Genetic Prognostic Factors and Follow-up in Uveal Melanoma

Thomas van den Bosch

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Genetic Prognostic Factors and Follow-up in Uveal Melanoma

Genetisch prognostische factoren en follow-up in uveamelanoom

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Contents

Aims and scope of this thesis

An important part of oncological research is to identify prognostic factors and predict which patients are at risk for (early) metastasis. This thesis aims to describe the known genetic alterations in uveal melanoma and define new chromosomal regions and markers involved with (micro-) metastasis and the response to local therapy. In **chapter 1** the current knowledge of clinical and molecular genetic aspects of uveal melanoma is reviewed. The similarities and dissimilarities regarding the genetic background and genetic differences between uveal melanoma and cutaneous melanoma are discussed in **chapter 2.** The following chapters, **chapter 3-8**, describe the cytogenetic and molecular genetic research regarding prognostic factors and follow-up in primary uveal melanoma samples and cell lines.

In the final **chapter 9,** a general discussion including an overview of our results and recent techniques and developments in the ocular oncology field is presented.


Genetic
factors
patterns
therapy
BAP1
enucleation
chromosomal
prominence
pathology
MLPA
tumor
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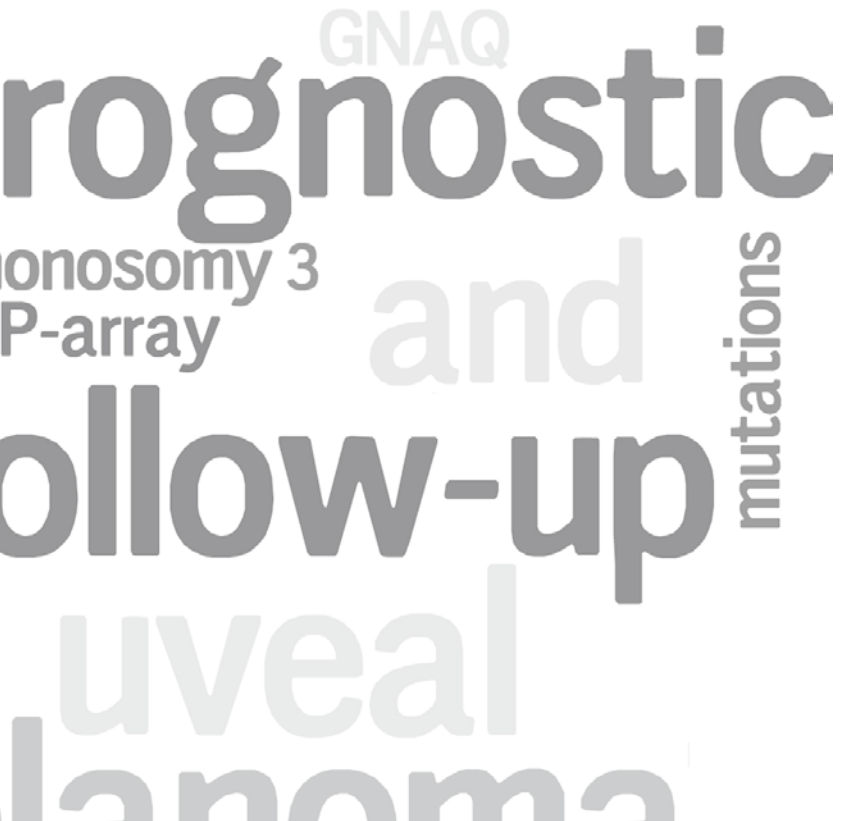
Part 1.

Genetic aberrations in melanocytic malignancies



Chapter 1

Genetics of uveal melanoma

T. van den Bosch, J. van Beek, E. Kiliç, N.C. Naus, D. Paridaens, A. de Klein

Advances in Malignant Melanoma - Clinical and Research Perspectives, Armstrong AW (Ed.), ISBN: 978-953-307-575-4, InTech, online open access

Introduction

Uveal melanoma is the most common type of primary eye cancer in adults, affecting 0.7/100,000 of the Western population yearly (Egan et al., 1988). The melanoma originates from neural crest derived melanocytes of the uvea (choroid, ciliary body and iris) and despite enucleation or conservative treatment, half of all patients die of, most often late appearing, metastatic disease (15-year survival: 53%) (Diener-West et al., 1992; Gamel et al., 1993; Kujala et al., 2003). To detect the (micro) metastasizing cells in an early phase is one of the main challenges in the (uveal melanoma) oncology field and a prerequisite for proper patient selection in future therapeutic interventions. Several clinical, histological and genetic markers have been identified to predict poor prognosis in uveal melanoma patients and genetic markers as chromosome 3 loss or the expression of a specific set of genes have proven to be far out the most significant. This will not only facilitate diagnosis and prediction of prognosis but will also assist in selecting patients for adjuvant therapy and the monitoring of circulating tumor cells. Alternatively, some of the tumor markers as *GNAQ/GNA11*, or *BAP1* may serve as targets for new types of intervention tackling that specific pathway.

In this chapter, the most recent cytogenetic and molecular genetic approaches will be discussed along with the most important findings and their attribution to current and future management of patients with uveal melanoma.

Clinical aspects of uveal melanoma

Diagnosis

The diagnosis of uveal melanoma is based on ophthalmic examination using ancillary tests (ultrasonography, transillumination, optical coherence tomography, occasionally fluorescein angiography, computed tomography, magnetic resonance imaging, and photography for follow-up) (Figure 1). Approximately 30% of patients have no symptoms at time of diagnosis (Damato 2010). Upon diagnosis of the primary tumor, patients are screened for metastases by liver enzyme tests and liver ultrasound and at that moment, less than 2% patients have detectable metastases (Shields, J. A. et al., 1991).

The primary uveal melanoma is located either in the choroid (72%), in the ciliary body (23%) or in the iris (5%). Choroidal melanomas usually present as a discoid, dome-shaped or mushroom-shaped subretinal mass, whereas ciliary body melanomas regularly present as sessile or dome-shaped lesions. Iris melanomas may also present as dome-shaped lesions or diffuse lesions and are the least common type of uveal melanoma. Iris melanomas tend to present at a smaller size, probably because pigmented lesions of the iris are usually visible to the patient at an early stage, which adds to a favorable prognosis. Iris melanomas may cause blockage of the drainage angle and lead to secondary elevation of intraocular

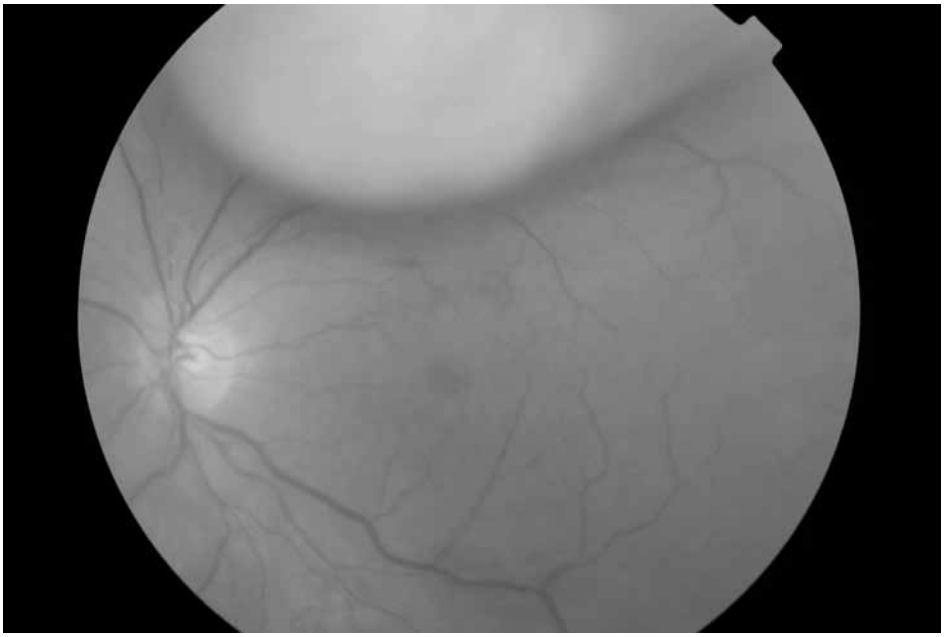


Figure 1: Fundus photography showing a superiorly located uveal melanoma of the left eye
(color page 186)

pressure (Shields, C. L. et al., 2001). In contrast to iris melanomas, melanomas located in the ciliary body are associated with a high metastatic potential (Schmittel et al., 2004).

If enucleation or biopsy is performed, the diagnosis is confirmed by histopathological examination. Melanomas consist of spindle, epitheloid cells or a mix of both cell types, and hematoxylin-and-eosin (H&E) staining is used to differentiate between these cell types. Periodic-acid Schiff (PAS) staining helps to identify microvascular patterns (three closed loops located back to back). Additional melanocytic markers that can be used in immunohistochemistry are S-100 or HMB-45.

Predisposing factors

Men and women are equally affected by uveal melanoma and most patients are aged 60 years or older. Certain phenotypes have been described, predisposing to uveal melanoma. Caucasian race for instance, is the most important one known to date. Uveal melanoma is approximately 150 times more common in Caucasians than in Africans (Margo et al., 1998; Singh et al., 2005). Furthermore, blue or gray eyes as well as fair skin type and inability to tan have been suggested to predispose to uveal melanoma (Gallagher et al., 1985; Schmidt-Pokrzywniak et al., 2009; Tucker et al., 1985). Although these facts may point towards a possible role of UV-radiation in the development of uveal melanoma, current evidence regarding UV-radiation is still inconclusive (Li et al., 2000; Manning et al., 2004; Marshall

et al., 2006; Singh et al., 2004; Vajdic et al., 2002). There is however a tendency for iris melanomas to occur in the lower half of the iris, which has been explained by the increased sunlight exposure of this area (Shields, J.A. & Shields 2007).

