrected for multiple testing using the Benjamini and Hochberg correction. Substrates with a corrected P-value of 0.05 or less were assumed to be significant.

Western blot analysis

Cell lysates (10 μ g) were separated on 12.5% SDS–PAGE gels, and proteins were transferred to Hybond-polyvinylidene difluoride membranes (Amersham Biosciences, Buckinghamshire, UK). The membranes were blocked with 5% skim milk in a PBS-Tween 0.1% solution and probed at room temperature for 1 h with antibodies specific to each antigen: phospho-Src (Tyr527; dilution 1:1000), phospho-Src family (Tyr416; dilution 1:1000) and Src (36D10; dilution 1:1000) antibody (all from Cell Signaling Technology, Hertfordshire, UK). An antibody against actin (Abcam, Cambridge, UK) was used as a loading control. Membranes were subsequently incubated at room temperature with horseradish peroxidase-conjugated IgG anti-mouse or anti-rabbit secondary antibodies for 1 h. Supersignal West Femto ECL (Pierce) was used to visualize protein bands on the membrane.

siRNA treatment

Sub-confluent cell cultures were grown without antibiotics 24 h before transfection in RPMI 1640 medium. A mixture of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and two different siRNA constructs (40 nM) were incubated in the standard medium with reduced serum (1%), according to the manufacturer's instructions. The siRNA constructs (Stealth) were predesigned and validated ($\approx$ 70% knockdown) by the manufacturer (Invitrogen). After 24 and 48 h, the cells were harvested and RNA and protein lysates were prepared.

WST-1 assay

Cell proliferation in response to PP1 (10 and 50 μ M) was measured by mitochondrial function using the WST-1 proliferation reagent (Sigma-Aldrich) as described earlier (Narayanan et al., 2005). This assay measures tetrazolium reductase activity in the mitochondria, which serves as a measure of cell viability.

In short, 96-well plates were filled with 1250 UM cells per well. At 1 or 3 days (tumor 1–5) and 1 or 6 days (92.1; OCM-1, -3 and -8; Mel-202, -270, -285, -290, OMM-1, -2.3 and -2.5) after treatment, the WST-1 reagent was added and absorbance was measured at 450nm on a multiwell spectrophotometer. The median and standard error of eight wells were taken at each time and dosage point.

Quantitative PCR

The cell lines (92.1; OCM-1, -3 and -8; Mel-202, -270, -285, -290, OMM-1, -2.3 and -2.5) were analysed for Src gene expression. Primers for Src and the reference gene, β -actin, were developed with Beacon Designer software (Premier Biosoft, Palo Alto, CA, USA). Primer sequences for Src: 5'-GCTGCGGCTGGAGGTCAAG-3' (forward) and 5'-AGACATCGTGCCAGGCTTCAG-3' (reverse). Primer sequence for β -actin: 5'-CGGGACCTGACTGACTACCTC-3' (forward) and 5'-CTCCTTAATGTCACGCACGATTTC-3' (reverse). The PCR reaction settings were 95°C for 5 min, then 40 cycles at 96°C for 15 s and at 60°C for 45 s. The DNA melting point of the amplicons was acquired by measuring the fluorescence of SYBR Green (Bio-Rad, Hercules, CA, USA) during a linear temperature transition from 70 to 97°C at 0.2°C each for 10 s with accompanying software (Bio-Rad).

Results

ERK1/2 activation in UM

An antibody array was applied to investigate the MAPK pathway in 10 UM cell lines, in three primary UM and three UM metastasis. We observed a uniform HSP27 phosphorylation, with the exception of in three UM cell lines (OCM1, -3, -8). UMs displaying activated ERK1/2 as well as phosphorylated HSP27 were most common, whereas signals for phosphorylated ERK1/2 were low in metastasis tissue (MET1-3) and metastatic UM cell lines (OMM1, OMM2.3 and OMM2.5) (Figure 6.1). Remarkably, two of the metastatic cell lines (OMM2.3, OMM2.5) are derived from the same patient as cell line Mel270 but contained far less activated ERK1/2.

Differential kinase activity in UM

Reduction of ERK1/2 activation in metastatic cell lines compared with that in primary UM cell lines provides a model to identify the underlying mechanism of ERK1/2 activation in the absence of *BRAF* and *NRAS* mutations. To investigate whether a kinase is differentially activated between primary UM cell lines and metastatic UM cell lines, we used peptide-based tyrosine kinase arrays (Lemeer et al., 2007). The UM cell lines displayed a high kinase activity, whereas the metastatic UM cell lines displayed a low kinase activity, although the same amount of lysate was incubated (Figure 6.2A). After normalisation, we could analyse the kinase data and identify nine substrates that were significantly differentially phosphorylated between primary and metastatic UM cell lines (Figure 6.2B, Table 6.1.). Primary UM and metastatic tissue also showed differential phosphorylation of these nine peptides, although not as clearly as observed in the cell lines (Figure 6.2C).

Figure 6.1. The MAPK activation in primary UM and UM metastases was studied with a MAPK antibody array. We observed uniform HSP27 phosphorylation in both cell lines and tissue samples, except in OCM1, -3 and -8 (A). Activated ERK1/2 was normalised with HSP27 and shown to be low in UM metastases (MET1-3), whereas metastatic cell lines just passed the background (OMM1, -2.3, -2.5) (B).

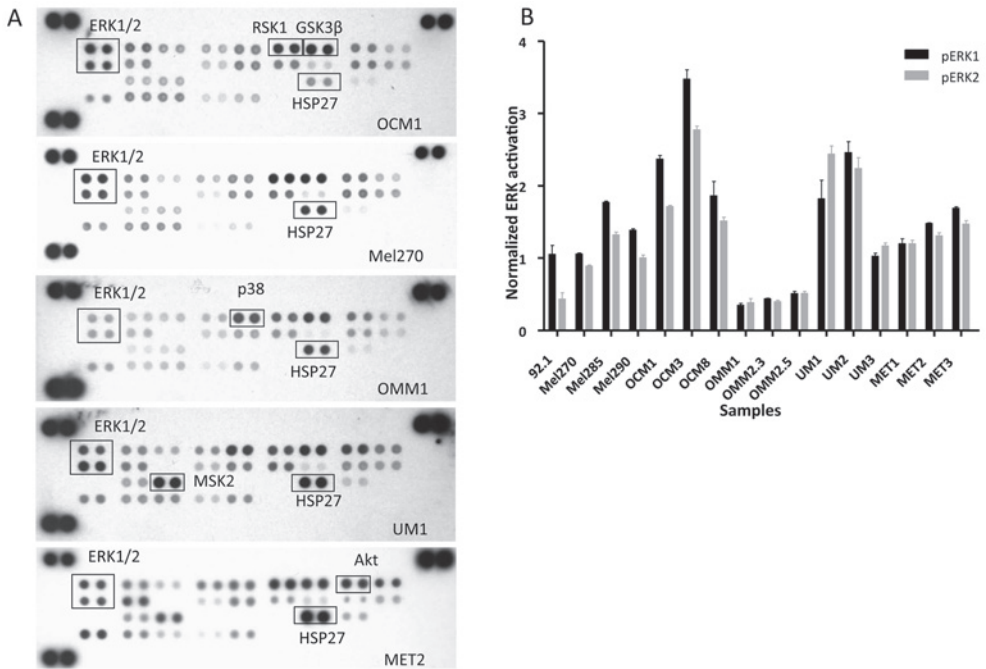
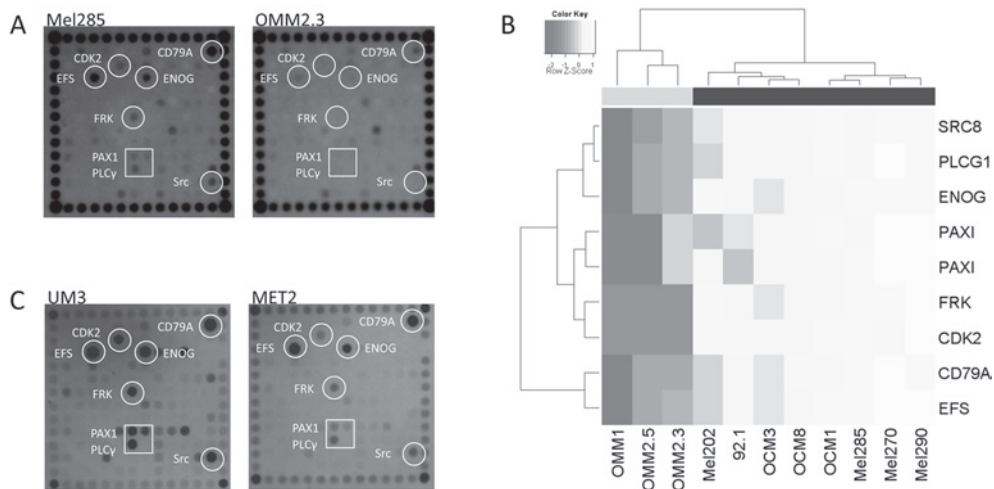


Table 6.1. Tyrosine kinase substrates on the kinase array that were differentially phosphorylated between primary UM cell lines and metastatic cell lines.

Substrate	UniProt ID	Position	Log Fold Change	Adj. P value	Kinase
CDK2	P24941	T14/Y15	5.3	0.00005	Lyn
FRK	P42685	Y387	6.2	0.0002	unknown
SRC8	Q14247	Y499	4.6	0.006	Src
ENOG	P09104	Y43	4.7	0.01	Src
EFS	O43281	Y253	3.1	0.01	Src
PLCG1	P19174	Y771	4.2	0.01	Syk, Sky, GFRs
CD79A	P11912	Y182/Y188	3.1	0.01	Lyn
PAXI	P49023	Y118	4.0	0.02	FAK, Src, Brk
PAXI	P49023	Y31	4.3	0.03	FAK, Src, Brk

Figure 6.2. Tyrosine kinase activity was measured with an array of peptide substrates. Two representative examples of a UM cell line and a metastatic cell line (**A**). Analysis with eBayes identified nine substrates, representing eight proteins, to be significantly ($P=0.01$) differentially phosphorylated between UM and metastatic cell lines (**B**). UM (UM1-3) tyrosine kinase activity is high compared with liver metastasis (MET1-3) (two representative arrays are shown) (**C**).



Candidate kinase: Src

We identified nine peptides derived from eight proteins that were differentially phosphorylated between primary and metastatic cell line lysates. On the basis of a literature search, we identified candidate tyrosine kinases for eight out of nine peptides (Table 6.1) (Cooper et al., 1983; Thomas and Brugge, 1997; Koike et al., 2003; Diella et al., 2004). Among the candidates, Src and Src family members were most prominent. To validate the candidacy of Src, we performed *in vitro* inhibition experiments with the Src-kinase specific inhibitors PP1 and the PP1 analogue, PP2. We added PP1 and PP2 (10 mM) to lysates of primary UM tissue and of a primary UM cell line and measured the inhibitory effect of these Src inhibitors using kinase array (Figure 6.3A). A total of seven out of nine substrates that identified Src in the first screen displayed a significantly reduced phosphorylation when PP1 or PP2 were added to lysates of UM1 and Mel270 (Figure 6.3A). The PLCG1 peptide and one of the PAX1 (Y31) substrates did not reach significance but were still phosphorylated at a reduced level after PP1 and PP2 treatments. The peptide representing FAK1 Y576/Y577 is a genuine substrate for Src, which was not detected in the UM cell line comparison, but phosphorylation was significantly down regulated by PP1 and PP2 treatments. In the control experiment, in which we added the inactive analogue of PP1 (PP3) to cell lysates, we did not observe a loss of kinase activity (not shown).

The kinase activity of metastasis tissue and UM tissue differed marginally (Figure 6.2C), and incubation with PP1 (10 mM) resulted in a decimation of kinase activity similar to the inhibition that we observed in UM tissue (Figure 6.3B). To validate Src activity, Mel270 was transfected with two siRNA constructs that target Src and reduced kinase activity (Figure 6.3B).

Figure 6.3. UM1 (06-12) and Mel270 treatment with Src inhibitors (PP1/PP2) identified eight substrates with a significant reduction in phosphorylation **(A)**. The inhibition of EFS peptide phosphorylation by genetic (Src siRNA) and pharmacological means (PP1) in cell line Mel270, and PP1 treatment of cell lysates of UM (UM1) and metastasis tissue (MET3) **(B)**.