Specific conditions as ocular and oculodermal melanocytosis (Nevus of Ota) (Gonder et al., 1982; Singh et al., 1998), neurofibromatosis type I (Wiznia et al., 1978), dysplastic nevus syndrome (Albert et al., 1985) are all associated with an increased incidence of uveal melanoma. Although uveal melanoma is rarely hereditary, several familial cancer syndromes have been reported: xeroderma pigmentosa, Li-Fraumeni syndrome and familial breast and ovarian cancer (Travis et al., 1996; Wooster et al., 1994). The low incidence of familial uveal melanoma cases limits approaches such as linkage analysis for the identification of susceptibility genes (Singh et al., 1996; Triozzi et al., 2008).

Clinical prognostic factors

The predictive value of classic prognostic parameters such as age, tumor size, tumor location, histological cell type and presence of vascular loops has been analyzed in several retrospective studies (Coleman et al., 1993; Mooy & De Jong 1996). These parameters were complemented by the more recent identification of other clinical, histological (tumor-infiltrating lymphocytes, protein biomarkers) and genetic parameters (chromosomal aberrations, expression profiling)(Kujala et al., 2003; Naus et al., 2002; Patel, B. C. et al., 1998; Petrausch et al., 2008; Sisley et al., 2006; Tschentscher et al., 2003; van den Bosch et al., 2010; van Gils et al., 2008b). Tumor size (largest tumor diameter) is the most significant clinical prognostic parameter and because of its ease of determination with ultrasonography, most often used for therapy-planning. The 5-year mortality rate in patients with tumors below 10 mm in diameter is approximately 15% and increases to 53% for tumors larger than 15 mm in diameter (Gamel et al., 1993). Tumors located in the ciliary body correlate with progressive disease (Schmittel et al., 2004). The same holds true for tumors that show scleral invasion, optic nerve invasion, or extra ocular extension (Damato 2010; McLean et al., 2004).

Histological presence of epitheloid cells and closed vascular patterns are also strongly associated with early death from uveal melanoma (Folberg et al., 1993; Maniotis et al., 1999; Seddon et al., 1983). These histological prognostic factors as well as genetic factors are less frequently used for primary therapy planning as tumor tissue is required for the pathological and genetic assessment of present risk factors. In most cases, enucleation enables research on tumor tissue from relatively large-sized tumors. More frequent use of in-vivo biopsy prior to therapy may help assessing genetic risk factors, also in smaller tumors that may be treated conservatively. Several groups have already proven fine-needle biopsy to be a reliable technique yielding sufficient tumor tissue for cytogenetic analysis(Midena et al., 2006; Naus et al., 2002; Shields, C. L. et al., 2011).

Clinical, histological, and cytogenetic factors can be used to identify patients with high risk of metastases from uveal melanoma (Eskelin et al., 2000). As micrometastases are thought to arise early in the disease and precede clinically detectable macrometastases, present prognostic factors may thus be used to identify patients with micrometastatic disease.

Metastasis

Uveal melanomas metastasize almost exclusively by haematogenous route, and about 90% of patients with metastatic disease have hepatic metastases (Bedikian et al., 1995; Gragoudas et al., 1991). Other, less frequent sites for metastases include lung, skin, bone and brain(Collaborative Ocular Melanoma Study 2001; Diener-West et al., 2004; Gragoudas 2006; Landreville et al., 2008). Involvement of regional nodes is rare and is attributed to the absence of draining lymphatics of the eye. Extraocular extension of tumor tissue though, may result in occasional metastatic involvement of lymph nodes.

The 15-year disease specific survival rates for patients with uveal melanoma is: 53% (Gamel et al., 1993). Shields et al (Shields, C. L. et al., 2011) recently reported a 3-year peak mortality of 24%. This could indicate a possible state of tumor dormancy or latency where circulating tumor cells remain silent and undetectable for the first 2 years after diagnosis (Klein 2011). Metastatic disease only rarely responds to treatment, and is usually fatal within 2-9 months after onset of symptoms (Diener-West et al., 2005; Eskelin et al., 2003). If the liver is involved, survival is most of the time shorter than 3 months. Treatment by systemic or intra-hepatic chemotherapy or partial hepatectomy rarely prolongs life (Augsburger et al., 2009). This highlights the urgent need for new and more effective therapies.

Fine needle biopsies and tumor heterogeneity

In previous research we have substantiated that specific regions on chromosome 1 and 3 are important in the etiology of uveal melanoma (Kilic et al., 2005). Both our genetic and expression profiling studies point towards certain areas on the genome, that are important in tumor development and progression (van Gils et al., 2008a; van Gils et al., 2008b). As most cytogenetic and molecular genetic studies up till now involve patient samples from large tumors treated by enucleation, no specific knowledge is currently available for patients who receive conservative treatment such as stereotactic radiation therapy. Even though the melanomas treated by stereotactic radiotherapy are smaller than treated by enucleation, still 25% of these tumors metastasize (van den Bosch T, manuscript submitted). This implies that also smaller-sized tumors have the typical chromosomal aberrations required for dissemination of the disease. Cytogenetic and molecular genetic analyzes of smaller tumors will most likely give more insight into tumor evolution and may enable identification of less complex chromosomal aberrations in uveal melanoma. In-vivo biopsy will be crucial for gaining tissue of small-sized tumors.

Previous results, with fluorescent in situ hybridization (FISH) on fine-needle aspiration biopsies (FNAB) acquired tumor tissue, showed that adequate FNAB material can be collected in a reliable and safe way for FISH analysis (Naus et al., 2002). The risk of local metastasis due to biopsy taking was found not to be increased with the FNAB technique (Char et al., 1996), and even a lower risk is reported if a transvitreal route is chosen for FNAB (Glasgow et al., 1988; Karcioglu et al., 1985). Tissue structure is also recognizable in contrast to the single cells that have been aspirated with FNAB. Bechrakis et al. combined a vitrectomy with a biopsy and showed that in 97% of the biopsies histological diagnosis was possible(Bechrakis et al., 2002). So there is a growing preference using this technique, especially since it is a more controllable approach and yields more material, on which in addition to cytogenetic and molecular genetic techniques histological examination will be possible.

Heterogeneity of monosomy 3 (complete loss of a copy of chromosome 3) in uveal melanoma has been studied by FISH analysis on paraffin-embedded tumor material, and on single-cell suspensions of fresh tumor tissue and showed that a difference in percentage of monosomy 3 may be present in some cases. However, our earlier results, where FISH on FNAB and tumor samples were compared, shows this to lead to misclassification in less than 1% of cases (Naus et al., 2002). Tumor heterogeneity was further investigated and it was concluded that analysis of tumor biopsies in uveal melanoma gives an accurate prediction of the high-risk characteristics (Mensink et al., 2009b). In a more thorough study, we showed that hyperdiploidy (60-70 chromosomes) often resulted in copy number loss of chromosome 3, with loss of heterozygosity of one allele (Mensink manuscript submitted).

Therapy

Until the late eighties, the only treatment available was enucleation of the affected eye. Nowadays, conservative treatment protocols such as brachytherapy, thermotherapy, or radiation therapy may be used to treat small and medium-sized tumors with conservation of the eye additionally. The large-sized melanomas however, e.g. large in diameter and/or thickness (also known as tumor prominence or height), are preferably still treated by enucleation (Shields, J. A. et al., 1996). The survival rate of patients with metastatic disease remains extremely poor as none of the current therapies proves to be effective. Several different cytotoxic agents such as dacarbazine have been administered alone, or in combination with other chemotherapeutic drugs or interferon-alfa-2b to high-risk patients after primary therapy. These regimens however, have not led to improved outcomes for these patients yet (Triozzi et al., 2008). Despite improvements in treatment protocols for primary tumor and metastatic disease, and despite the fact that hardly any of the patients have clinically detectable metastasis at presentation, still half of all patients die of metastatic disease (Kujala et al., 2003).

Unfortunately no effective therapy exists for the treatment of metastatic disease at this moment, but new protocols involving combinations of chemotherapy and immunotherapy have been initiated recently. Systemic therapy may be more effective if administered early after diagnosis treating micrometastatic rather than macrometastatic disease. In the latter case, multiple mechanisms of resistance against systemic interventions may be present (Triozzi et al., 2008). With this in mind a new adjuvant immunotherapy protocol has been developed, where clinical, histological, and cytogenetic factors are used to identify high-risk uveal melanoma patients and to treat these patients by immunization with their own trained dendritic cells to prevent future metastatic disease. This multicenter trial is performed by the ROMS in collaboration with Radboud University Nijmegen.

Molecular aspects of uveal melanoma

Cancer development is often associated with genomic instability and acquisition of genomic heterogeneity (Bayani et al., 2007), generating both clonal and non-clonal tumor cell populations (Katona et al., 2007; Ye et al., 2007). Several mutations in the cell cycle can lead to aneuploidy: during mitosis, spindle checkpoint processes such as chromosome attachment to the spindle and chromosome segregation are vulnerable to changes leading to single point mutations or even gross chromosomal rearrangements(Kops et al., 2005; Olaharski et al., 2006). There is a delicate balance between a possible benefit from the accumulation of genetic and epigenetic alterations and a lethal genetically unstable state of the cells.(Nguyen & Ravid 2006). Polyploidy is also well known in cancer and it tends to occur in tumors with a more aggressive phenotype (Castedo et al., 2006; Kaneko & Knudson 2000).

Research is focusing on finding pathways involved in carcinogenesis, thereby trying to understand tumor onset and early development and transition to metastatic disease. Highly invasive tumors are compared with poorly invasive ones, primary tumors with its metastases, and therapy-resistant tumors with responsive ones in order to search for differentially expressed genes and specific chromosomal regions or genes involved in these processes.

Cytogenetic and molecular genetic techniques

A wide variety of cytogenetic and molecular genetic techniques are available and others still in development. Short term cultured uveal melanoma specimens are very suitable for classic cytogenetic analysis and spectral karyotyping (SKY), and these samples generally display a relatively simple karyotype with recurrent chromosomal anomalies. (Figure 2)

Fluorescent in situ hybridization, comparative genomic hybridization (CGH) and quantitative PCR techniques can be applied on fresh or frozen tissues, cell lines, and archival formalin-fixed paraffin-embedded samples. Currently micro array based CGH, SNP analysis and gene expression analysis are the most frequently applied techniques. A drawback of

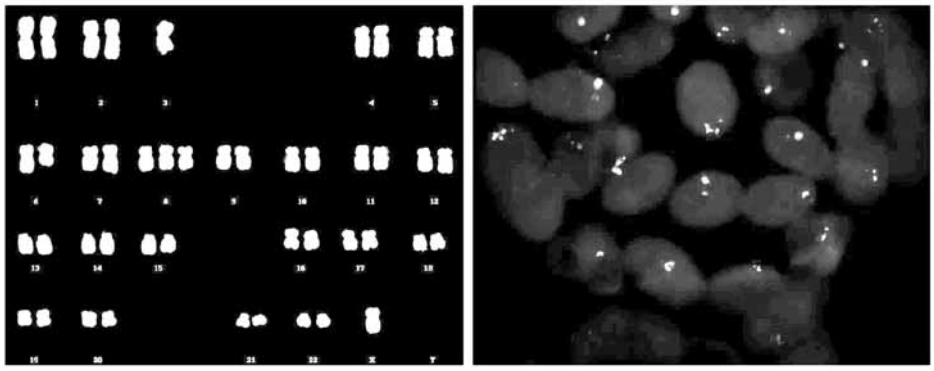


Figure 2: Karyogram showing loss of chromosome 3, isodisomy of 6p, and gain of chromosome 8 (left), FISH nuclei showing one signal for chromosome 3p (red) and centromere 3 (green) (right)
(color page 186)

array-based approaches is that the analyzed signal represents the average value of all cells in the tumor sample, requiring a high signal-to-noise ratio to quantitatively and reliably detect low-level DNA copy number changes on individual array elements(Albertson et al., 2003). The great advantage is that expression and copy number information on thousands of gene and chromosome locations can be obtained from a single mRNA or DNA sample in just one experiment.

Recently next generation sequencing (NGS) has been applied on primary uveal melanoma samples resulting in the detection of mutations, showing single or multiple base pair changes. A brief summary of the current findings is outlined below (The technical aspects of these techniques have been reviewed recently by us (Mensink et al., 2009a) and others (Harbour 2009).

Chromosomal anomalies as prognostic markers

Specific chromosomal anomalies, as deletion of chromosome 1p, monosomy of chromosome 3 or gain of chromosome 8q, strongly correlate with decreased survival in uveal melanoma patients. Monosomy of chromosome 3 is the most frequently found non-random chromosomal aberration in uveal melanoma and is predominantly found in metastasizing tumors(Prescher et al., 1996). In univariate analysis, monosomy 3 was the most significant predictor ($p < 0.0001$) of poor prognosis in uveal melanoma, followed by tumor location and diameter (Prescher et al., 1996). It is considered to be a primary event, because it is seen in combination with all other chromosomal aberrations in uveal melanoma such as loss of chromosome 1p, gain of 6p and gain of 8q (Prescher et al., 1995). In the majority of tumors with chromosome 3 loss there is complete monosomy, although occasionally isodisomy is acquired(Aalto et al., 2001; Scholes et al., 2001; White, V. A. et al., 1998). Rarely, melanomas with partial aberrations on chromosome 3 or translocations have been

described, making it difficult to map putative tumor suppressor genes. Loss of heterozygosity studies demonstrate common regions of allelic loss located at 3p25 and on the long arm spanning spanning from 3q24 to 3q26 (Onken et al., 2007; Parrella et al., 2003).

Concomitant loss of chromosomes 1p and 3 has a stronger correlation with metastasizing disease than either one of them separately (Kilic et al., 2005) (Figure 3). The common deleted region on chromosome 1 ranges from 1p34.3 to 36.2 (Aalto et al., 2001; Hausler et al., 2005; Hughes et al., 2005). The association with chromosome 8q gain was slightly less significant than for monosomy of chromosome 3, but a strong inverse correlation ($p < 0.0001$) of dosage effect of additional copies of 8q on survival was observed(Sisley et al., 1997). Gain of chromosome 8, or acquisition of an isochromosome 8q, is suggested to be a secondary event in uveal melanoma, because variable copy numbers of 8q can be present in one tumor (Horsman & White 1993; Prescher et al., 1994). It occurs frequently in tumors that have lost one copy of chromosome 3 and it is an independent prognostic factor of progressive disease (Patel, K. A. et al., 2001; Sisley et al., 1997; White, V. A. et al., 1998). The shortest region of overlapping gain spans from 8q24.1 to 8qter (Hughes et al., 2005; Sisley et al., 2006).

In a series of large posterior uveal melanomas, presence of a chromosome 6p abnormality was predictive of a more favorable outcome (White, V. A. et al., 1998). These tumors with gain of chromosome 6p have been proposed to represent a separate group of uveal

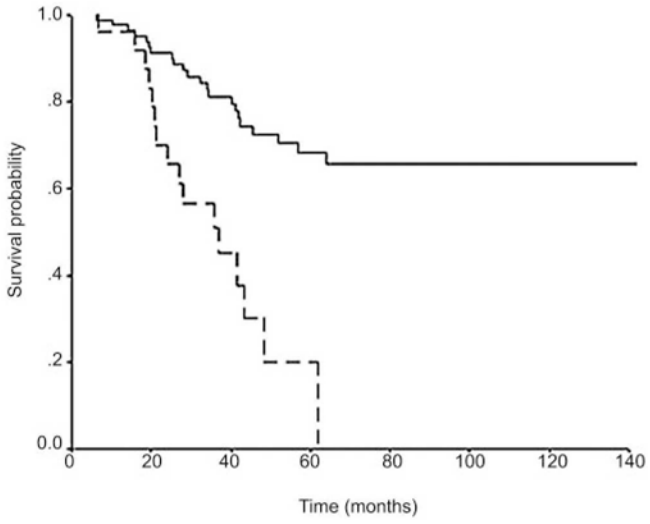


Figure 3: Kaplan-Meier survival curve of loss of chromosome 1p36 and chromosome 3 versus all other aberrations. Dashed line: tumors with concurrent loss of 1p36 and chromosome 3; solid line: tumors with any other chromosomal aberration ($P < 0.001$) (Figure adapted from Kilic et al., 2005)

melanoma with an alternative genetic pathway in carcinogenesis, because gain of 6p is found frequently in tumors with disomy 3 (Ehlers et al., 2008; Hoglund et al., 2004; Landreville et al., 2008; Sisley et al., 1997).

Abnormalities of other chromosomes have also been detected in uveal melanoma. However, they often lead to contradictory results regarding the prognostic impact. Chromosome 18q22 has been suggested to play a prognostic role (White, J. S. et al., 2006), but this could not be confirmed by other groups (Mensink et al., 2008). Chromosome 9p21(Scholes et al., 2001) and chromosome 16q (Vajdic et al., 2003) have been described to be important in uveal melanoma as well.

SNP arrays can be used to define copy number changes in tumors from signal intensities reflecting the amount of hybridized DNA (Bignell et al., 2004) (Figure 4), and for determining and mapping of chromosomal regions with loss of heterozygosity. The great advantage is that information on thousands of locations or genes can be obtained in a single experiment with a high resolution. With SNP-array we and others were able to confirm the frequently found alterations by FISH on chromosome 1p, 3, 6p, 6q, 8p and 8q. Other frequently found chromosomal alterations are: +7, -9p, -10 or +10, -11q23-q25 (van den Bosch et al., 2010).

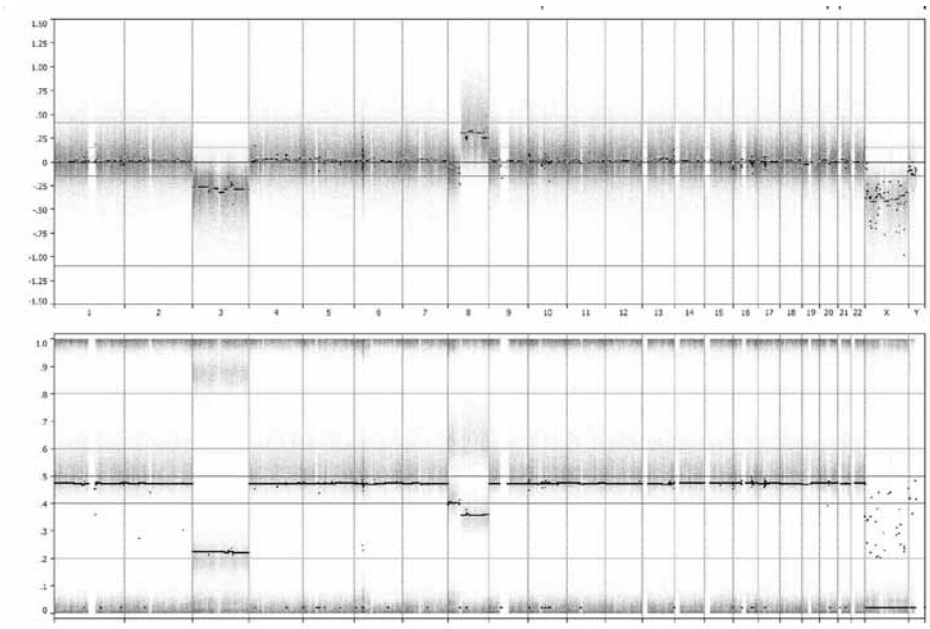


Figure 4: SNP-array results of a male patient with uveal melanoma, showing monosomy 3, loss of 8p, and gain of chromosome 8q (Log-R ratio upper panel). The lower panel represents the B- allele frequency showing allelic imbalance of chromosome 3 and 8 **(color page 187)**

Partial losses of chromosome 3 are very rare and therefore also rarely reported in the available literature (Parrella et al., 2003; Trolet et al., 2009; Tschentscher et al., 2001). In nearly all cases, complete loss of one copy of chromosome 3 is found, even in the smaller-sized melanomas that had been enucleated. With the recent high-resolution SNP-array's, no specific small regions of loss on chromosome 3 have been found.