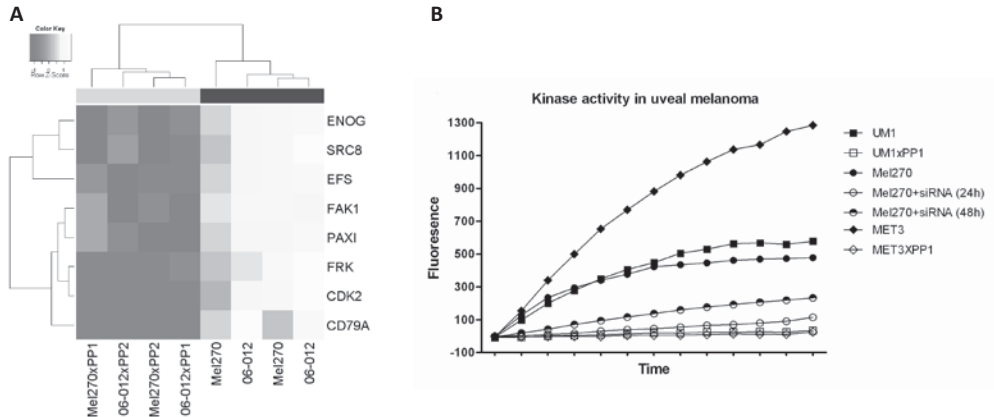
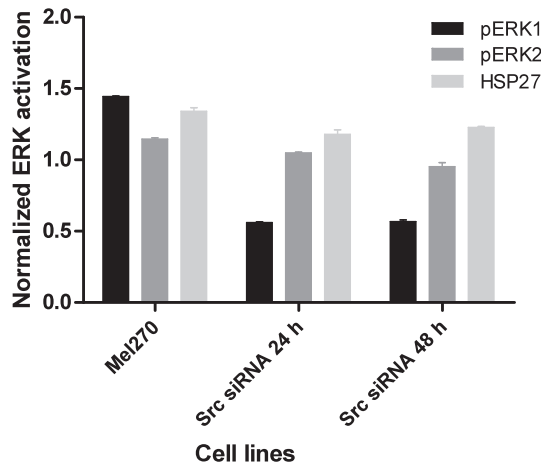


Figure 6.4. ERK1/2 activation in Mel270 24 and 48 h after transfection with Src siRNA. Phosphorylated HSP27 is included as reference signal.



Regulation of ERK1/2 and growth

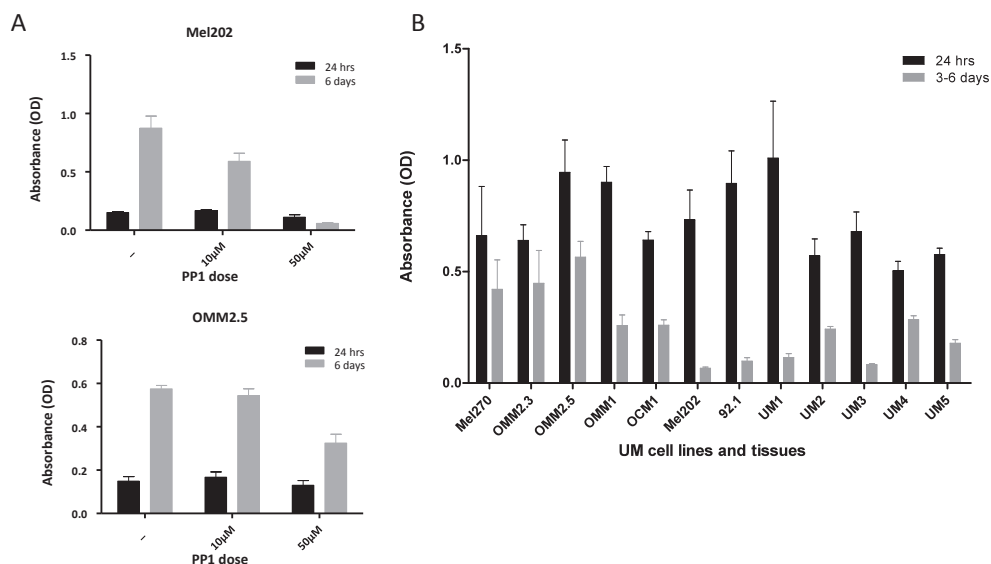
To investigate whether Src contributes to ERK1/2 activation in Mel270, we analysed the two Src siRNA-transfected cell cultures with the MAPK antibody array. At 24 and 48 h after transfection with Src siRNA, we observed a reduced ERK1 phosphorylation, whereas ERK2 phosphorylation was minimally affected (Figure 6.4). Whether Src inhibition and consequently a lowered ERK activation in UM cell lines is

associated with a reduced growth was investigated with the WST-1 viability assay (Figure 6.5). All UM cell lines showed a PP1-dose and time (1–6 days)-dependent reduction in cell viability but the magnitude of the response differed widely. In general, the metastatic UM cell lines were less affected by PP1. We also determined the growth inhibition rate of PP1 in cultures of five primary UM cell cultures and observed an increased sensitivity to PP1 treatment compared with the cell lines. We had to take samples at day 3 of PP1 treatment because, thereafter, massive cell death occurred (Figure 6.5B).

Src protein is reduced in metastasis cell line

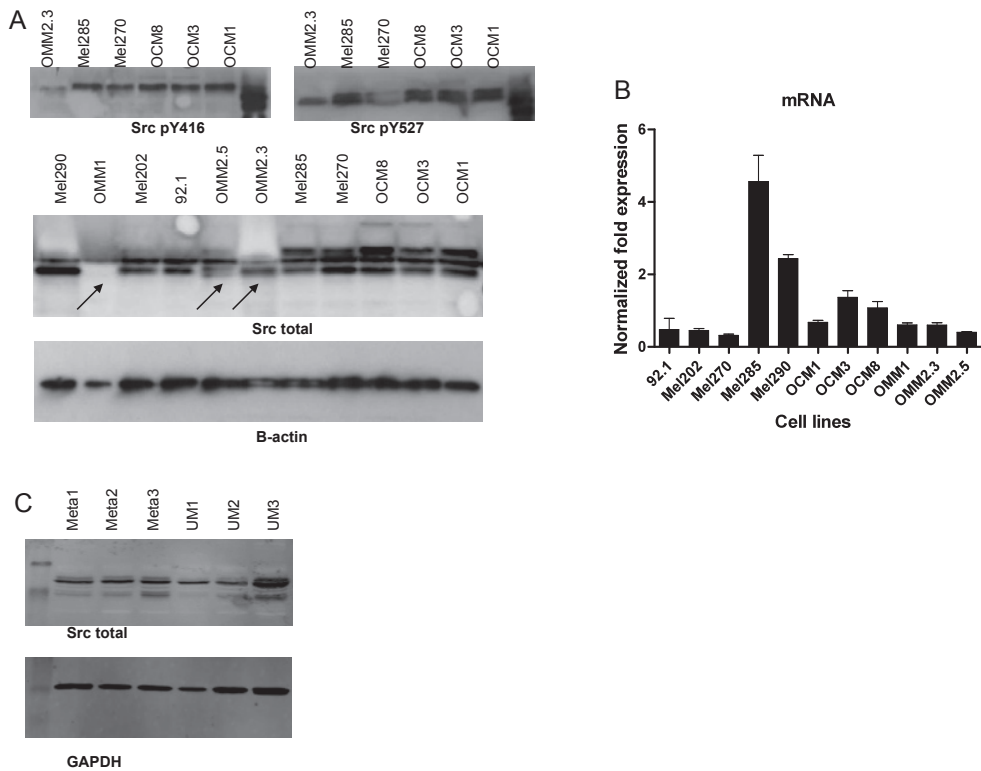
Src is regulated by the phosphorylation of tyrosine residues at position 416 (Y416) and 527 (Y527). The expression of phosphorylated Src Y416, which is associated with an active conformation, was low in the metastatic UM cell lines (Figure 6.6A). Surprisingly, the phosphorylation of Y527, which is associated with an inactive conformation, was also low, and a subsequent analysis indicated that Src expression is low in metastatic UM cell lines. Therefore, the difference between kinase activity in metastatic cell lines (OMM1, OMM2.3 and OMM2.5) and UM cell lines (OCM1, OCM3, OCM8, Mel202, Mel270, Mel285, Mel290 and 92.1) seems to be the result of a difference in Src expression.

Figure 6.5. UM cell lines and primary cultures were cultured with PP1 (10 mM and 50 mM). After 24 h and at 3 days (UM cultures) and 6 days (cell lines), viability was tested with the WST-1 assay. Two representative cell culture experiments for which all time points and conditions are shown (A). Growth inhibition by PP1 (50 mM) after 24 h and at 3 and 6 days was normalised to the control culture of each individual cell line (B).



To investigate the origin of a lowered Src expression, we performed a gene expression analysis (Figure 6.6B). Src gene expression varied widely in the cell lines and in the metastatic cell lines, but a correlation between protein and gene expression was not observed in UM cell lines. A western analysis of Src expression in UM and metastasis tissue revealed a very high Src expression in only one out of three primary UM, whereas all three metastasis tissues displayed medium expression of Src protein (Figure 6.6C).

Figure 6.6. Western analysis of Src in the UM cell lines for activating phosphorylation (Y416), inactivating phosphorylation (Y527) and total Src expression (**A**). Src gene expression measured by qPCR varied widely but did not correlate with a variation in protein expression (**B**). UM and metastasis tissue all displayed a medium Src kinase expression, except for UM3, which presents a high level of expression (**C**).



Discussion

Constitutive activation of ERK1/2 has often been reported for UM (Rimoldi et al., 2003; Weber et al., 2003; Zuidervaart et al., 2005). Using a more quantitative approach, we distinguished a decrease in active ERK1/2 in metastatic cell lines and in fresh liver metastasis, suggesting a loss of ERK1/2 activation during UM progression. The latter is unexpected as ERK1/2 activation is generally associated with mitogen signalling and is known to determine malignant potential *in vitro*. However, in endometrial and breast cancer, ERK1/2 activation has been associated with a good prognosis (Milde-Langosch et al., 2005; Mizumoto et al., 2007). A possible explanation is provided by the observation that ERK1/2 is involved in oncogene and stress-induced senescence (Serrano et al., 1997; Stott et al., 1998). This mechanism is thought to be an important defense for cells that are at risk of neoplastic transformation and need to be circumvented by tumor cells in order to proliferate. Loss of activated ERK1/2 may not only relieve the associated inhibitory mechanisms in a direct manner but may also require alternative mitogenic signals to take over in UM metastasis.

Metastatic and UM cell lines provide a unique model to identify the mechanisms that regulate ERK1/2 activation in UM. Earlier work already showed that ERK1/2 phosphorylation in UM depends on the MAPK pathway, although mutations in the usual suspects (e.g., *BRAF* and *NRAS*) are lacking (Calipel et al., 2006). We investigated the possibility of a tyrosine kinase with differential activities in UM and UM metastasis to be responsible for ERK1/2 activation, using an array of kinase activity assays. Src was revealed as a differentially activated tyrosine kinase and this was supported by incubation with Src-specific kinase inhibitors, PP1 and PP2. Moreover, by treating cell lysates instead of cell cultures, we minimized the secondary effects of the inhibitors. However, PP1 and PP2 affect most of the Src family of tyrosine kinases, and the observed reduction in kinase activity therefore does not specifically mark Src. Multi-target inhibitors are a problem in molecular analysis but may be beneficial in clinical application, as, in CM, a switch from Src to Yes signalling has been reported in brain metastases (Summy and Gallick, 2003). To specifically inhibit Src, we targeted the Src gene expression with a siRNA approach. We detected a reduced kinase activity in Mel270 on transfection and this was correlated with a reduced ERK1 activation. The ERK2 activation seemed unaffected, which could be because of the limited efficacy of siRNA treatment, or it could indicate the activity of another, yet unidentified, kinase. A low Src protein expression in conjunction with a loss of ERK1/2 activation in metastatic UM cell lines, however, supports the hypothesis that, in UM, Src kinase is involved in ERK1/2 activation. Gene expression analysis revealed no significant differences between metastatic and UM cell lines, and thereby indicated that posttranscriptional mechanisms are most likely involved in Src down regulation. Src is both a kinase as well as a client protein for the chaperone HSP90 that is expressed in UM (Missotten et al., 2003). Whether HSP90 is reduced in metastases and whether treatment with HSP90 inhibitors depends on Src signalling is part of future investigation (Babchia et al., 2008; Faingold et al., 2008). The inhibition of Src kinase activity resulted in a strong growth reduction in all UM cultures, whereas in UM cell lines, the response varied more widely. The genetic background of the cell lines might play a role in the observed

variation. However, all UM cell lines displayed Src kinase activity and PP1 sensitivity, irrespective of *c-kit* upregulation (Mel270) or the *BRAF* V600E (OCM1) and *GNAQ* Q209L (Mel202) mutation status (Lefevre et al., 2004; Van Raamsdonk et al., 2008). Tissue of UM and UM liver metastasis displayed more or less comparable Src levels. The incubation of lysates with Src inhibitors resulted in a comparable reduction of kinase activity in UM and metastasis tissue. The possibility that there exist Src negative clones in liver metastasis can, however, not be ruled out on the basis of these data. Clinical trials targeting Src kinase activity in UM should therefore anticipate this potential risk.

In conclusion, we have identified a differential ERK1/2 activation in UM and metastatic UM cell lines. Using tyrosine kinase activity profiling, we identified Src as a determinant of ERK1/2 activation and showed that Src expression and kinase activity, together with ERK1/2 activation, are reduced in UM metastases cell lines.

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References

- Babchia N, Calipel A, Mouriaux F, Faussat AM, Mascarelli F (2008) HSP90 inhibitors 17-AAG and 17-DMAG target wild-type B-Raf signaling for the proliferation of human uveal melanoma cell lines. *Invest Ophthalmol Vis Sci* 49: 2348–2356
- Calipel A, Mouriaux F, Glotin AL, Malecaze F, Faussat AM, Mascarelli F (2006) Extracellular signal-regulated kinase-dependent proliferation is mediated through the protein kinase A/B-Raf pathway in human uveal melanoma cells. *J Biol Chem* 281: 9238–9250
- Chen PW, Murray TG, Salgaller ML, Ksander BR (1997) Expression of MAGE genes in ocular melanoma cell lines. *J Immunother* 20: 265–275
- Cohen Y, Goldenberg-Cohen N, Parrella P, Chowers I, Merbs SL, Peer J, Sidransky D (2003) Lack of BRAF mutation in primary uveal melanoma. *Invest Ophthalmol Vis Sci* 44: 2876–2878
- Cooper JA, Reiss NA, Schwartz RJ, Hunter T (1983) Three glycolytic enzymes are phosphorylated at tyrosine in cells transformed by Rous sarcoma virus. *Nature* 302: 218–223
- Cruz III F, Rubin BP, Wilson D, Town A, Schroeder A, Haley A, Bainbridge T, Heinrich MC, Corless CL (2003) Absence of BRAF and NRAS mutations in uveal melanoma. *Cancer Res* 63: 5761–5766
- Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, Teague J, Woffendin H, Garnett MJ, Bottomley W, Davis N, Dicks E, Ewing R, Floyd Y, Gray K, Hall S, Hawes R, Hughes J, Kosmidou V, Menzies A, Mould C, Parker A, Stevens C, Watt S, Hooper S, Wilson R, Jayatilake H, Gusterson BA, Cooper C, Shipley J, Hargrave D, Pritchard-Jones K, Maitland N, Chenevix-Trench G, Riggins GJ, Bigner DD, Palmieri G, Cossu A, Flanagan A, Nicholson A, Ho JW, Leung SY, Yuen ST, Weber BL, Seigler HF, Darrow TL, Paterson H, Marais R, Marshall CJ, Wooster R, Stratton MR, Futreal PA (2002) Mutations of the BRAF gene in human cancer. *Nature* 417: 949–954
- Diella F, Cameron S, Gemund C, Linding R, Via A, Kuster B, Sicheritz-Ponten T, Blom N, Gibson TJ (2004) Phospho.ELM: a database of experimentally verified phosphorylation sites in eukaryotic proteins. *BMC Bioinformatics* 5: 79
- Edmunds SC, Cree IA, Di Nicolantonio F, Hungerford JL, Hurren JS, Kelsell DP (2003) Absence of BRAF gene mutations in uveal melanomas in contrast to cutaneous melanomas. *Br J Cancer* 88: 1403–1405
- Egan KM, Seddon JM, Glynn RJ, Gragoudas ES, Albert DM (1988) Epidemiologic aspects of uveal melanoma. *Surv Ophthalmol* 32: 239–251
- Faingold D, Marshall JC, Anteck A, Odashiro AN, Bakalian S, Fernandes BF, Burnier Jr MN (2008) Immune expression and inhibition of heat shock protein 90 in uveal melanoma. *Clin Cancer Res* 14: 847–855
- Goding CR (2000) Mitf from neural crest to melanoma: signal transduction and transcription in the melanocyte lineage. *Genes Dev* 14: 1712–1728
- Hofmann UB, Kauczok-Vetter CS, Houben R, Becker JC (2009) Overexpression of the KIT/SCF in uveal melanoma does not translate into clinical efficacy of imatinib mesylate. *Clin Cancer Res* 15: 324–329
- Janssen CS, Sibbett R, Henriquez FL, McKay IC, Kemp EG, Roberts F (2008) The T1799A point mutation is present in posterior uveal melanoma. *Br J Cancer* 99: 1673–1677
- Kan-Mitchell J, Mitchell MS, Rao N, Liggett PE (1989) Characterization of uveal melanoma cell lines that grow as xenografts in rabbit eyes. *Invest Ophthalmol Vis Sci* 30: 829–834
- Koike A, Kobayashi Y, Takagi T (2003) Kinase pathway database: an integrated protein-kinase and NLP-based protein-interaction resource. *Genome Res* 13: 1231–1243
- Lefevre G, Glotin AL, Calipel A, Mouriaux F, Tran T, Kherrouche Z, Maurage CA, Auclair C, Mascarelli F (2004) Roles of stem cell factor/ c-Kit and effects of Glivec/STI571 in human uveal melanoma cell tumorigenesis. *J Biol Chem* 279: 31769–31779
- Lemeer S, Jopling C, Naji F, Ruijtenbeek R, Slijper M, Heck AJ, den Hertog J (2007) Protein-tyrosine kinase activity profiling in knock down zebrafish embryos. *PLoS ONE* 2: e581
- Luyten GP, Naus NC, Mooy CM, Hagemeijer A, Kan-Mitchell J, Van Drunen E, Vuzevski V, de Jong PT, Luiders TM (1996) Establishment and characterization of primary and metastatic uveal melanoma cell lines. *Int J Cancer* 66: 380–387
- Maat W, Kilic E, Luyten GP, de Klein A, Jager MJ, Gruis NA, van der Velden PA (2008) Pyrophosphorolysis detects B-Raf mutations in primary uveal melanoma. *Invest Ophthalmol Vis Sci* 49: 23–27

- Milde-Langosch K, Bamberger AM, Rieck G, Grund D, Hemminger G, Muller V, Loning T (2005) Expression and prognostic relevance of activated extracellular-regulated kinases (ERK1/2) in breast cancer. *Br J Cancer* 92: 2206–2215
- Missotten GS, Journee-de Korver JG, Wolff-Rouendaal D, Keunen JE, Schlingemann RO, Jager MJ (2003) Heat shock protein expression in the eye and in uveal melanoma. *Invest Ophthalmol Vis Sci* 44: 3059–3065
- Mizumoto Y, Kyo S, Mori N, Sakaguchi J, Ohno S, Maida Y, Hashimoto M, Takakura M, Inoue M (2007) Activation of ERK1/2 occurs independently of KRAS or BRAF status in endometrial cancer and is associated with favorable prognosis. *Cancer Sci* 98: 652–658
- Mooy CM, Van der Helm MJ, Van der Kwast TH, de Jong PT, Ruiter DJ, Zwarthoff EC (1991) No N-ras mutations in human uveal melanoma: the role of ultraviolet light revisited. *Br J Cancer* 64: 411–413
- Narayanan R, Kenney MC, Kamjoo S, Trinh TH, Seigel GM, Resende GP, Kuppermann BD (2005) Trypan blue: effect on retinal pigment epithelial and neurosensory retinal cells. *Invest Ophthalmol Vis Sci* 46: 304–309
- Reddy KB, Nabha SM, Atanaskova N (2003) Role of MAP kinase in tumor progression and invasion. *Cancer Metastasis Rev* 22: 395–403
- Rimoldi D, Salvi S, Lienard D, Lejeune FJ, Speiser D, Zografos L, Cerottini JC (2003) Lack of BRAF mutations in uveal melanoma. *Cancer Res* 63: 5712–5715
- Satyamoorthy K, Li G, Gerrero MR, Brose MS, Volpe P, Weber BL, Van Belle P, Elder DE, Herlyn M (2003) Constitutive mitogen-activated protein kinase activation in melanoma is mediated by both BRAF mutations and autocrine growth factor stimulation. *Cancer Res* 63: 756–759
- Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW (1997) Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* 88: 593–602
- Singh AD, Topham A (2003) Incidence of uveal melanoma in the United States: 1973–1997. *Ophthalmology* 110: 956–961
- Smyth GK (2004) Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* 3, (Article 3)
- Soparker CN, O'Brien JM, Albert DM (1993) Investigation of the role of the ras protooncogene point mutation in human uveal melanomas. *Invest Ophthalmol Vis Sci* 34: 2203–2209
- Stott FJ, Bates S, James MC, McConnell BB, Starborg M, Brookes S, Palmero I, Ryan K, Hara E, Vousden KH, Peters G (1998) The alternative product from the human CDKN2A locus, p14(ARF), participates in a regulatory feedback loop with p53 and MDM2. *EMBO J* 17: 5001–5014
- Summy JM, Gallick GE (2003) Src family kinases in tumor progression and metastasis. *Cancer Metastasis Rev* 22: 337–358
- Thomas SM, Brugge JS (1997) Cellular functions regulated by Src family kinases. *Annu Rev Cell Dev Biol* 13: 513–609
- van der Velden PA, Metzelaar-Blok JA, Bergman W, Monique H, Hurks H, Frants RR, Gruis NA, Jager MJ (2001) Promoter hypermethylation: a common cause of reduced p16(INK4a) expression in uveal melanoma. *Cancer Res* 61: 5303–5306
- van Elsas A, Zerp S, van der FS, Kruse-Wolters M, Vacca A, Ruiter DJ, Schrier P (1995) Analysis of N-ras mutations in human cutaneous melanoma: tumor heterogeneity detected by polymerase chain reaction single-stranded conformation polymorphism analysis. *Recent Results Cancer Res* 139: 57–67
- van Raamsdonk CD, Bezroukove V, Green G, Bauer J, Gaugler L, O'Brien JM, Simpson EM, Barsh GS, Bastian BC (2008) Frequent somatic mutations of GNAQ in uveal melanoma and blue naevi. *Nature* 457(7229): 599–602
- Waard-Siebinga I, Blom DJ, Griffioen M, Schrier PI, Hoogendoorn E, Beverstock G, Danen EH, Jager MJ (1995) Establishment and characterization of an uveal-melanoma cell line. *Int J Cancer* 62: 155–161
- Weber A, Hengge UR, Urbanik D, Markwart A, Mirmohammadsaegh A, Reichel MB, Wittekind C, Wiedemann P, Tannapfel A (2003) Absence of mutations of the BRAF gene and constitutive activation of extracellular-regulated kinase in malignant melanomas of the uvea. *Lab Invest* 83: 1771–1776
- Zuidervaart W, van Nieuwpoort F, Stark M, Dijkman R, Packer L, Borgstein AM, Pavey S, van der Velden PA, Out C, Jager MJ, Hayward NK, Gruis NA (2005) Activation of the MAPK pathway is a common event in uveal melanomas although it rarely occurs through mutation of BRAF or RAS. *Br J Cancer* 92: 2032–2038

Summary and general discussion

Nederlandse samenvatting (Summary in Dutch)

Nawoord (Acknowledgements)

Biography

List of publications



Introduction

At present, molecular testing of uveal melanoma has developed to such a level that one can predict the prognosis of the patient with great accuracy. The course of the clinical disease or patient survival has not been altered, because effective treatment regimens against metastatic disease or measures to prevent progress are at present lacking. In order to treat or prevent growth of metastases, we need to understand the molecular pathways underlying uveal melanoma tumor development and tumor progression. The aim of this thesis is therefore to characterize the molecular mechanisms and the pathways that drive tumor growth and progression in uveal melanoma.