For the fact that most uveal melanomas have complete loss of chromosome 3, either FISH, q-PCR, or SNP-array may be used for analyzing chromosome status in patients with uveal melanoma. There is thus no advantage of either SNP-array or FISH for only detecting chromosome 3 alterations in uveal melanoma. SNP-array though, allows for testing of multiple loci on different chromosomes compared to just one or two loci per chromosome with FISH or q-PCR. SNP-array is a fast measure for genome-wide assessment of chromosomal aberrations whereas FISH takes more time as multiple experiments would be necessary to assess multiple chromosomes or loci. There is one major difference between SNP-array and FISH, being that SNP-array enables assessment of heterozygosity, potentially revealing regions with loss of heterozygosity or allelic imbalances. This feature may provide us with information especially in disomic chromosome 3 cases where one allele is lost and the remaining one is copied by mistake (isodisomy) or uniparental disomic states. These processes may lead to loss of heterozygosity and may also help find an answer for patients with disomy 3 who still developed metastasis.

Gene expression profiling

By analyzing uveal melanomas on the basis of gene expression patterns, two different classes of uveal melanomas can be determined. Class II tumors represent a high-risk of developing metastasis in the future while the class I tumors have a more favorable prognosis(Tschentscher et al., 2003; van Gils et al., 2008b). In the class II tumors, a global down-regulation of neural crest and melanocyte-specific genes is found, together with an up-regulation of epithelial genes. If the prognostic value of gene expression studies is compared to the clinico-pathologic or cytogenetic markers, the classification on basis of a set of classifier genes by gene expression is far-out superior (Petrausch et al., 2008; Tschentscher et al., 2003; Worley et al., 2007). These tumors furthermore exhibit epithelial features, such as cell morphology, and up-regulation of the E-cadherin pathway (Worley et al., 2007). RNA extraction of enucleated tumor or a fine-needle biopsy is feasible and can be used for transcriptomic analysis on uveal melanoma samples, this service is in fact currently commercially available (Onken et al., 2010). Mentioned expression studies yielded similar sets of classifier genes, however, these classifier genes are merely markers of the underlying cause whereas genes involved in tumor progression and metastatic potential still have to be discovered. (van Gils et al., 2008b), If these are genes encoding cell surface markers, they could be a target for cell therapy aimed at an immunological response to eliminate tumor cells.

Next Generation Sequencing

The introduction of Next Generation Sequencing provides chromosomal mutational analysis up to the base-pair level. This sequencing technique therefore has the highest resolution possible and with the possibility for fast genome-wide testing in multiple tumor samples at once, is a very precise and reliable technique for mutational analysis. A limitation is the fact that preceding PCR amplification is required most of the time, which may introduce mutations due to the error rate of the polymerase enzyme. The most recent genes involved in uveal melanoma were found by sequence analysis, such as *BAP1* and *GNAQ*. These genes are discussed in more detail further on.

Epigenetic regulation

Epigenetic mechanisms are known to alter genomes by other ways than direct changes in DNA sequence. For example, genes and promoters may have their functions silenced by methylation processes. In uveal melanoma, methylation of *CDKN2A* is present in 4 to 32% (Merbs & Sidransky 1999), *RASSF1* in 13 to 70% (Maat et al., 2007), *RARB* up to 7% and *TIMP3* in 9% (van der Velden et al., 2003; van Gils et al., 2008b). *RASEF* is targeted by LOH in combination with methylation in primary uveal melanoma; there is only low percentage methylation (Maat et al., 2008). *hTERT*, an important gene in carcinogenesis, is methylated in up to 52% of uveal melanoma, whereas *FHIT* and *APC* are never hypermethylated (Merhavi et al., 2007; Moulin et al., 2008; Zeschnigk et al., 2003). In none of these studies hypermethylation of a gene correlated with metastatic disease. When we looked for regions containing blocks of up or down regulated genes using LAP analysis specific regions with (significantly) low or high expression on the genome were apparent (van Gils et al., 2008b). These local over or under expression could be the result of small deletions or amplifications. Alternatively epigenetic mechanisms as hyper- or hypomethylation could be an explanation.

Genes involved in uveal melanoma

Deregulation of the RAS-RAF-MEK-ERK or mitogen-activated protein kinase (MAPK) pathway is common in many human malignancies (Inamdar et al., 2010). Mutations of specific members of these key molecular signaling pathways have been implicated in tumorigenesis of cutaneous melanoma. Many of these often well-known oncogenes (e.g. *NRAS*, *BRAF*) or tumor suppressor genes (*PTEN*, *CDKN2A*) have also been analyzed in uveal melanoma and occasionally mutations in these genes were found. Although no relation with development of metastatic disease was found, the presence of somatic mutations in these genes can provide a starting point for early detection of metastatic cells in blood or therapeutic intervention. More promising are the recent findings of two frequently mutated members of the MAP-kinase pathway, *GNAQ* and *GNA11*, and the *BAP1* gene.

The MAP-kinase pathway: GNAQ and GNA11

Recently Van Raamsdonk and coworkers demonstrated that approximately 80% of uveal melanomas carried activating mutations in either *GNAQ* or *GNA11*, turning these into oncogenes(Herlyn & Nathanson 2010; Van Raamsdonk et al., 2009; Van Raamsdonk et al., 2010). These genes belong to a subfamily of genes encoding for the G-protein α subunit involved in MAPK cell signaling. Mutations in the G-protein α stimulatory subunit of *GNAQ* were found in 46% of uveal melanoma cases, whereas mutations of *GNA11* were found in 35% of uveal melanoma cases with wild-type *GNAQ*. Both of these genes showed to be mutually exclusive and were by their oncogenic conversion suggested to be the cause of constitutive MAP-kinase pathway activation. This in turn leads to cell proliferation even in the absence of extracellular stimuli.

Activating somatic mutations of *GNAQ* at codon 209 were found by Onken et al, in 31 of 58 (54%) posterior uveal melanomas, and in two of nine (22%) iris melanomas(Onken et al., 2008; Romano et al., 2011). Iris melanomas thus less often show *GNAQ* mutations, but can occasionally have mutant *BRAF* (Henriquez et al., 2007; Onken et al., 2008; Sisley et al., 2011; Van Raamsdonk et al., 2009). Conjunctival melanomas on the other hand often have *BRAF* involvement but do not have *GNAQ* mutations(Dratviman-Storobinsky et al., 2010). These specific *GNAQ/GNA11* mutations are also found in 83% of blue naevi of the skin (Van Raamsdonk et al., 2009) and are present in all stages of progression(Sisley et al., 2011). These mutations therefore are thought to occur early in tumorigenesis, which is underlined by the fact they are not correlated with either molecular class or metastasis in general (Bauer et al., 2009; Onken et al., 2008).

The RAS-RAF-MEK-ERK pathway

Mutations in the RAS-RAF-MEK-ERK (MAPK) pathway are thought to be early or initiating events in tumorigenesis (Onken et al., 2008). In general, this pathway is activated by autocrine growth factor stimulation or by mutation of *BRAF* or *RAS* genes(Dhomen & Marais 2009; Fensterle 2006; Mercer & Pritchard 2003; Zuidervaart et al., 2005) resulting in excessive cell proliferation. A single substitution (p.V600E) appears to account for more than 90% of all *BRAF* mutations in cutaneous melanoma and this mutation is also frequently found in benign and premalignant nevi thereby suggesting these to be early events in tumorigenesis (Davies et al., 2002; Pollock et al., 2003). *BRAF* and *NRAS* are both activators of the MAPK pathway but mutations of these genes are very rare in uveal melanoma (Cohen et al., 2003; Kilic et al., 2004; Mooy et al., 1991; Saldanha et al., 2004). However, activation of the MAPK pathway appears to be a common event through *GNAQ*/ *GNA11*-mutation induced G-protein signaling and possibly also by activation of ERK, a downstream kinase in the pathway(Calipel et al., 2006; Weber et al., 2003). It has been suggested that MAPK activation

in uveal melanoma may arise via crosstalk with the PI3K-PTEN-AKT pathway(Zuidervaart et al., 2005).

The PI3K-PTEN-AKT pathway

The tumor suppressor gene phosphatase and tensin homolog (*PTEN*), is involved in the PI3K pathway as negative regulator of AKT. Loss of function of *PTEN* by deletion or mutation, leads to activation of AKT and over expression of the PI3K-PTEN-AKT pathway preventing apoptosis(Ehlers et al., 2008; Ibrahim & Haluska 2009). Inactivation of *PTEN* is reported in 15% of uveal melanoma cases and has been linked to an increase in aneuploidy but also poor clinical outcome (Abdel-Rahman et al., 2006; Ehlers et al., 2008). This may suggest a role in later stages of tumor growth and development. Activating mutations of *AKT3* may also lead to activation of this pathway, though mutations of *AKT3* have not been reported in uveal melanoma up till now.

The metastasis-associated gene BAP1

Somatic mutations in the ubiquitin carboxyl-terminal hydrolase of *BRCA1*-associated protein 1 (*BAP1*) were found in 84% of class II uveal melanomas(Harbour et al., 2010). The *BAP1*-gene is located on chromosome 3p21.1 and the encoded protein is part of the ubiquitin proteasome system that has been implicated in other cancer types as well, such as lung, breast and renal cell carcinoma (Harbour et al., 2010; Jensen et al., 1998; Patel, M. et al., 2011; Wood et al., 2007). *BAP1* is reported to participate in multiprotein complexes involved in regulation of expression of several other genes that regulate various cellular processes (Patel, M. et al., 2011). Somatic *BAP1* mutation was only found in 1 out of 26 investigated class I tumors against 26 out of 31 class II tumors. These mutations are thus suggested to occur later in uveal melanoma progression than for instance *GNAQ* mutations(Harbour et al., 2010).

Other investigated genes

Several candidate genes were proposed in uveal melanoma recently, such as *DDEF1, NBS1, HDM2*, *LZST-1*, *APITD1*, *CCND1* and *BCL-2* (van den Bosch et al., 2010). For most of these genes, a definite role in tumorigenesis or progression towards metastasis has to be validated. In 65% of uveal melanoma cases, *CCND1* is reported to be overexpressed resulting in activation of cyclin-dependent kinases(Coupland et al., 1998; Coupland et al., 2000; Ehlers & Harbour 2006). The *CCND1* overexpression is associated with large tumor size, epitheloid cytology, and poor prognosis(Coupland et al., 2000). Elevated expression of *BCL-2* is observed in uveal melanoma but also in normal melanocytes. This overexpression is reported to block apoptosis(Brantley & Harbour 2000; Chana et al., 1999; Coupland et al., 2000; Jay et al., 1996) and is suggested to be responsible for the resistance to chemotherapy or irradiation of melanocytes (Ehlers & Harbour 2006; McGill et al., 2002).

Gene targeted therapy

The survival rates for patients with metastasized uveal melanoma, treated by chemotherapeutic drugs or combination chemotherapy regimens remain disappointingly low and toxicity may be significant (Sullivan & Atkins 2010). Conventional cytotoxic chemotherapeutics are toxic to all cells including normal cells, and therefore targeted therapy may be more valuable in the treatment of these patients(Triozzi et al., 2008). As the molecular basis for tumor development and progression is emerging, therapy aimed at interfering with specific molecular pathways may be important (Triozzi et al., 2008).

MAPK pathway activation appears to be important in uveal melanoma, therefore inhibition of this pathway or intermediates of this pathway represent a promising target(Sisley et al., 2011). *GNAQ*^{Q209} mutations are exclusive to melanocytic tumor cells thereby enabling very specific therapy targeted at mutant cells only. *GNAQ* mutant cell lines appeared highly sensitive to inhibitors of MEK (Van Raamsdonk et al., 2009) and phase II clinical trials testing this hypothesis are currently underway(Sullivan & Atkins 2010).

Inhibitors of members of the RAS-RAF-MEK-ERK pathway such as the small-molecule inhibitor PLX4032, showed promising results in patients with cutaneous melanoma containing *BRAF* mutations. Tumor shrinkage was found in 80% of patients who received PLX4032 and progression-free survival was found to increase by an average of 7 months (Bollag et al., 2010; Flaherty et al., 2010; Herlyn & Nathanson 2010). These mutations are however rarely encountered in uveal melanoma patients. Downstream effectors of *GNAQ* and *GNA11* remain to be elucidated and are highly potential targets for therapy. Care needs to be taken as interference with the normal function of these proteins, could be harmful. *GNAQ* protein activity for instance, appeared to be crucial for cardiomyocyte survival in animal models(Sisley et al., 2011). This problem may however be solved if inhibitors could be designed that specifically interfere with mutant *GNAQ* only. As MEK inhibitors proved to be of value for uveal melanoma, other ways to circumvent the *GNAQ* protein blocking problem may lie in designing inhibitors of intermediates of the MAPK pathway such as BRAF, RAS, MEK and ERK. IGF-1R and the downstream molecule mTOR, may also be involved in the PI3K-PTEN-AKT pathway and RAS-RAF-MEK-ERK pathway, and also serve as potential targets for inhibitor-therapy, which is currently being tested (Patel, M. et al., 2011).

Since *BAP1* functions as a tumor suppressor gene in uveal melanoma, future targeted therapy should be aimed at restoring one or more functions that are lost when *BAP1* is inactivated. This is, at present, more difficult than designing inhibitory agents. One of the primary functions of *BAP1* appears to be the deubiquination of histone H2A, and histone deacetylase (HDAC) inhibitors may have therapeutic potential by countering the accumulation of histone H2A following loss or inactivation of *BAP1* (Harbour, J.W. et al., 2012). Secondly, genetic

changes of BAP1 could influence the *BRCA1* functions such as the *BRCA*-dependent DNA damage response. However, exposure of multiple myeloma cell lines with a mutated or deleted *BAP1* gene to ionizing radiation did not lead to consistent differences in *RAD51* or *BRCA1* complex formation. (Bott, M. et al., 2011) In addition, PARP inhibitors did not confer a different sensitivity in *BAP1* mutant cell lines compared to *BAP1* wild-type cells. Whether the this is also the case in UM cells remains to be resolved.

New immunotherapeutic approaches are also currently tested, such as those administering patients immunomodulatory monoclonal antibodies (e.g. CTLA4 antibodies) or vaccinating patients with their own dendritic cells trained to identify (circulating) tumor cells and initiate tumor cell destruction by presenting tumor particles to cytotoxic T cells. We use this Dendritic Cell Therapy to treat high-risk patients after they have received local therapy, such as enucleation or irradiation, in order to eliminate circulating tumor cells and micrometastatic lesions. Results have to be awaited from this phase I study that already showed promising results in cutaneous melanoma patients (De Vries, IJ. et al, 2005). Combination strategies such as immunotherapy and gene therapy may be more effective than single therapy regimes but these combinations have to be researched in the future.

Conclusion

Uveal melanoma is a rare but aggressive intra-ocular malignancy leading to metastatic spread in approximately 50% of patients. Current therapies have up till now unfortunately not resulted in improved survival. Patients with metastases from uveal melanoma still have a poor prognosis, with no effective therapy available yet. Even chemotherapeutic agents administered alone or in combination, have not resulted in a change in survival rates for these patients. Recent cytogenetic and molecular genetic research identified several genetic prognostic factors, capable of making reliable predictions of prognosis in patients with uveal melanoma. These genetic factors prove to be even more important predictors than clinical and pathological factors and have already been implemented in the current ocular oncology clinical practice.

Next generation genetic techniques such as SNP-array, next generation sequencing and gene-expression profiling shed light on chromosomal regions, genes, gene expression, and molecular pathways involved in uveal melanoma tumor progression and development. More knowledge has been gained by these recent techniques combined with fine-needle aspiration biopsy tumor sampling, identifying the molecular genetic make-up of small and medium- sized melanomas as well as the large melanomas. Uveal melanoma has hereby been identified as a heterogeneous type of malignancy showing variations in chromosome 3 alterations within tumors and different genes altered in different patients. With the molecular

background of large and smaller-sized uveal melanoma emerging, patients may be selected on this molecular basis for future therapy.

Gene-targeted therapy is recently been tested in the clinical setting, facilitating interference with specific molecular pathways or signaling molecules, either as single agent or in combination with immunotherapy or chemotherapy. These developments may serve as first steps towards more specific and patient-tailored therapy not limited to treatment of patients with metastatic disease alone. These therapies may also be valuable for patients with recent diagnosis of uveal melanoma, attacking micrometastatic disease as early as possible. There is great optimism that more specific and thus more effective therapies in the next several years will lead to advanced patient management, and thereby improved survival rates for patients with this deadly disease.

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Chapter 2

Genetics of uveal melanoma and cutaneous melanoma: two of a kind?

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Abstract

Cutaneous melanoma and uveal melanoma both derive from melanocytes but show remarkable differences in tumorigenesis, mode of metastatic spread, genetic alterations and therapeutic response. In this review we discuss the differences and similarities along with the genetic research techniques available and the contribution to our current understanding of melanoma. The several chromosomal aberrations already identified, prove to be very strong predictors of decreased survival in CM and UM patients. Especially in UM, where the overall risk of metastasis is high (45%), genetic research might aid clinicians in selecting high risk patients for future systemic adjuvant therapies.

Introduction

Cutaneous melanoma (CM) has shown to be one of the life-threatening malignancies with the fastest rise in incidence over the last decades. The highest incidence of CM is observed in Australia (60-70 per 100,000 individuals). In Europe and the USA the incidence is lower (10-15 and 20-30 per 100,000[1,2], respectively). CM accounts for more than 90% of all melanomas[3], whereas uveal melanoma (UM) is only encountered in 5%[4]. Nevertheless, UM is the most frequently occurring intra-ocular malignancy (85%) in the Western world. Although CM and UM both derive from melanocytes these two distinct tumors show remarkable differences in tumorigenesis, mode of metastatic spread, genetic alterations and therapeutic response[5,6]. CM can occur anywhere on the body but is predominantly observed in sun-exposed body parts. This partly explains the high incidence of CM in the light-skinned residents of Australia and New-Zealand. UM can occur anywhere along the uveal tract but tend to occur more frequently in the choroid (80%) and the ciliary body (15%) (Figure 1). The incidence of UM appears to be relatively stable with around 7 new patients per 1 million individuals yearly in the Western world. UV-light exposure has shown not to be of specific risk in UM. Although recently, Schmidt *et al* [7] demonstrated a positive interaction between UM and individuals with light colored eyes who sustained frequent UV-radiation. In addition, the tendency of iris melanomas to occur in the lower half of the iris has been explained by the increased sunlight exposure of this area[8]. Other known risk factors for CM and UM are fair skin type (CM and UM), familial occurrence of melanoma (CM)[9], a high number of melanocytic naevi (CM), light colored eyes (UM) and oculodermal melanocytosis (UM)[10,11].

Diagnostics

Clinical examination of suspicious lesions remain an important modality in diagnosing CM and UM. As for the diagnosis in CM, dermatologists rely mostly on clinical examination and reserve (excisional) biopsy for tumors of uncertain origin. Only UM of the iris may be diagnosed by external examination relating to possible early-stage detection. For detection of UM of the choroid or ciliary body, a thorough ophthalmic examination including indirect ophthalmoscopy and ultrasonography of the retina has to be conducted. Tumor growth can lead to retinal detachment and result in extra ocular extension of the tumor. At this point,

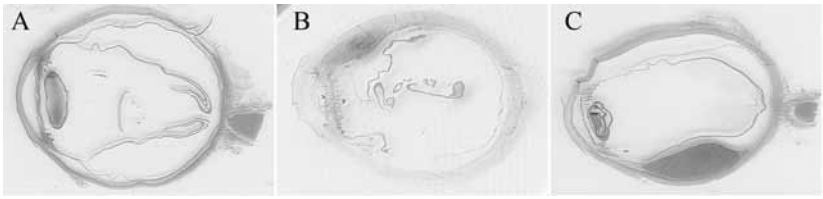

Figure 1: Uveal melanoma located in: iris (A), ciliary body (B), choroid (C) **(color page 187)**

defects in visual field or central vision may be present. Early symptoms of tumor growth however, can be vague or absent to the patients' notion.