Molecular markers

Monosomy 3 was the first recognized molecular predictor of metastatic disease (Sisley et al., 1990; Horsthemke et al., 1992; Prescher et al., 1995; White et al., 1998). The possibility to identify patients at high risk of developing metastases makes intense monitoring of these patients useful, and may improve early detection and treatment of metastatic disease. Hence, tumor tissue should be available for prognostification. For patients treated with enucleation, tumor tissue is readily available. However, patients are preferably treated with an eye-conserving therapy that does not generate tumor tissue for analysis. Alternatively, a tumor sample can be obtained by fine needle aspiration biopsies (FNABs) from the tumor base by a scleral approach, or from the apex of the tumor via a transvitreal approach. Chromosome analysis and gene expression analysis of the tumor FNAB can be used to identify patients at risk.

This approach relies on the assumption that uveal melanomas represent a genetically homogeneous entity. In **Chapter 2**, we tested this assumption by analyzing tumor heterogeneity. We showed that some uveal melanomas are heterogeneous for the number of copies of chromosome 3: while one area of the tumor may show monosomy 3, another may not. Since then, other studies have confirmed tumor heterogeneity in uveal melanomas and a follow-up study showed that any number of cells with loss of chromosome 3 is associated with a bad prognosis (Bronkhorst et al., 2011). This implies that one may miss cells with monosomy 3 in uveal melanoma if they occur in low numbers. A negative test on a biopsy therefore does not exclude the presence of monosomy 3 somewhere else in the tumor.

More recently, disparate results for samples obtained from the tumor base and the apex were revealed (Schoenfield et al., 2009). This confirmed the presence of heterogeneity in uveal melanoma and illustrated the potential risk for the analysis of biopsies. An additional study showed that one in ten uveal melanomas overall and one in five melanomas that were less than 3.5 mm in thickness seemed to be heterogeneous when sampled from more than one location (Augsburger et al., 2008). Before prognostic biopsies become everyday clinical practice, procedures should be developed to minimize false negative testing (Kivelä et al., 2009).

Methylation analysis

The correlation of monosomy 3 with decreased survival and metastatic disease may imply the presence of a tumor suppressor gene at this chromosome (Prescher et al., 1996; Kilic et al., 2005). Assessment of minimally deleted regions in malignant uveal melanoma has helped to identify two susceptibility regions (Tschenstcher et al., 2001; Parella et al., 2003). Tumor suppressor genes have not been identified at these loci but proximal of the 3p25 locus, the *Bap1* (3p21.1) gene has very recently been identified as a tumor suppressor gene (Harbour et al., 2010). A tumor suppressor role was furthermore supported by germline *Bap1* mutations that predispose to mesothelioma and melanocytic malignancies (Wesner et al., 2011; Testa et al., 2011). In virtually the same region as *Bap1*, we identified the *RASSF1* (Ras association domain family 1) gene (3p21.3) as a potential tumor suppressor gene that is silenced by methylation in uveal melanoma (Maat et al., 2007).

Hypermethylation of promoter-associated CpG islands has emerged as an important epigenetic mechanism leading to the transcriptional silencing of tumor suppressor genes in cancer development, including the development of uveal melanoma (Jones and Baylin, 2002; van der Velden et al., 2001; van der Velden et al., 2003; van Dinten et al., 2005). Studies on other malignancies have identified the *RASSF1* gene, located on chromosome 3p21.3, as an important regulator of cell proliferation (Hamilton et al., 2005; Pfeifer and Dammann, 2005; Choi et al., 2006). Hypermethylation of the CpG island in the promoter region of a major alternative transcript of this gene, *RASSF1a*, occurs frequently in various carcinomas, including those of the breast, prostate, and lung, and in cutaneous malignant melanoma (Spugnardi et al., 2003; Kang et al., 2004; Yeo et al., 2005; Fukasawa et al., 2006). In **chapter 3** we performed methylation analysis of uveal melanoma cell lines, primary tumor tissue and metastases. This analysis revealed a methylated promoter region of *RASSF1a* in 50% of primary tumors. Part of the primary samples and a metastatic lesion showed methylated as well as unmethylated DNA in the same sample, indicating tumor heterogeneity. Development of metastatic disease correlated with the presence of a hypermethylated *RASSF1a* promoter region. It has been suggested that the *RASSF1a* protein acts at the level of cell cycle control and mitosis but also in apoptosis checkpoints (Shivakumar et al., 2002). Recently it was shown that ectopic expression of *RASSF1a* in uveal melanoma results in senescence while depletion of *RASSF1a* in uveal melanocytes promotes proliferation (Calipel et al., 2011). Loss of expression of *RASSF1a* in uveal melanoma due to hypermethylation and monosomy 3 may therefore promote tumor development by escaping cell cycle control and promoting mitosis.

Epigenetic regulation of *RASEF*, a potential tumor-suppressor gene in uveal melanoma

An alternative for the analysis of somatic changes in tumors is the analysis of the germline in families that display a predisposition to uveal melanoma development. While in cutaneous melanoma, familial occurrence is a well-known phenomenon; familial occurrence of uveal melanoma is quite rare. However, families presenting both cutaneous and uveal melanomas without a genetic link to the familial melanoma locus at 9p21 may represent a different syndrome. Linkage analysis in such uveal and

cutaneous melanoma families identified the 9q21 region as a locus for a potential tumor suppressor gene involved in the development of hereditary melanoma (Jönsson et al., 2005). In addition, LOH analysis in two uveal melanomas from members of the families in which linkage was identified indicated 9q21 to be the possible region for a tumor suppressor gene. The 9q21 region harbors the *RASEF* gene, which is potentially involved in the RAS pathway that is prominent in the development of melanoma (Padua et al., 1984; Rimoldi et al., 2003).

In **Chapter 4** we analyzed the *RASEF* gene for mutations and in line with the findings of Jönsson et al., we did not detect any mutations in the *RASEF* gene other than a known single nucleotide polymorphism (SNP) (Sweetser et al., 2005). Our data indicated that tumors often showed heterogeneity with regard to methylation of the *RASEF* gene. The combination of a homozygous genotype (TT) and a methylated *RASEF* gene was associated with death due to metastases. Cell lines that did not express *RASEF* contained a methylated promoter, whereas all cell lines with expression lacked this methylation, confirming epigenetic regulation. The primary uveal melanomas displayed heterogeneity for *RASEF* methylation but never reached levels above ~ 50% methylation, and most commonly only a part of the CpG's present in the promoter region was methylated. We conclude that homozygosity in combination with methylation is the mechanism that targets *RASEF* in uveal melanoma, appointing *RASEF* as a bona fide tumor suppressor that is epigenetically silenced in uveal melanoma. Allelic imbalance at this locus supports a tumor-suppressor role for *RASEF*; however, analysis of *RASEF* in proliferation, survival, and migration of uveal melanoma is needed to confirm this.

Mitogen-activated protein kinase (MAPK) pathway

Another candidate uveal melanoma gene at 9q21 is the *GNAQ* gene that was found to be mutated in almost half of the uveal melanoma (Van Raamsdonk et al., 2009). *GNAQ* is part of the heterotrimeric G-protein and represents the GTP-binding part that couples GPCR intracellular signaling. Recently also the homolog of *GNAQ*, i.e. *GNA11*, was shown to be mutated in a large portion of the uveal melanoma that did not contain a *GNAQ* mutation (van Raamsdonk et al., 2010). *GNAQ* and *GNA11* are most likely to signal via protein kinase C and will lead to activation of the RAF-MEK-ERK or mitogen-activated protein kinase (MAPK) pathway (Patel et al., 2011). The MAPK pathway plays an important role in the development of many types of cancer, as well as in melanocytic neoplasia (Davies et al., 2002; Cohen et al., 2003).

In cutaneous melanocytes, activation of the MAPK pathway has been shown to occur by a variety of mechanisms, including endocrine and autocrine growth factor stimulation and mutation of the *N-RAS* and *B-RAF* genes. In contrast to these findings, *B-RAF* mutations have been reported only rarely in uveal melanoma. A possible explanation for this apparent lack of *B-RAF* mutations is that uveal melanomas are genetically heterogeneous, and therefore mutations are not present in each cell, similar to our findings in previous studies on the heterogeneous distribution of monosomy of chromosome 3 and

methylation of *RASSF1a* (see **Chapter 2** and **Chapter 3**). To detect mutations in a background of normal DNA, we used pyrophosphorolysis-activated polymerization to investigate whether *B-RAF* mutations are indeed present in uveal melanoma and to test tumor heterogeneity. In **Chapter 5** we conclude that *B-RAF* mutations occur in uveal melanoma, although the clinical relevance of such mutations in a minor percentage of cells still has to be determined. Our data reveal that the *B-RAF* mutation frequency in uveal melanoma is higher than earlier anticipated and add to the rare reports on *B-RAF* mutations in uveal melanoma. The relative scarcity of the *B-RAF* mutation excludes an elemental role for this mutation in uveal melanoma.

Tyrosine kinase activity profiling

Fairly little is known about the molecular pathogenesis of uveal melanoma as compared to cutaneous melanoma and with the detection of the *GNAQ* and *GNA11* mutations, the question remains how these mutations stimulate proliferation. Activation of the classical MAPK pathway is seen in many types of cancer and activation of this RAS-RAF-MEK-ERK route may well be essential in stimulating cell division in uveal melanoma too. Analysis with immunohistochemistry and western blot showed activation of ERK1/2 in most uveal melanomas, supporting the idea of activation of the MAPK pathway (Rimoldi et al., 2003; Weber et al., 2003; Zuidervaart et al., 2005). Also, the pharmacological inhibition of MAPK/ERK kinases 1 and 2 (MEK1/2) and the genetic targeting of *B-RAF* with siRNA resulted in a reduced proliferation of uveal melanoma cell lines (Levefre et al., 2004; Calipel et al., 2006). This indicates that although mutations are absent in this pathway, RAS-RAF-MEK-ERK signaling is essential for uveal melanoma growth and suggests that an upstream factor is involved in autonomous uveal melanoma proliferation.

In **chapter 6** we analyzed mechanisms involved in RAS-RAF-ERK activation in cell lines with differential ERK activation. We compared a uveal melanoma cell line, obtained from a primary uveal melanoma, with two cell lines derived from two metastases from the same patient. Compared to the primary uveal melanoma cell line, the two metastatic cell lines displayed a reduced ERK1/2 activation. We were able to identify Src as a crucial upstream tyrosine kinase for ERK1/2 activation in the primary uveal melanoma. In order to validate the candidacy of Src as regulator, we applied genetic (siRNA) and pharmaceutical inhibitors of Src. A strong reduction of active ERK1 was observed in conjunction with marked growth inhibition.

Most uveal melanomas present mutations in the *GNAQ* and *GNA11* genes that are correlated with MAPK activation (van Raamsdonk et al., 2009). We identified Src as a crucial upstream tyrosine kinase for ERK1/2 activation in uveal melanoma and hence Src is expected to be involved in *GNAQ/GNA11* signaling. Reduced ERK1/2 activation in metastatic cell lines and in fresh liver metastases suggests a loss of ERK1/2 activation during uveal melanoma progression. The latter is unexpected, as ERK1/2 activation is generally associated with malignant potential in vitro. However, in endometrial and breast cancer, ERK1/2 activation has been associated with a good prognosis (Milde-Langosch et al., 2005; Mizumoto et

al., 2007). A possible explanation is provided by the observation that ERK1/2 is involved in oncogene and stress-induced senescence (Serrano et al., 1997; Stott et al., 1998). This mechanism is thought to be an important defense for cells that are at risk of neoplastic transformation and need to be circumvented by tumor cells in order to proliferate. Loss of activated ERK1/2 may not only relieve the associated inhibitory mechanisms in a direct manner but may also require alternative mitogenic signals to take over in uveal melanoma metastasis. Overall, we can conclude that Src is a differentially activated tyrosine kinase and that the Src family plays an important role in cell proliferation in primary uveal melanoma.

Future studies

Identifying the molecular pathways that are involved in proliferation of uveal melanoma may lead to effective therapies of metastases of this malignancy. The identification of mutations such as *GNAQ* and *GNA11* that may initiate tumor growth, and unraveling the molecular basis of the association between loss of chromosome 3 and metastasis formation may assist in the selection of new drugs. Furthermore, as shown in this thesis, tumors are quite often heterogeneous and not all cells from the primary tumor may react in the same way. Additionally, cells that are present in metastases may be a subclone of the primary tumor and have different characteristics.

Using material from the patient's primary tumor and metastases may be essential to determine that tumor's specificity to new treatments, leading to individual targeted therapies, to obtain long-term survival.

References

- Augsburger JJ, Corrêa ZM, Trichopoulos N, Shaikh A. Size overlap between benign melanocytic choroidal nevi and choroidal malignant melanomas. *Invest Ophthalmol Vis Sci.* 2008 Jul;49(7):2823-8.
- Bronkhorst IH, Maat W, Jordanova ES, Kroes WG, Schalijs-Delfos NE, Luyten GP, Jager MJ. Effect of heterogeneous distribution of monosomy 3 on prognosis in uveal melanoma. *Arch Pathol Lab Med.* 2011 Aug;135(8):1042-7.
- Calipel A, Lefevre G, Pouponnot C, Mouriaux F, Eychène A, Mascarelli F. Mutation of B-Raf in human choroidal melanoma cells mediates cell proliferation and transformation through the MEK/ERK pathway. *J Biol Chem.* 2003 Oct 24;278(43):42409-18.
- Calipel A, Mouriaux F, Glotin AL, Malecize F, Faussat AM, Mascarelli F. Extracellular signal-regulated kinase-dependent proliferation is mediated through the protein kinase A/B-Raf pathway in human uveal melanoma cells. *J Biol Chem.* 2006 Apr 7;281(14):9238-50.
- Calipel A, Abonnet V, Nicole O, Mascarelli F, Coupland SE, Damato B, Mouriaux F. Status of RASSF1A in Uveal Melanocytes and Melanoma Cells. *Mol Cancer Res.* 2011 Sep;9(9):1187-98.
- Choi YL, Kang SY, Shin YK, Choi JS, Kim SH, Lee SJ, Bae DS, Ahn G. Aberrant hypermethylation of RASSF1A promoter in ovarian borderline tumors and carcinomas. *Virchows Arch.* 2006 Mar;448(3):331-6.
- Cohen C, Zavala-Pompa A, Sequeira JH, Shoji M, Sexton DG, Cotsonis G, Cerimele F, Govindarajan B, Macaron N, Arbiser JL. Mitogen-activated protein kinase activation is an early event in melanoma progression. *Clin Cancer Res.* 2002 Dec;8(12):3728-33.
- Cohen Y, Goldenberg-Cohen N, Parrella P, Chowers I, Merbs SL, Pe'er J, Sidransky D. Lack of BRAF mutation in primary uveal melanoma. *Invest Ophthalmol Vis Sci.* 2003 Jul;44(7):2876-8.
- Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, Teague J, Woffendin H, Garnett MJ, Bottomley W, Davis N, Dicks E, Ewing R, Floyd Y, Gray K, Hall S, Hawes R, Hughes J, Kosmidou V, Menzies A, Mould C, Parker A, Stevens C, Watt S, Hooper S, Wilson R, Jayatilake H, Gusterson BA, Cooper C, Shipley J, Hargrave D, Pritchard-Jones K, Maitland N, Chenevix-Trench G, Riggins GJ, Bigner DD, Palmieri G, Cossu A, Flanagan A, Nicholson A, Ho JW, Leung SY, Yuen ST, Weber BL, Seigler HF, Darrow TL, Paterson H, Marais R, Marshall CJ, Wooster R, Stratton MR, Futreal PA. Mutations of the BRAF gene in human cancer. *Nature.* 2002 Jun 27;417(6892):949-54.
- Edmunds SC, Cree IA, Dí Nicolantonio F, Hungerford JL, Hurren JS, Kelsell DP. Absence of BRAF gene mutations in uveal melanomas in contrast to cutaneous melanomas. *Br J Cancer.* 2003 May 6;88(9):1403-5.
- Folberg R. The molecular classification of uveal melanocytic lesions: an important discovery in context. *J Mol Diagn.* 2010 Jul;12(4):391-3.
- Fukasawa M, Kimura M, Morita S, Matsubara K, Yamanaka S, Endo C, Sakurada A, Sato M, Kondo T, Horii A, Sasaki H, Hatada I. Microarray analysis of promoter methylation in lung cancers. *J Hum Genet.* 2006;51(4):368-74.
- Hamilton DW, Lusher ME, Lindsey JC, Ellison DW, Clifford SC. Epigenetic inactivation of the RASSF1A tumour suppressor gene in ependymoma. *Cancer Lett.* 2005 Sep 8;227(1):75-81.
- Harbour JW, Onken MD, Roberson ED, Duan S, Cao L, Worley LA, Council ML, Matatall KA, Helms C, Bowcock AM. Frequent mutation of BAP1 in metastasizing uveal melanomas. *Science.* 2010 Dec 3;330(6009):1410-3.
- Horsthemke B, Prescher G, Bornfeld N, Becher R. Loss of chromosome 3 alleles and multiplication of chromosome 8 alleles in uveal melanoma. *Genes Chromosomes Cancer.* 1992 Apr;4(3):217-21.
- Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. *Nat Rev Genet.* 2002 Jun;3(6):415-28. Review.
- Jönsson G, Bendahl PO, Sandberg T, Kurbasic A, Staaf J, Sunde L, Crüger DG, Ingvar C, Olsson H, Borg A. Mapping of a novel ocular and cutaneous malignant melanoma susceptibility locus to chromosome 9q21.32. *J Natl Cancer Inst.* 2005 Sep 21;97(18):1377-82.
- Kang GH, Lee S, Lee HJ, Hwang KS. Aberrant CpG island hypermethylation of multiple genes in prostate cancer and prostatic intraepithelial neoplasia. *J Pathol.* 2004 Feb;202(2):233-40.
- Kiliç E, Brüggewirth HT, Verbiest MM, Zwarthoff EC, Mooy NM, Luyten GP, de Klein A. The RAS-BRAF kinase pathway is not involved in uveal melanoma. *Melanoma Res.* 2004 Jun;14(3):203-5.
- Kiliç E, Naus NC, van Gils W, Klaver CC, van Til ME, Verbiest MM, Stijnen T, Mooy CM, Paridaens D, Beverloo HB, Luyten GP, de Klein A. Concurrent loss of chromosome arm 1p and chromosome 3 predicts a decreased disease-free survival in uveal melanoma patients. *Invest Ophthalmol Vis Sci.* 2005 Jul;46(7):2253-7.

- Kivelä T. Hot issues in the current management of eye cancer. *Future Oncol.* 2009 Dec;5(10):1541-4.
- Lefevre G, Glotin AL, Calipel A, Mouriaux F, Tran T, Kherrouche Z, Maurage CA, Auclair C, Mascarelli F. Roles of stem cell factor/c-Kit and effects of Glivec/STI571 in human uveal melanoma cell tumorigenesis. *J Biol Chem.* 2004 Jul 23;279(30):31769-79.
- Maat W, van der Velden PA, Out-Luiting C, Plug M, Dirks-Mulder A, Jager MJ, Gruis NA. Epigenetic inactivation of RASSF1a in uveal melanoma. *Invest Ophthalmol Vis Sci.* 2007 Feb;48(2):486-90.
- Malaponte G, Libra M, Gangemi P, Bevelacqua V, Mangano K, D'Amico F, Mazzarino MC, Stivala F, McCubrey JA, Travali S. Detection of BRAF gene mutation in primary choroidal melanoma tissue. *Cancer Biol Ther.* 2006 Feb;5(2):225-7.
- Milde-Langosch K, Bamberger AM, Rieck G, Grund D, Hemminger G, Müller V, Löning T. Expression and prognostic relevance of activated extracellular-regulated kinases (ERK1/2) in breast cancer. *Br J Cancer.* 2005 Jun 20;92(12):2206-15.
- Mizumoto Y, Kyo S, Mori N, Sakaguchi J, Ohno S, Maida Y, Hashimoto M, Takakura M, Inoue M. Activation of ERK1/2 occurs independently of KRAS or BRAF status in endometrial cancer and is associated with favorable prognosis. *Cancer Sci.* 2007 May;98(5):652-8.
- Padua RA, Barrass N, Currie GA. A novel transforming gene in a human malignant melanoma cell line. *Nature.* 1984 Oct 18-24;311(5987):671-3.
- Parrella P, Fazio VM, Gallo AP, Sidransky D, Merbs SL. Fine mapping of chromosome 3 in uveal melanoma: identification of a minimal region of deletion on chromosomal arm 3p25.1-p25.2. *Cancer Res.* 2003 Dec 1;63(23):8507-10.
- Patel M, Smyth E, Chapman PB, Wolchok JD, Schwartz GK, Abramson DH, Carvajal RD. Therapeutic implications of the emerging molecular biology of uveal melanoma. *Clin Cancer Res.* 2011 Apr 15;17(8):2087-100.
- Pfeifer GP, Dammann R. Methylation of the tumor suppressor gene RASSF1A in human tumors. *Biochemistry (Mosc).* 2005 May;70(5):576-83. Review.
- Prescher G, Bornfeld N, Friedrichs W, Seeber S, Becher R. Cytogenetics of twelve cases of uveal melanoma and patterns of nonrandom anomalies and isochromosome formation. *Cancer Genet Cytogenet.* 1995 Mar;80(1):40-6.
- Prescher G, Bornfeld N, Hirche H, Horsthemke B, Jöckel KH, Becher R. Prognostic implications of monosomy 3 in uveal melanoma. *Lancet.* 1996 May 4;347(9010):1222-5.
- Rimoldi D, Salvi S, Liénard D, Lejeune FJ, Speiser D, Zografos L, Cerottini JC. Lack of BRAF mutations in uveal melanoma. *Cancer Res.* 2003 Sep 15;63(18):5712-5.
- Schoenfield L, Pettay J, Tubbs RR, Singh AD. Variation of monosomy 3 status within uveal melanoma. *Arch Pathol Lab Med.* 2009 Aug;133(8):1219-22.
- Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell.* 1997 Mar 7;88(5):593-602.
- Shivakumar L, Minna J, Sakamaki T, Pestell R, White MA. The RASSF1A tumor suppressor blocks cell cycle progression and inhibits cyclin D1 accumulation. *Mol Cell Biol.* 2002 Jun;22(12):4309-18.
- Sisley K, Rennie IG, Cottam DW, Potter AM, Potter CW, Rees RC. Cytogenetic findings in six posterior uveal melanomas: involvement of chromosomes 3, 6, and 8. *Genes Chromosomes Cancer.* 1990 Sep;2(3):205-9.
- Spugnardi M, Tommasi S, Dammann R, Pfeifer GP, Hoon DS. Epigenetic inactivation of RAS association domain family protein 1 (RASSF1A) in malignant cutaneous melanoma. *Cancer Res.* 2003 Apr 1;63(7):1639-43.
- Stott FJ, Bates S, James MC, McConnell BB, Starborg M, Brookes S, Palmero I, Ryan K, Hara E, Vousden KH, Peters G. The alternative product from the human CDKN2A locus, p14(ARF), participates in a regulatory feedback loop with p53 and MDM2. *EMBO J.* 1998 Sep 1;17(17):5001-14.
- Sweetser DA, Peniket AJ, Haaland C, Blomberg AA, Zhang Y, Zaidi ST, Dayyani F, Zhao Z, Heerema NA, Boultonwood J, Dewald GW, Paietta E, Slovak ML, Willman CL, Wainscoat JS, Bernstein ID, Daly SB. Delineation of the minimal commonly deleted segment and identification of candidate tumor-suppressor genes in del(9q) acute myeloid leukemia. *Genes Chromosomes Cancer.* 2005 Nov;44(3):279-91.
- Testa JR, Cheung M, Pei J, Below JE, Tan Y, Sementino E, Cox NJ, Dogan AU, Pass HI, Trusa S, Hesdorffer M, Nasu M, Powers A, Rivera Z, Comertpay S, Tanji M, Gaudino G, Yang H, Carbone M. Germline BAP1 mutations predispose to malignant mesothelioma. *Nat Genet.* 2011 Aug 28;43(10):1022-5.