The overall survival is known to be dependent of the tumor thickness (CM) and largest tumor diameter (UM) at time of diagnosis. Therefore, clinicians still concentrate on early detection of CM and UM. This resulted in an average tumor thickness of 0.76 mm in CM at time of diagnosis nowadays. This was shown to relate to an overall 10 year survival of 90%[12] of these small lesions. Similarly, UMs with a diameter of under 4 mm relate to a 5-year survival of 84%, The 5-year survival rate for medium-sized UM (4-8 mm in diameter) is 68%, and 47% with large size UM(over 8 mm in diameter)[13]. The survival of CM and UM patients with metastatic disease is however equally bad with a dismal mean of 2-7 months[14-16].

Therapy

The most frequently used therapeutic option in CM is excision of the primary tumor and enucleation of the tumor containing eye in case of large UM. Most small and medium-sized UMs are currently managed by eye-saving treatments such as observation (small inactive tumors), episcleral brachytherapy or charged-particle radiotherapy and several other variants of radiotherapy. In CM, radiotherapy is only used for palliative purposes as CM cells appear to be relatively radio-resistant. Adjuvant systemic therapy is mainly used in patients at high-risk of metastasis or in patients who already have developed metastasis. The response rates of chemotherapeutic agents in metastasized CM and UM are however as low as 7-25%[17-20].

Metastasis

Both malignancies display a strong tendency to metastasize[3]. Although the mode of metastatic spread is different: CMs tend to metastasize by both hematogenous and lymphogenous route and by local invasion. CMs are known to be able to give rise to metastases in: skin (13-38%), distant lymph nodes (5-34%), distant subcutaneous tissues (32%), lung (18-36%), liver (14-20%), CNS (2-20%) and/or bone (4-17%)[21]. In UM, metastatic spread is almost exclusively by hematogeneous route to remote organs of which the liver is involved in almost all cases (90%)[15,22]. The reason why UM is not involved in metastatic spread by lymphogenous route is thought to be a direct result of the absence of draining lymphatics of the eye[6,23]. It is however still unknown why the liver is especially affected by metastases although there are reports about sporadic metastases in lung (24%) and bone (16%)[24-27].

Eventually 45% of UM patients die of metastasis regardless of enucleation or radiotherapy[16]. This has led to theories about the early presence of micrometastasis in the disease process, which remain dormant for years before they give rise to clinically detectable macrometastasis[28]. The exact duration of this proposed state of dormancy and cues

for metastatic development remain uncertain. Shields *et al* [29,30] reported tumors with a size of just 1.0 mm to be capable of metastasizing, hence the need for highly specific and sensitive prognostic markers to predict which patient is at risk of developing metastasis. In the quest for significant prognostic markers in UM, already several have been identified. Age (over 60 years), largest basal tumor diameter (over 18 mm), tumor cell type (epitheloid cellularity) and closed vascular patterns correlate with early metastatic disease and shorter survival[31-33]. In CM, tumor thickness (increasing Breslow thicknesses), level of invasion, age (old age), gender (males), anatomic site of primary tumor (head/neck or trunk), number of metastatic lymph nodes and ulceration on histopathological research appeared to be independent significant prognostic factors of early metastasis[21]. These factors are summarized in a staging system known as the TNM-staging system. This system relies on tumor stage at time of diagnosis which has shown to be the most important prognostic factor in CM and is now widely used for prognostic purposes and clinical decision making[34].

Tumor Research Methods

Genetic analysis of tumor material, either from excised CM or from enucleated eyes has led to the identification of genetic prognostic markers for both types of melanoma. In the past years several cytogenetic- and molecular genetic techniques have been used to investigate the genomic background of melanomas. With conventional karyotyping, we and others were able to identify chromosomal gains, losses and translocations in UM (Table 1). Comparative genomic hybridization (CGH) allows a complete copy number analysis of the entire genome by comparative hybridization of differentially labeled genomic sample and reference DNA to normal human metaphase spreads. Both these techniques have a low resolution of 5-20 MB. Fluorescence in situ hybridization (FISH) provides a higher test resolution and even clonal gains and losses present in only a low percentage of tumor cells can be detected[35,36]. Furthermore, FISH has high test specificity and although time consuming, still is a frequently

Table 1. Overview of techniques used in (molecular) cytogenetics

Method	Resolution	Provides genome wide testing?	Detection balanced anomalies?	Detection unbalanced anomalies?
Karyotype G-banding	~ 5 – 10 Mb	+	+	+
FISH	~ 100 kb	-	+	+
SKY	~ 1 - 2 Mb	+	+	+
MSI	<1 kb	-	-	+
CGH	~ 5 – 20 Mb	+	-	+
MLPA	~ 1 – 40 kb	-	-	+
SNP/ CGH array	> 100 kb	+	-	+

used technique in tumor research and diagnostics. Also paraffin embedded tissue sections can be assayed by FISH. A drawback to this technique however, is that only a small number of loci can be analyzed in one single experiment. Molecular genetic techniques such as multiplex ligation-dependent probe amplification (MLPA) and microsatellite instability analysis (MSI) require input of isolated DNA and enable analysis of multiple loci in one experiment with a high resolution. MLPA is a polymerase chain reaction (PCR)-based technique which functions through the simultaneous hybridization of multiple (up to 50) probes to tumor DNA. Each probe with unique length is only amplified when ligated to its unique probe-counterpart. This provides high specificity of hybridized probes. The final amount of DNA, after several PCR-cycles, is dependent of its initial quantity and eventually copy number changes can be quantitated by relative quantification(RQ). MLPA has proven to be a suitable test for detection of chromosomal anomalies in tumor material[37].

For loss of heterozygosity (LOH) analysis, MSI is frequently used. With this technique, specific markers are required which are allowed to hybridize to the so-called microsatellites within genomic intronic DNA. These microsatellites are tandem repeats of simple polymorphic sequences that are randomly distributed and allow detection of the presence or absence of two different alleles. A drawback to this technique is that only a limited number of markers can be analyzed in a single experiment.

Microarray-based CGH, single-nucleotide polymorphism (SNP) arrays and gene expression analysis are among the most frequently applied array-based techniques nowadays. All these techniques are based on series of DNA segments (oligonucleotides or bacterial artificial chromosomes; BACs) orderly arranged on a chip, to which fluorescently labeled DNA or RNA can be hybridized. This enables the analysis of copy number status or gene expression of one entire genome very rapidly. Nowadays, there are chips available which enable analysis of structural variation at high level of detail with up to 1.2 million markers. The use of SNP arrays can also provide evaluation of loss of heterozygosity or isodisomy of parts of the genome. Drawbacks to array technology are its cost, which is about tenfold compared to FISH, and the inability to detect balanced anomalies and genomic abnormalities in frequencies below 10% of analyzed nuclei. Table 1 provides an overview of the differences in resolution and detection limits among the cytogenetic and array-based techniques. The different research techniques previously mentioned, certainly contributed to our understanding of melanoma by identification of chromosomes and genes involved in the disease. In the following section we will discuss the most important chromosomal and genetic alterations UM and CM.

Chromosomal Aberrations in UM

Chromosome 3

The most frequently encountered chromosomal aberration in UM is loss of one of the two copies of chromosome 3 (monosomy 3 or -3). Monosomy 3 is observed in approximately 50% of cases[38-41] and appears rather specific for UM as this chromosomal anomaly is rarely encountered in CM or other cancer types[42](Figure 2). Several groups have already shown there is a strong correlation between monosomy 3 and the development of metastatic disease[43-46]. In addition, monosomy 3 strongly relates to several clinical and histopathological parameters such as: epithelioid cytology, closed vascular patterns, large tumor diameter, and ciliary body involvement[41,44,47,48]. Also, monosomy 3 is thought to represent an early event in tumorigenesis because the alteration is frequently seen in combination with all other known chromosomal abnormalities[49]. In 5-10% of cases one copy of chromosome 3 is lost and the remaining copy is duplicated. This isodisomic state of chromosome 3 appears to be prognostically equivalent to monosomy 3[50]. Rarely, partial deletions of chromosome 3 are found[26] and although this has hampered fine mapping studies a common region of allelic loss on 3p25 and on 3q24-q26 could be defined[50,51]. Most likely these regions harbor putative tumor suppressor genes but no specific genes have yet been identified.

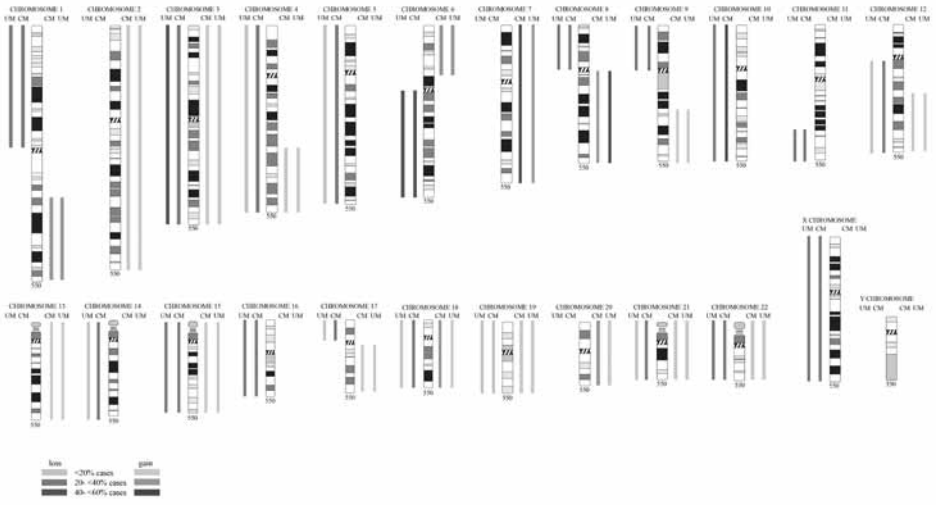

Figure 2: Chromosomal aberrations in cutaneous melanoma (CM) and uveal melanoma (UM): based on all cases in the Mitelman Database of Chromosomal Aberrations in cancer, by Höglund [42]. **(color page 188)**

Gene expression profiling on UM tumor material does show promising results. By this technique UMs were found to cluster naturally in two distinct molecular classes (class I or class II) based on classifier gene sets[52]. Both classes appear to have clinical prognostic relevance; patients with class I tumors rarely die of metastases, while patients with class II tumors have a high risk of death due to metastases[33,53]. Onken *et al* [54] reported an eight-year survival of 95% for patients with class I UM and 31% for patients with class II UM. Moreover, class II tumors display the previously identified poor prognostic factors: monosomy 3, epitheloid cytology and closed vascular patterns. The strong significant relation between molecular class and survival indicates that array technology clearly outperforms clinical and histopathological parameters[53-56].