- Tschentscher F, Prescher G, Horsman DE, White VA, Rieder H, Anastassiou G, Schilling H, Bornfeld N, Bartz-Schmidt KU, Horsthemke B, Lohmann DR, Zeschnigk M. Partial deletions of the long and short arm of chromosome 3 point to two tumor suppressor genes in uveal melanoma. *Cancer Res.* 2001 Apr 15;61(8):3439-42.
- Van der Velden PA, Metzelaar-Blok JA, Bergman W, Monique H, Hurks H, Frants RR, Gruis NA, Jager MJ. Promoter hypermethylation: a common cause of reduced p16(INK4a) expression in uveal melanoma. *Cancer Res.* 2001 Jul 1;61(13):5303-6.
- Van der Velden PA, Zuidervaart W, Hurks MH, Pavey S, Ksander BR, Krijgsman E, Frants RR, Tensen CP, Willemze R, Jager MJ, Gruis NA. Expression profiling reveals that methylation of TIMP3 is involved in uveal melanoma development. *Int J Cancer.* 2003 Sep 10;106(4):472-9. Erratum in: *Int J Cancer.* 2004 Oct 10;111(6):982.
- Van Dinten LC, Pul N, van Nieuwpoort AF, Out CJ, Jager MJ, van den Elsen PJ. Uveal and cutaneous melanoma: shared expression characteristics of melanoma-associated antigens. *Invest Ophthalmol Vis Sci.* 2005 Jan;46(1):24-30.
- Van Raamsdonk CD, Bezrookove V, Green G, Bauer J, Gaugler L, O'Brien JM, Simpson EM, Barsh GS, Bastian BC. Frequent somatic mutations of GNAQ in uveal melanoma and blue naevi. *Nature.* 2009 Jan 29;457(7229):599-602.
- Van Raamsdonk CD, Griewank KG, Crosby MB, Garrido MC, Vemula S, Wiesner T, Obenauf AC, Wackernagel W, Green G, Bouvier N, Sozen MM, Baimukanova G, Roy R, Heguy A, Dolgalev I, Khanin R, Busam K, Speicher MR, O'Brien J, Bastian BC. Mutations in GNA11 in uveal melanoma. *N Engl J Med.* 2010 Dec 2;363(23):2191-9.
- Weber A, Hengge UR, Urbanik D, Markwart A, Mirmohammadsaegh A, Reichel MB, Wittekind C, Wiedemann P, Tannapfel A. Absence of mutations of the BRAF gene and constitutive activation of extracellular-regulated kinase in malignant melanomas of the uvea. *Lab Invest.* 2003 Dec;83(12):1771-6.
- White VA, McNeil BK, Horsman DE. Acquired homozygosity (isodisomy) of chromosome 3 in uveal melanoma. *Cancer Genet Cytogenet.* 1998 Apr 1;102(1):40-5.
- Wiesner T, Obenauf AC, Murali R, Fried I, Griewank KG, Ulz P, Windpassinger C, Wackernagel W, Loy S, Wolf I, Viale A, Lash AE, Pirun M, Socci ND, Rütten A, Palmedo G, Abramson D, Offit K, Ott A, Becker JC, Cerroni L, Kutzner H, Bastian BC, Speicher MR. Germline mutations in BAP1 predispose to melanocytic tumors. *Nat Genet.* 2011 Aug 28;43(10):1018-21.
- Yeo W, Wong WL, Wong N, Law BK, Tse GM, Zhong S. High frequency of promoter hypermethylation of RASSF1A in tumorous and non-tumorous tissue of breast cancer. *Pathology.* 2005 Apr;37(2):125-30.
- Zuidervaart W, van Nieuwpoort F, Stark M, Dijkman R, Packer L, Borgstein AM, Pavey S, van der Velden P, Out C, Jager MJ, Hayward NK, Gruis NA. Activation of the MAPK pathway is a common event in uveal melanomas although it rarely occurs through mutation of BRAF or RAS. *Br J Cancer.* 2005 Jun 6;92(11):2032-8.

Summary and general discussion

Nederlandse samenvatting (Summary in Dutch)

Nawoord (Acknowledgements)

Biography

List of publications



Introductie

Met behulp van moleculair onderzoek is het mogelijk om met een grote mate van nauwkeurigheid de prognose van patiënten met een oogmelanoom te bepalen. Ondanks deze vooruitgang is het beloop van de ziekte of de duur van de overleving van de patiënt nauwelijks verbeterd, omdat een effectieve therapie tegen uitzaaiingen of de mogelijkheid om uitzaaiingen te voorkomen, ontbreekt. Om dit te kunnen veranderen is kennis vereist over de manier waarop deze tumoren groeien en hoe ze kunnen uitzaaien ofwel metastaseren. Het doel van dit onderzoek was daarom om de mechanismen die betrokken zijn bij de ontwikkeling van een oogmelanoom beter te begrijpen.

Moleculaire markers

Monosomie 3, ofwel het verlies van één kopie van chromosoom 3 was de eerste moleculaire voorspeller van metastasering (Sisley et al., 1990; Horsthemke et al., 1992; Prescher et al., 1995; White et al., 1998). De mogelijkheid om patiënten die een hoog risico lopen om uitzaaiingen te ontwikkelen te selecteren voor frequente controle, maakt vroegdetectie van metastasen en eerdere behandeling mogelijk. Voor vroegopsporing is echter wel tumormateriaal nodig voor analyse. Voor patiënten die behandelend worden door middel van verwijdering van de oogbol is dit geen probleem, maar voor de patiënten die behandeld worden met oog-sparende therapie zoals lokale bestraling, is tumormateriaal niet voorhanden. Een alternatieve manier om toch tumorweefsel in handen te krijgen en een uitspraak over risico of prognose te kunnen doen, is door middel van het nemen van een biopt met een dunne naald (FNAB; Fine Needle Aspiration Biopsy).

Deze aanpak berust op de aanname dat oogmelanomen een genetisch homogene eenheid vormen. In **hoofdstuk 2** van dit proefschrift hebben we deze aanname onderzocht en aangetoond dat oogmelanomen ook heterogeen kunnen zijn wat betreft de verdeling van chromosoom 3: In het ene gebied kan een kopie verloren zijn gegaan, terwijl dat in het ander gebied of gedeelte van de tumor niet het geval is. Sinds deze bevinding zijn er meerdere studies geweest die deze heterogeniteit bevestigd hebben en een follow-up studie heeft laten zien dat de aanwezigheid van cellen met maar één kopie van chromosoom 3, hoe beperkt dat aantal ook kan zijn, toch geassocieerd wordt met een slechte prognose (Bronkhorst et al., 2011). Dit betekent dat je deze cellen kunt missen en dat een negatieve testuitslag van een biopt de aanwezigheid van deze cellen in de tumor, niet uitsluit. Voordat deze zogenaamde prognostische biopten in de praktijk kunnen worden aangeboden zullen er eerst goede manieren moeten worden gevonden om fouten te minimaliseren (Kivelä et al., 2009).

Methylatie onderzoek

De correlatie tussen monosomie 3 en de beperkte overleving door uitzaaiingen, kan betekenen dat er zich op chromosoom 3 een tumor-suppressor gen bevindt (Prescher et al., 1996; Kilic et al., 2005). Twee gebieden werden hiervoor aangewezen, maar er werden geen tumor-suppressor genen geïdentificeerd

(Tschentscher et al., 2001; Parella et al., 2003). Net naast één van deze gebieden werd recent het Bap1 (3p21.1) gen ontdekt (Harbour et al., 2010). In ongeveer dezelfde regio identificeerden wij het *RASSF1* gen (Ras association domain family 1), dat uitgeschakeld wordt door middel van methylatie (Maat et al., 2007). Hypermethylatie van CpG eilanden in de promotorregio van tumor suppressor genen en onderdrukking van expressie heeft zich ontwikkeld tot een belangrijk epigenetisch mechanisme in de ontwikkeling van kanker dat ook in oogmelanomen is beschreven (van der Velden et al., 2001; Jones and Baylin, 2002; van der Velden et al., 2003; van Dinten et al., 2005).

Hypermethylatie van CpG eilanden in de promotor regio van een belangrijk alternatief transcript van dit gen, *RASSF1a*, wordt in verschillende carcinomen gevonden (Spugnardi et al., 2003; Kang et al., 2004; Yeo et al., 2005; Fukasawa et al., 2006). In **hoofdstuk 3** wordt aangetoond dat een gemethyleerd *RASSF1a* in 50% van de primaire tumoren voorkomt en dat dit correleert met de ontwikkeling van uitzaaiingen. Een deel van de primaire monsters en een uitgezaaide laesie vertonen tumor heterogeniteit en presenteren zowel ongemethyleerd, als gemethyleerd DNA in hetzelfde monster. Er zijn aanwijzingen dat het *RASSF1a* eiwit de celcyclus controleert en dat verlies dientengevolge de celdeling kan bevorderen (Shivakumar et al., 2002; Hamilton et al., 2005; Pfeifer and Damman, 2005; Choi et al., 2006; Calipel et al., 2011).

Epigenetische regulatie van *RASEF*

Een alternatief voor de analyse van somatische veranderingen in tumoren, is de analyse van het erfelijk materiaal in gezinnen die een predispositie voor oogmelanomen hebben. Terwijl in huidmelanomen het familiair voorkomen een bekend fenomeen is, is het familiair voorkomen van oogmelanomen vrij zeldzaam. Er bestaan echter families waar zowel huid- en oogmelanomen voorkomen, die niet in verband staan met het familiair melanoom locus op 9p21. Linkage-analyse in deze oog- en huid-melanoom families wijzen de 9q21 regio aan als een locus voor een mogelijke tumor suppressor gen dat betrokken is bij de ontwikkeling van het erfelijke melanoom. Daarnaast identificeert heterozygotie (LOH) analyse in twee oogmelanomen, de 9q21 regio als mogelijke omgeving voor een tumor suppressor gen (Jönsson et al., 2005).

Deze regio herbergt het *RASEF*-gen, dat mogelijk betrokken is in de *RAS*-pathway, die prominent aanwezig is in de ontwikkeling van melanomen (Padua et al., 1984; Rimoldi et al., 2003). In **hoofdstuk 4** hebben we het *RASEF* gen onderzocht voor mutaties en in overeenstemming met de bevindingen van Jönsson et al., konden wij geen mutaties detecteren in het gen, anders dan een bekend polymorfisme (Jönsson et al., 2005; Sweetser et al., 2005). Epigenetische regulatie was echter aanwezig en cellijnen zonder *RASEF* expressie bleken een gemethyleerd gen te bevatten.

Onze resultaten met tumorweefsel gaven aan dat tumoren vaak heterogeen zijn met betrekking tot methylatie van het *RASEF* gen. Integratie van genetische en epigenetische analyse toonde aan dat de combinatie van een homozygoot genotype en een gemethyleerd *RASEF* gen, geassocieerd

was met overlijden als gevolg van uitzaaiingen. Wij hypothetiseren dat in oogmelanomen verlies van een allel, in combinatie met methylering, het mechanisme is dat *RASEF* kan uitschakelen. Allelische imbalance op dit locus ondersteunt een tumor-suppressor rol voor *RASEF*, maar verdere analyse van *RASEF* in zowel proliferatie, overleving en metastasering is nodig om dit te bevestigen.

Mitogen-activated protein kinase (MAPK) route

Een ander kandidaat melanoom-gen op 9q21 is het *GNAQ* gen, dat in bijna de helft van de oogmelanomen gemuteerd is (Van Raamsdonk et al., 2009). *GNAQ* is onderdeel van een complex eiwit dat mede zorg draagt voor intracellulaire signalering. Recent bleek dat ook de homoloog van *GNAQ*, *GNA11* genaamd, een mutatie bevat in de oogmelanomen die geen *GNAQ* mutatie bezitten (van Raamsdonk et al., 2010). *GNAQ* en *GNA11* signaleren via Protein-kinase C (PKC) en dit zou kunnen resulteren in activatie van de MAPK (mitogen-activated protein kinase) route (Patel et al., 2011). De MAPK route speelt een belangrijke rol in de ontwikkeling van vele vormen van kanker en dit geldt ook voor de tumoren die afstammen van melanocyten (Davies et al., 2002; Cohen et al., 2003).