Chromosome 8

Gain of 8q (+8q) is found in around 40% of UM cases and proved to be an independent significant prognostic marker for decreased survival[43,45]. It frequently occurs in combination with monosomy 3, either as +8q or as isodisomy 8q, and this combination also shows a strong relation with metastatic disease[43-45,57]. Abnormalities of chromosomes 3 and 8 are more common in ciliary body-located UMs, whilst alterations of the long arm of chromosome 8 tend to relate to choroid-derived UMs[43,46,48,49]. However, in the study by Kilic *et al* [58], chromosome 8q abnormalities were shown to correlate with large tumor diameter but there was no significant relation found between gain of 8q and the metastatic phenotype by univariate analysis. Gain of 8q is also frequently observed in different copy numbers in different UMs, therefore this is speculated to be a late event following the initiation of monosomy 3. The common region of amplification was found to range from 8q24.1 to 8q24.3[59,60]. Although gain of chromosome 8q is observed in 25% of CMs, the co-occurrence of monosomy 3 and gain of 8q, as in UM, is rarely observed in CM. Several oncogenes on chromosome 8q were hinted as possible factors in UM pathogenesis, among these genes are: *MYC* (on 8q24), *NBS*1 (on 8q21) and *DDEF*1(on 8q24)[46,61-64]. A potential metastasis suppressor gene located on 8p21, named *LZTS*1, has been pointed out by Onken *et al* [23].

Other Chromosomal Aberrations in UM

Kilic *et al* [65] showed loss of 1p36 in combination with monosomy 3 to be of prognostic significance: these aberrations occurring together display a stronger correlation with decreased survival than monosomy 3 or loss of 1p36 alone (-1p36 by itself is not of prognostic significance). One of the suggested tumor suppressor genes in the 1p36 region, *APITD1* was found to be not of significance in patients survival[66]. The common deleted regions on chromosome 1 were found to range from 1p34.3 to 36.2[48,67]. Alterations of chromosome 6 are frequently encountered in both UM & CM (discussed later), but show less prognostic value compared to monosomy 3 or gain of 8q in UM[42,46]. Of these alterations, gain of

DNA-material on the short arm of chromosome 6 (+6p) is found in 25-29% of UM and relates to spindle cell cytology and low risk for development of metastasis[33,42,49,68,69]. Hughes *et al* [60] reported the shortest region of overlap on the p-arm on chromosome 6 to be restricted to 6p22.3-p25. The simultaneous occurrence of +6p and -3, however, is rarely observed. Loss of DNA material on the long arm of chromosome 6 (-6q), observed in 25-38%, possibly represents another late event in tumorigenesis and correlates with worse prognosis[39,42,48,69,70]. The region of common deletion on the long arm was found to range from 6q16.1 to 22.3[60]. Infrequently, abnormalities of the other chromosomes such as loss of 9p, loss of chromosome 10, loss of 11q23-q25 and gain of chromosomes 7 and 10 have been reported[39,40,44,46,47] but a possible role in tumorigenesis and/ or development of metastasis in UM has yet to be evaluated.

Genes

Much less is known about genes involved in the development and progression to metastasis in UM compared to CM. This is mainly the result of the lower incidence of UM and the small quantities of tumor sample available for research. While there are many different potential tumor genes identified in CM every year, UM lags behind. However, several candidate genes were proposed in UM recently, such as *GNAQ, DDEF1, NBS1, HDM2, BCL-2* and *CCND1*. For most of these genes, a definite role in tumorigenesis or progression towards metastasis has to be validated.

G protein alpha subunit q (*GNAQ*) is the first gene found to be mutated frequently in UM. Several groups have shown that approximately 46% of UMs carry mutations in the *GNAQ* gene[27,81,104](Table 2) turning *GNAQ* into an oncogene. This oncogenic conversion leads to constitutive activation of the MAP-kinase pathway which results in a situation in which the cell is provided continuous growth signals in the absence of extracellular stimuli[113] and thus, cell proliferation. So far, *GNAQ* status has not been linked to disease free survival, so it could represent an early event in tumorigenesis[27,104]. This mutation is also found in 83% of blue naevi of the skin[81].

Furthermore, the *DDEF1*-gene has been described in UM. It is located on 8q24 and found to be mutated in 50% of UMs leading to overexpression[69]. High expression of *DDEF1* was shown to result in more motile low grade UM cells by Ehlers *et al* [63] and could therefore be important in metastatic development[63,114,115]. The *NBS1*-gene, is found to be overexpressed in 50% of UM[62]. The encoded protein product is postulated to be part of a complex involved in DNA-repair[102]. It is theorized that overexpressed *NBS1* could allow UM progression by promoting the repair of DNA damage which occurs more frequently in advanced tumors with increased genetic instability. High expression of the *HDM2*-gene on 12q15, is found in 97% of UM[69]. High *HDM2* expression was shown

Table 2. Commonest known genetic changes in CM and UM

CM						UM					
	Gene	**Mechanism**	**Location**	**Cases (%)**	**Reference**		**Gene**	**Mechanism**	**Location**	**Cases (%)**	**Reference**
Proto oncogenes	NRAS	mutation	1p13	15-25	[71,72]	Proto oncogenes	NRAS	mutation	1p13	*	[95-98]
	AKT3	amplification	1q44	40-67	[73]		AKT3	amplification	1q44	-	
	BRAF	mutation	7q34	36-61	[74-76]		BRAF	mutation	7q34	48*2	[81,99-101]
	NBS1	amplification	8q21	*	[77]		NBS1	amplification	8q21	50	[62,102]
	MYC	amplification	8q24	1-40	[78-80]		MYC	amplification	8q24	43	[103]
	DDEF1	amplification	8q24	-			DDEF1	amplification	8q24	50	[63,69]
	GNAQ	mutation	9p21	83*1	[81]		GNAQ	mutation	9p21	46	[81,104]
	CCND1	amplification	11q13	6-44	[82-84]		CCND1	amplification	11q13	65	[69,105-107]
	HDM2	amplification	12q15	-			HDM2	amplification	12q15	97	[69,105,106]
	BCL-2	amplification	18q21	>90%	[85,86]		BCL-2	amplification	18q21	100	[105,108,109]
Tumor suppressor genes	LZTS1	deletion	8p21	-		Tumor suppressor genes	LZTS1	deletion	8p21	-	[23]
	CDKN2A-sporadic	deletion, mutation	9p21	*	[87]		CDKN2A-sporadic	deletion, mutation	9p21	*	[110]
	CDKN2A-familial	deletion, mutation	9p21	30-80	[88-91]		CDKN2A-familial	deletion, mutation	9p21	*	[110]
	PTEN	deletion, mutation	10q23	10-40	[92-94]		PTEN	deletion, mutation	10q23	15	[111,112]

- no data available

* rarely observed or sporadic reports in literature

*1 observed in 83% of blue naevi

*2 observed in 48% of iris melanomas

to inhibit p53 and its function of eliminating abnormal cells[105,106]. An elevated expression of *BCL-2*, located on 18q21, is observed in UM but also in normal melanocytes. This overexpression is reported to block apoptosis[105,106,108,109] and is suggested to be responsible for the resistance to chemotherapy or irradiation of melanocytes[69,116]. In 65% of UM cases, *CCND1* is reported to be overexpressed. Overexpression of *CCND1* leads to activation of cyclin dependent kinases (CDKs) which consequently phosphorylate and inactivate Rb[69,106,107]. The *CCND1* overexpression is associated with large tumor size, epitheloid cytology and poor prognosis[106].

Chromosomal Aberrations in CM

CMs display a more complex karyotype compared to UM. The most frequently observed chromosomal aberration in CM is monosomy 10. This aberration is found in approximately 60% of CM cases and appears to be significantly more frequent compared to UM, where monosomy 10 is found in 27% of cases[42](Figure 2). Because monosomy 10 could include loss of tumor suppressor genes, much research has been aimed at identifying possible tumor suppressor genes involved. Phosphatase and tensin homolog (*PTEN*) is one of the identified tumor suppressor genes, located on 10q23, with strong evidence for a role in CM tumorigenesis[92](Table 2). *PTEN* is thought to be inactivated by deletion or mutation and through loss of its negative regulatory effect on AKT, lead to activation of the AKT-pathway and consequently prevent apoptosis[85,111]. The actual inactivation of *PTEN* is observed in up to 30-40% of CM cell lines[92,93], but only in 10% of primary CMs. *PTEN* inactivation or downregulation is mainly found in tumors with an increase in aneuploidy, suggesting that it is a late event in tumor progression[27,111]. In UM, inactivation of *PTEN* is reported in 15% of cases and has been linked to an increase in aneuploidy but also poor clinical outcome[111,112]. The other frequently reported chromosomal aberrations involved in CM are: -1p, +1q, -4, -5, -6q, +7, -9p, -11q, -12q, -14, -15, -16, -17p, +18, +20, -21 and -22[42]. Some of them will be discussed here along with the most well-known genes, involved in tumorigenesis and/ or metastatic development.

Chromosome 1

Rearrangements of the distal part of the short arm of chromosome 1, leading to loss or gain of 1p are reported in 28% and respectively 33% of CMs. Several regions along chromosome 1 are of specific interest because they harbor the *NRAS*- and *AKT*3-gene. *NRAS* is located in the 1p13-region and shown to be activated by mutation in 15-25% of CMs[71,72]. *NRAS* is believed to be also involved in the MAP-kinase pathway. Activation of *NRAS* leads to activation of the MAP-kinase pathway and as a result cellular proliferation. Additionally, *NRAS* binds and activates lipid kinase phosphoinositide-3 kinase (PI3K), thereby activating the AKT-pathway and preventing apoptosis[85]. A direct activating mutation of the *AKT*3-gene located on 1q44 is found in 40-67% of CMs[73]. Overexpression of *AKT*3 renders cells less

sensitive to apoptotic stimuli and as mentioned before, *PTEN* inactivation can lead to the selective activation of AKT in CMs[92]. Different groups have shown *NRAS* mutations to be very rare in UM[95-98].