In melanocyten van de huid vindt activatie van de MAPK route plaats door een verscheidenheid van mechanismen, waaronder stimulatie door groeifactoren en mutatie van de *N-RAS* en *B-RAF* genen. Mutaties in *B-RAF* die kenmerkend zijn voor huidmelanomen worden slechts zelden aangetroffen in oogmelanomen (Calipel et al., 2003; Kiliç et al., 2004; Zuidervaart et al., 2005; Calipel et al., 2006). Een mogelijke verklaring voor het schijnbare gebrek aan *B-RAF* mutaties is dat oogmelanomen genetisch heterogeen zijn en de mutaties waarschijnlijk niet in elke cel aanwezig zijn. Dit is vergelijkbaar met onze bevindingen in eerdere onderzoeken naar de heterogene verdeling van monosomie van chromosoom 3 en methylatie status van het *RASSF1a* gen (hoofdstuk 2 en hoofdstuk 3). Om te onderzoeken of *B-RAF* mutaties, eventueel in een achtergrond van normaal DNA, inderdaad aanwezig zijn in oogmelanomen en om de heterogeniteit van de tumor te onderzoeken, gebruikten we een zeer gevoelige mutatie specifieke PCR (pyrophosphorolysis-geactiveerde polymerisatie; PAP). In **hoofdstuk 5** concluderen we dat *B-RAF* mutaties, frequenter dan aangenomen, voorkomen in oogmelanomen. Hoewel de klinische relevantie van deze *B-RAF* mutaties in een klein percentage van de tumorcellen nog nader moet worden bepaald, lijkt de relatieve schaarste van *B-RAF* mutatie bevattende cellen, een belangrijke rol voor deze mutatie in oogmelanomen uit te sluiten.

Tyrosine kinase-activiteit

In vergelijking met het huidmelanoom is er vrij weinig bekend over de moleculaire pathogenese van oogmelanomen. Ook met de detectie van *GNAQ* en *GNA11* mutaties, blijft het de vraag hoe deze mutaties de proliferatie precies stimuleren. Activatie van de klassieke MAPK route is te zien in vele soorten van kanker en activatie van deze route is vermoedelijk van essentieel belang bij de stimulatie van de celdeling in het oogmelanoom (Rimoldi et al., 2003; Weber et al., 2003; Zuidervaart et al., 2005). Remming van de MAPK route met farmacologische en genetische middelen resulteerde in een

verminderde proliferatie van oogmelanoom-cellijnen (Levefre et al., 2004; Calipel et al., 2006). Dit geeft aan dat, hoewel mutaties in deze route afwezig zijn, MAPK signalering essentieel is voor groei van het oogmelanoom en suggereert tevens dat een in de route hoger gelegen factor betrokken is bij de proliferatie van deze tumoren.

In **hoofdstuk 6** analyseerden we het mechanisme dat betrokken is bij MAPK-activering in oogmelanoom cellijnen. We vergeleken een oogmelanoom-celijn verkregen uit een primair oogmelanoom met twee cellijnen afkomstig van twee uitzaaiingen van dezelfde patiënt. In vergelijking met de primaire oogmelanoom-celijn, vertoonden de twee gemetastaseerde cellijnen een verminderde MAPK activiteit. We waren in staat om Src te identificeren als een cruciale hoger gelegen tyrosine kinase betrokken bij MAPK activatie in het primaire oogmelanoom. Om de bevindingen te valideren, pasten we genetische remming (siRNA) en farmaceutische remming van Src toe. Een sterke vermindering van MAPK activatie en celgroei werden waargenomen en bevestigden daarmee de rol van Src in oogmelanomen.

Verminderde MAPK activatie in gemetastaseerde cellijnen en in verse levermetastasen duidt op een verlies van MAPK activatie bij de progressie van het oogmelanoom. Dit laatste is onverwacht, omdat MAPK activatie over het algemeen geassocieerd is met het maligne karakter van tumorcellen. Echter, in sommige tumoren zoals endometrium- en borstkanker, wordt MAPK activatie ook in verband gebracht met een goede prognose (Milde-Langosch et al., 2005; Mizumoto et al., 2007). Een mogelijke verklaring wordt geleverd door de observatie dat de MAPK route betrokken is bij oncogen en stress-geïnduceerde cel-inactivatie (senescence) (Serrano et al., 1997; Stott et al., 1998). De gedachte is, dat dit mechanisme een belangrijk verdedigingsmechanisme is voor cellen die risico lopen om te ontaarden, dat omzeild moet worden door de tumorcellen om te kunnen vermenigvuldigen. Verlies van een geactiveerde MAPK route in metastasecellen zoals in dit proefschrift beschreven, kan daardoor ook voordelen voor de tumorcel opleveren en uiteindelijk metastasering bevorderen.

Toekomst

De identificatie van de moleculaire pathways die betrokken zijn bij de ontwikkeling van het oogmelanoom, kunnen in de toekomst leiden tot effectieve therapieën voor het gemetastaseerde oogmelanoom. De identificatie van mutaties, zoals *GNAQ* en *GNA11*, die invloed hebben op de groei van de tumor en het ontrafelen van de moleculaire basis van het verband tussen het verlies van chromosoom 3 en metastasering, kan helpen bij de zoektocht naar nieuwe geneesmiddelen. Zoals weergegeven in dit proefschrift, zijn tumoren vaak heterogeen en zullen niet alle cellen van de tumor op dezelfde manier reageren. Zo kunnen cellen in metastasen een subkloon zijn van de primaire tumor en daarom verschillende karakteristieken hebben. Onderzoek met behulp van materiaal van de primaire tumor van patiënt en diens metastasen zal essentieel zijn om nieuwe therapieën te ontwikkelen, hetgeen kan leiden tot individueel gerichte therapie om lange-termijn overleving mogelijk te verbeteren.

References

- Bronkhorst IH, Maat W, Jordanova ES, Kroes WG, Schalijs-Delfos NE, Luyten GP, Jager MJ. Effect of heterogeneous distribution of monosomy 3 on prognosis in uveal melanoma. *Arch Pathol Lab Med*. 2011 Aug;135(8):1042-7.
- Calipel A, Lefevre G, Poupponnot C, Mouriaux F, Eychène A, Mascarelli F. Mutation of B-Raf in human choroidal melanoma cells mediates cell proliferation and transformation through the MEK/ERK pathway. *J Biol Chem*. 2003 Oct 24;278(43):42409-18.
- Calipel A, Mouriaux F, Glotin AL, Malecize F, Faussat AM, Mascarelli F. Extracellular signal-regulated kinase-dependent proliferation is mediated through the protein kinase A/B-Raf pathway in human uveal melanoma cells. *J Biol Chem*. 2006 Apr 7;281(14):9238-50.
- Calipel A, Abonnet V, Nicole O, Mascarelli F, Coupland SE, Damato B, Mouriaux F. Status of RASSF1A in Uveal Melanocytes and Melanoma Cells. *Mol Cancer Res*. 2011 Sep;9(9):1187-98.
- Choi YL, Kang SY, Shin YK, Choi JS, Kim SH, Lee SJ, Bae DS, Ahn G. Aberrant hypermethylation of RASSF1A promoter in ovarian borderline tumors and carcinomas. *Virchows Arch*. 2006 Mar;448(3):331-6.
- Cohen Y, Goldenberg-Cohen N, Parrella P, Chowers I, Merbs SL, Pe'er J, Sidransky D. Lack of BRAF mutation in primary uveal melanoma. *Invest Ophthalmol Vis Sci*. 2003 Jul;44(7):2876-8.
- Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, Teague J, Woffendin H, Garnett MJ, Bottomley W, Davis N, Dicks E, Ewing R, Floyd Y, Gray K, Hall S, Hawes R, Hughes J, Kosmidou V, Menzies A, Mould C, Parker A, Stevens C, Watt S, Hooper S, Wilson R, Jayatilake H, Gusterson BA, Cooper C, Shipley J, Hargrave D, Pritchard-Jones K, Maitland N, Chenevix-Trench G, Riggins GJ, Bigner DD, Palmieri G, Cossu A, Flanagan A, Nicholson A, Ho JW, Leung SY, Yuen ST, Weber BL, Seigler HF, Darrow TL, Paterson H, Marais R, Marshall CJ, Wooster R, Stratton MR, Futreal PA. Mutations of the BRAF gene in human cancer. *Nature*. 2002 Jun 27;417(6892):949-54.
- Fukasawa M, Kimura M, Morita S, Matsubara K, Yamanaka S, Endo C, Sakurada A, Sato M, Kondo T, Horii A, Sasaki H, Hatada I. Microarray analysis of promoter methylation in lung cancers. *J Hum Genet*. 2006;51(4):368-74.
- Hamilton DW, Lusher ME, Lindsey JC, Ellison DW, Clifford SC. Epigenetic inactivation of the RASSF1A tumour suppressor gene in ependymoma. *Cancer Lett*. 2005 Sep 8;227(1):75-81.
- Harbour JW, Onken MD, Roberson ED, Duan S, Cao L, Worley LA, Council ML, Matattall KA, Helms C, Bowcock AM. Frequent mutation of BAP1 in metastasizing uveal melanomas. *Science*. 2010 Dec 3;330(6009):1410-3.
- Horsthemke B, Prescher G, Bornfeld N, Becher R. Loss of chromosome 3 alleles and multiplication of chromosome 8 alleles in uveal melanoma. *Genes Chromosomes Cancer*. 1992 Apr;4(3):217-21.
- Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. *Nat Rev Genet*. 2002 Jun;3(6):415-28. Review.
- Jönsson G, Bendahl PO, Sandberg T, Kurbasic A, Staaf J, Sunde L, Crüger DG, Ingvar C, Olsson H, Borg A. Mapping of a novel ocular and cutaneous malignant melanoma susceptibility locus to chromosome 9q21.32. *J Natl Cancer Inst*. 2005 Sep 21;97(18):1377-82.
- Kang GH, Lee S, Lee HJ, Hwang KS. Aberrant CpG island hypermethylation of multiple genes in prostate cancer and prostatic intraepithelial neoplasia. *J Pathol*. 2004 Feb;202(2):233-40.
- Kiliç E, Brüggewirth HT, Verbiest MM, Zwarthoff EC, Mooy NM, Luyten GP, de Klein A. The RAS-BRAF kinase pathway is not involved in uveal melanoma. *Melanoma Res*. 2004 Jun;14(3):203-5.
- Kiliç E, Naus NC, van Gils W, Klaver CC, van Til ME, Verbiest MM, Stijnen T, Mooy CM, Paridaens D, Beverloo HB, Luyten GP, de Klein A. Concurrent loss of chromosome arm 1p and chromosome 3 predicts a decreased disease-free survival in uveal melanoma patients. *Invest Ophthalmol Vis Sci*. 2005 Jul;46(7):2253-7.
- Kivelä T. Hot issues in the current management of eye cancer. *Future Oncol*. 2009 Dec;5(10):1541-4.
- Lefevre G, Glotin AL, Calipel A, Mouriaux F, Tran T, Kherrouche Z, Maurage CA, Auclair C, Mascarelli F. Roles of stem cell factor/c-Kit and effects of Glivec/STI571 in human uveal melanoma cell tumorigenesis. *J Biol Chem*. 2004 Jul 23;279(30):31769-79.
- Maat W, van der Velden PA, Out-Luiting C, Plug M, Dirks-Mulder A, Jager MJ, Gruis NA. Epigenetic inactivation of RASSF1a in uveal melanoma. *Invest Ophthalmol Vis Sci*. 2007 Feb;48(2):486-90.
- Milde-Langosch K, Bamberger AM, Rieck G, Grund D, Hemminger G, Müller V, Löning T. Expression and prognostic relevance of activated extracellular-regulated kinases (ERK1/2) in breast cancer. *Br J Cancer*. 2005 Jun 20;92(12):2206-15.
- Mizumoto Y, Kyo S, Mori N, Sakaguchi J, Ohno S, Maida Y, Hashimoto M, Takakura M, Inoue M. Activation of ERK1/2 occurs independently of KRAS or BRAF status in endometrial cancer and is associated with favorable prognosis. *Cancer Sci*. 2007 May;98(5):652-8.

- Padua RA, Barrass N, Currie GA. A novel transforming gene in a human malignant melanoma cell line. *Nature*. 1984 Oct 18;243(5987):671-3.
- Parrella P, Fazio VM, Gallo AP, Sidransky D, Merbs SL. Fine mapping of chromosome 3 in uveal melanoma: identification of a minimal region of deletion on chromosomal arm 3p25.1-p25.2. *Cancer Res*. 2003 Dec 1;63(23):8507-10.
- Patel M, Smyth E, Chapman PB, Wolchok JD, Schwartz GK, Abramson DH, Carvajal RD. Therapeutic implications of the emerging molecular biology of uveal melanoma. *Clin Cancer Res*. 2011 Apr 15;17(8):2087-100.
- Pfeifer GP, Dammann R. Methylation of the tumor suppressor gene RASSF1A in human tumors. *Biochemistry (Mosc)*. 2005 May;70(5):576-83. Review.
- Prescher G, Bornfeld N, Friedrichs W, Seeber S, Becher R. Cytogenetics of twelve cases of uveal melanoma and patterns of nonrandom anomalies and isochromosome formation. *Cancer Genet Cytogenet*. 1995 Mar;80(1):40-6.
- Prescher G, Bornfeld N, Hirche H, Horsthemke B, Jöckel KH, Becher R. Prognostic implications of monosomy 3 in uveal melanoma. *Lancet*. 1996 May 4;347(9010):1222-5.
- Rimoldi D, Salvi S, Liénard D, Lejeune FJ, Speiser D, Zografos L, Cerottini JC. Lack of BRAF mutations in uveal melanoma. *Cancer Res*. 2003 Sep 15;63(18):5712-5.
- Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell*. 1997 Mar 7;88(5):593-602.
- Sisley K, Rennie IG, Cottam DW, Potter AM, Potter CW, Rees RC. Cytogenetic findings in six posterior uveal melanomas: involvement of chromosomes 3, 6, and 8. *Genes Chromosomes Cancer*. 1990 Sep;2(3):205-9.
- Spugnardi M, Tommasi S, Dammann R, Pfeifer GP, Hoon DS. Epigenetic inactivation of RAS association domain family protein 1 (RASSF1A) in malignant cutaneous melanoma. *Cancer Res*. 2003 Apr 1;63(7):1639-43.
- Stott FJ, Bates S, James MC, McConnell BB, Starborg M, Brookes S, Palmero I, Ryan K, Hara E, Vousden KH, Peters G. The alternative product from the human CDKN2A locus, p14(ARF), participates in a regulatory feedback loop with p53 and MDM2. *EMBO J*. 1998 Sep 1;17(17):5001-14.
- Sweetser DA, Peniket AJ, Haaland C, Blomberg AA, Zhang Y, Zaidi ST, Dayyani F, Zhao Z, Heerema NA, Boultonwood J, Dewald GW, Paietta E, Slovak ML, Willman CL, Wainscoat JS, Bernstein ID, Daly SB. Delineation of the minimal commonly deleted segment and identification of candidate tumor-suppressor genes in del(9q) acute myeloid leukemia. *Genes Chromosomes Cancer*. 2005 Nov;44(3):279-91.
- Tschentscher F, Prescher G, Horsman DE, White VA, Rieder H, Anastassiou G, Schilling H, Bornfeld N, Bartz-Schmidt KU, Horsthemke B, Lohmann DR, Zeschknig M. Partial deletions of the long and short arm of chromosome 3 point to two tumor suppressor genes in uveal melanoma. *Cancer Res*. 2001 Apr 15;61(8):3439-42.
- Van der Velden PA, Metzelaar-Blok JA, Bergman W, Monique H, Hurks H, Frants RR, Gruis NA, Jager MJ. Promoter hypermethylation: a common cause of reduced p16(INK4a) expression in uveal melanoma. *Cancer Res*. 2001 Jul 1;61(13):5303-6.
- Van der Velden PA, Zuidervaart W, Hurks MH, Pavey S, Ksander BR, Krijgsman E, Frants RR, Tensen CP, Willemze R, Jager MJ, Gruis NA. Expression profiling reveals that methylation of TIMP3 is involved in uveal melanoma development. *Int J Cancer*. 2003 Sep 10;106(4):472-9. Erratum in: *Int J Cancer*. 2004 Oct 10;111(6):982.
- Van Dinten LC, Pul N, van Nieuwpoort AF, Out CJ, Jager MJ, van den Elsen PJ. Uveal and cutaneous melanoma: shared expression characteristics of melanoma-associated antigens. *Invest Ophthalmol Vis Sci*. 2005 Jan;46(1):24-30.
- Van Raamsdonk CD, Griewank KG, Crosby MB, Garrido MC, Vemula S, Wiesner T, Obenaus AC, Wackernagel W, Green G, Bouvier N, Sozen MM, Baimukanova G, Roy R, Heguy A, Dolgalev I, Khanin R, Busam K, Speicher MR, O'Brien J, Bastian BC. Mutations in GNA11 in uveal melanoma. *N Engl J Med*. 2010 Dec 2;363(23):2191-9.
- Weber A, Hengge UR, Urbanik D, Markwart A, Mirmohammadsaegh A, Reichel MB, Wittekind C, Wiedemann P, Tannapfel A. Absence of mutations of the BRAF gene and constitutive activation of extracellular-regulated kinase in malignant melanomas of the uvea. *Lab Invest*. 2003 Dec;83(12):1771-6.
- White VA, McNeil BK, Horsman DE. Acquired homozygosity (isodisomy) of chromosome 3 in uveal melanoma. *Cancer Genet Cytogenet*. 1998 Apr 1;102(1):40-5.
- Yeo W, Wong WL, Wong N, Law BK, Tse GM, Zhong S. High frequency of promoter hypermethylation of RASSF1A in tumorous and non-tumorous tissue of breast cancer. *Pathology*. 2005 Apr;37(2):125-30.
- Zuidervaart W, van Nieuwpoort F, Stark M, Dijkman R, Packer L, Borgstein AM, Pavey S, van der Velden P, Out C, Jager MJ, Hayward NK, Gruis NA. Activation of the MAPK pathway is a common event in uveal melanomas although it rarely occurs through mutation of BRAF or RAS. *Br J Cancer*. 2005 Jun 6;92(11):2032-8.

Summary and general discussion

Nederlandse samenvatting (Summary in Dutch)

Nawoord (Acknowledgements)

Biography

List of publications



Nawoord

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Biography

Willem Maat werd op 3 november 1978 geboren te Gorinchem. Na het behalen van het eindexamen VWO op het Walburgcollege in Zwijndrecht ging hij, na uitgeloot te zijn voor de studie geneeskunde, in 1997 biologie studeren aan de Universiteit van Leiden.

In 1998 begon hij zijn studie geneeskunde aan de Erasmus Universiteit in Rotterdam. Reeds in het 2^e jaar werd bij het keuzeblok "refractiechirurgie" de interesse voor de oogheelkunde gewekt, wat gevolgd werd door de reguliere en keuze-coschappen aan diezelfde afdeling aan het einde van de opleiding (begeleider Dr. G.P.M. Luyten).

In 2004 behaalde hij het artsexamen en begon kort daarna met onderzoek aan de Afdeling Oogheelkunde van het Leids Universitair Medisch Centrum in Leiden. Al snel kwam hij in contact met Dr. Ing. P.A. van der Velden van de afdeling Dermatologie, waarna een vruchtbare samenwerking begon. Gedurende de periode oktober 2004 tot september 2006 werden meerdere studies verricht die onder andere beschreven zijn in dit proefschrift.

In oktober 2006 begon hij aan de opleiding tot oogarts (opleider Prof. C.C. Sterk, later gevolgd door Prof. Dr. G.P.M. Luyten) waarin het onderzoek werd afgerond. Sinds februari 2012 is hij verbonden als oogarts aan het Maasstad Ziekenhuis Rotterdam. Samen met Annemieke Boxma heeft hij twee prachtige kinderen: Thijs en Suuze.

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Nederlandse samenvatting (Summary in Dutch)

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List of Publications

A comparison of HLA genotype with inflammation in uveal melanoma.

Van Essen TH, Bronkhorst IH, [Maat W](#), Verduyn W, Roelen DL, Luyten GP, Jager MJ
Invest Ophthalmol Vis Sci. 2012 May;53(6):2640-6

Effect of heterogeneous distribution of monosomy 3 on prognosis in uveal melanoma.

Bronkhorst IH, [Maat W](#), Jordanova ES, Kroes WG, Schalijs-Delfos NE, Luyten GP, Jager MJ
Arch Pathol Lab Med. 2011 Aug;135(8):1042-7

Regulation of VEGF-A in uveal melanoma.

el Filali M, Missotten GS, [Maat W](#), Ly LV, Luyten GP, van der Velden PA, Jager MJ
Invest Ophthalmol Vis Sci. 2010 May;51(5):2329-37.

Episodic Src activation in uveal melanoma revealed by kinase activity profiling.

[Maat W](#), el Filali M, Dirks-Mulder A, Luyten GP, Gruis NA, Desjardins L, Boender P, Jager MJ, van der Velden PA
Br J Cancer. 2009 Jul 21;101(2):312-9.

Evidence for Natural Killer cell mediated protection from metastasis formation in uveal melanoma patients.

[Maat W](#), van der Slik AR, Verhoeven DH, Alizadeh BZ, Ly LV, Verduijn W, Luyten GPM, Mulder A, Jager MJ, van Bergen J
Invest Ophthalmol Vis Sci. 2009 Feb;50(6):288-95.

Methylation in uveal melanoma.

Van der Velden PA, [Maat W](#)
Br J Ophthalmol. 2009 Jan;93(1):132.

Epigenetic regulation identifies RASEF as a tumor-suppressor gene in uveal melanoma.

[Maat W](#), Beiboer SH, Jager MJ, Luyten GP, Gruis NA, van der Velden PA
Invest Ophthalmol Vis Sci. 2008 Apr;49(4):1291-8.

Triamcinolone acetonide and anecortave acetate do not stimulate uveal melanoma cell growth.

El Filali M, Homminga I, [Maat W](#), van der Velden PA, Jager MJ
Mol Vis. 2008 Sep 24;14:1752-9.

Monosomy of chromosome 3 and an inflammatory phenotype occur together in uveal melanoma.

Maat W, Ly LV, Jordanova ES, de Wolff-Rouendaal D, Schalij- Delfos NE, Jager MJ
Invest Ophthalmol Vis Sci. 2008 Feb;49(2):505-10.

Pyrophosphorolysis detects B-RAF mutations in primary uveal melanoma.

Maat W, Kilic E, Luyten GPM, Gruis NA, Jager MJ, van der Velden PA
Invest Ophthalmol Vis Sci. 2008 Jan;49(1):23-7.

Retained intraocular foreign body masquerading malignant melanoma of the iris.

Maat W, Missotten GS, Luyten GPM, de Keizer RJW
Graefes Arch Clin Exp Ophthalmol. 2008 Apr;246(4):623-4.

Expression of Wnt5a and its downstream effector B-catenin are associated with poorer survival in uveal melanoma.

Zuidervaart W, Pavey S, van Nieuwpoort FA, Packer L, Out C, Maat W, Jager MJ, Gruis NA, Hayward NK
Melanoma Res. 2007 Dec;17(6):380-6.

A new cell line from a recurrent conjunctival melanoma.

Keijser S, Maat W, Missotten GS, de Keizer RJW
Br J Ophthalmol. 2007 Nov;91(11):1566-7.

Epigenetic inactivation of RASSF1a in uveal melanoma.

Maat W, van der Velden PA, Plug MJ, Dirks- Mulder A, Out- Luiting C, Jager MJ, Gruis NA
Invest Ophthalmol Vis Sci. 2007 Feb;48(2):486-90.

The Heterogeneous Distribution of Monosomy 3 in Uveal Melanomas: Implications for Prognostication Based on Fine Needle Aspiration Biopsies.

Maat W, Jordanova ES, van Zelderen- Bhola SL, Wessels HW, Schalij- Delfos NE, Jager MJ
Arch Pathol Lab Med. 2007 Jan;131(1):91-6.

HLA class I and II genotype in uveal melanoma: relation to occurrence and prognosis.

Maat W, Haasnoot GW, Claas FHJ, Schalij- Delfos NE, Schreuder GMT, Jager MJ
Invest Ophthalmol Vis Sci. 2006;47(1):3-6.

Inflammation and atherosclerosis: mechanisms underlying vulnerable plaque.

Krams R, Segers D, Gourabi BM, Maat W, Cheng C, van Pelt C, van Damme LC, de Feyter P, de Korte CL, Serruys PW
J Interv Cardiol. 2003 Apr;16(2):107-13.