Chromosome 6

Alterations of chromosome 6 are reported in a total of 66% of CMs, of which +6p is observed in 24% and -6q in 42%[42]. Of these alterations, the 6q10-q27 region shows the highest frequency of rearrangements as a result of deletion, translocation or due to the formation of an isochromosome of its short arm. The region on the short arm of chromosome 6 that frequently show alterations spans from 6p21 to 6p25 and mainly results in gain of DNA material. Up till now, there have not been reports about possible over- or underexpressed genes on chromosome 6 involved in tumorigenesis. As mentioned before, both +6p and -6q are common in UM. The prognostic value of these alterations however, proved to be lower than in CM[42,46].

Chromosome 7

In 36% of CMs, gain of DNA-material on both arms of chromosome 7 is observed. Most frequently described are somatic mutations within the 7q34 region, where the *BRAF*-gene is located. Up to 60-70% of CMs are characterized by activating mutations in *BRAF*[74]. The *BRAF*-gene encodes a kinase involved in the MAP-kinase pathway which, by mutation, is thought to lead to constitutive activation of the aforementioned pathway[117] and cell proliferation. A single substitution (p.V600E) appears to account for more than 90% of all *BRAF* mutations[118]. The same mutation is also found in 80% of benign naevi and is therefore believed to be an early event in melanomagenesis[75]. There is however evidence from another study that indicates a role in later stages of tumor growth and development[76]. Mutations of *BRAF* were shown to be absent in UMs[95,99]. But in a small study, *BRAF* mutations were shown to occur in 48% of UM of the iris[100].

Chromosome 9

Chromosomal aberrations on chromosome 9 presenting as either deletions of the short arm, -9p10-24 (37% of CMs) or long arm, +9q22-34 (15% of CMs) have been reported. One of the best characterized genes in CM is *CDKN2*A, located on 9p21. Inactivating mutations, or loss, results in inactivation of the two encoding tumor suppressor genes *p16* and *p14*. Both genes were already related to high susceptibility for CM and were found in a total of 30-80% of familial CM[88-90]. These mutations are however rarely observed in sporadic CM[87] or UM[110].

Epigenetics

Over the last years, there have been growing interest for the role of epigenetics in CM and UM pathogenesis and metastasis. The most well-known epigenetic features are methylation and microRNAs (miRNAs). Both act through different mechanisms by which they are thought to alter normal gene transcription. Methylation is frequently reported to induce silencing of certain genes by direct methylation of DNA strands or hypermethylation of specific promoters. Because human cancers are theorized to cause global demethylation and promoter hypermethylation, it is thought this could lead to activation of imprinted genes and the inactivation of genes[119]. In CM, several genes commonly hypermethylated have been identified such as: *RASSF1A, APC, PYCARD, RARB, MGMT, DAPK, 3-OST-2, HOXB13, SYK, TIMP3A, CDKN2A, FHIT, SOCS1, SOCS2* and *PTEN*. In UM, the studies regarding gene/promoter methylation status are still limited but *CDKN2A* is found to be methylated in 33% of cases[120,121]. Similarly, *RASSF1* appears to be methylated in 13-70%[122] and *hTERT* in up to 52%[123]. It is not certain whether these methylated sites contribute to metastasis.

MiRNAs have recently come to the attention because of their inhibitory effect on translation of mRNAs into proteins. Although there are limited studies available on the role of miRNAs, several miRNAs have been marked as possibly involved in UM tumorigenesis and/or metastasis such as *let-7b*, *miR18a*, *miR-199a*, *miR495*, *miR549* and more[124,125]. Worley *et al* [124] and Radhakrishnan *et al* [125] reported differentially expressed sets of miRNAs that could accurately distinguish two different classes with a low and high risk potential for metastatic disease. These miRNAs were shown to bind to genes often found to be deleted in UM such as 8p22, but also 13q and 17p. In CM, many different miRNAs have been identified such as *miR-137*, *miR-182*, *miR-221*, *miR-222* and different subtypes of the *let-7* family[126-129]. These are thought to act as important factors in CM tumorigenesis and metastasis, further research is however required to analyze their exact role in CM.

The Relation Between CM and UM

Although there are many differences between CM and UM, they do share some features. First of all, both tumors derive from neural crest melanocytes which migrated to the epidermic tissue or the eye. This common origin is still observed on morphologic and gross histopathologic research of tumor material from CM and UM. The chromosomal regions frequently observed to be amplificated or deleted in both melanotic tumors do resemble each other although the exact frequencies in which they occur differ. For instance, monosomy 3 is observed in around 50% of UMs and in 25% of CMs. The same holds for gene expression status: many of the genes found to be frequently overexpressed or underexpressed in CM, are also observed in UM. Furthermore, both tumors are highly metastatic which is illustrated by the early initiation of metastases. UM however, is not known to spread by lymphogenous route as CM is. This is an important difference and possibly due to the

anatomical restrictions of the eye and the lymphatic system. Another difference concerns the role of UV-radiation, which appears to be an important risk factor for the development of CM but is not known as a risk factor for development of choroid-localized UM. There is however evidence regarding an interaction between UV-radiation and development of UM in the easily to sunlight exposed iris[7].

Maybe the two types of melanotic tumors are more similar than previously thought because of its common origin and are the differences merely a result of the exact location of the melanoma and its direct environment. Each location has its own array of carcinogens to which the tissue is subject to. For instance, the retina is less intensely exposed to UV-radiation than the skin. The epithelial environment the cutaneous melanocytes reside in, leads to the cells having more epithelial qualities of which downregulation of the molecule E-cadherin during local invasion is an example. Uveal melanocytes do not require this "mesenchymal to epithelial transition" because they are not in an epithelial environment. This could for part explain the differences in the spectrum of mutations between the two types of melanocytic tumors.

Conclusion

Despite all developments in diagnostics and therapeutics of primary UMs in the last 20-30 years, there have been no significant decrease in metastasis-related deaths[6,130]. The prognosis for patients with metastasized disease still is 2-7 months, regardless of systemic therapy. This is probably due to the early initiation of metastasis in both CM and UM, which underlines the need for early prognostication. This could, at least for part, be achieved by continuing the search for prognostic factors in CM and UM through genetic research on tumor material. Genetic research has showed us that CM and UM have aberrations in common but that these differ in frequency between the two tumors. Even so, both express many of the same genes but not all. In CM, alterations of chromosomes 1, 6, 7, 9, 10, 14, 16 and 21 are frequently observed and already several candidate genes and proteins involved in the tumorigenesis of CM have been identified. UMs were shown to frequently display chromosomal aberrations on chromosomes 1, 3 and 8. Of these, monosomy 3, gain of 8q and the combination of loss of 1p36 and monosomy 3 appeared to be significant prognostic factors for decreased survival. There have not been identified genes yet that are prognostically active in UM, and at this point developments in UM lag behind compared to CM. New insights in UM however, came about by gene expression profiling of UMs which were shown to cluster naturally in two classes with different prognosis[52-54]. Generally, array technology has proven to outperform clinical and histopathological parameters in determining a patients' prognosis. This led to the frequent usage of gene expression testing in the current clinical setting in an attempt to identify high risk patients. We do have to remind we do not yet know whether monosomy 3 and classifier genes are truly involved in tumor

progression and metastatic potential or that those are merely markers of the underlying cause. Additionally, we have to evaluate whether these results may aid clinicians in assessing eligibility of patients for future (adjuvant) systemic therapies. Most of the genetic research is conducted on relatively large UMs because small UMs are treated conservatively and this has biased UM research. Recent groups already reported about the suitability of fine-needle aspiration biopsy in harvesting of tumor material from patients treated with eye-saving modalities[131-134]. Also for this diagnostic option we have to evaluate whether this will be beneficial for patient care and can lead to predictions about prognosis for the individual patient. Some genetic markers have already proven its value in predicting prognosis next to clinical and histopathological markers and could lead to selection for patient-tailored therapies in the near future. The challenge will be to prove or disprove the cost-effectiveness of array technology and find additional genetic markers predictive of worse prognosis in CM and UM patients.

Concluding, much information has been gained by genetic research of melanonomas and further research could augment our knowledge. Because there are similarities between the two tumors, research on one of two tumors could provide clues for research on the other. Epigenetics, the whole new field in genetic research, does look like a promising ally in our quest to understanding of pathogenesis and metastasis in CM and UM and might provide us with valuable prognostic information in the near future.

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Part 2.

Radiotherapy in uveal melanoma

Chapter 3

Risk factors associated with secondary enucleation after fractionated stereotactic radiotherapy in uveal melanoma

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Abstract

Aims: To report on risk factors of secondary enucleation after fractionated stereotactic radiotherapy (fSRT) in uveal melanoma (UM).

Methods: In total 118 patients had received fSRT. The patients who had undergone secondary enucleation were either grouped in a 'treatment failure' group or a 'complications' group according to the reason for secondary enucleation. Both patient groups as well as the group of patients who had been successfully treated with fSRT, were compared for clinical, histopathological and cytogenetic data.

Results: The secondary enucleation rate was 16% after a median of 4.7 years of follow-up, with 5% due to treatment failure and 11% due to complications. Logistic regression showed the main predictive factors for secondary enucleation to be: large tumor height (odds ratio [OR], 1.5; 95% confidence interval [CI], 1.2–1.9 $p<0.001$); and optic disc involvement (OR, 6.4; 95% CI, 1.2–37.7 $p=0.030$). Gain of chromosome 8q was frequently present in the secondary enucleation group (56%); most frequently in the treatment failure group ($p=0.013$). Mitotic figures were frequent in tumors with gain of chromosome 8q ($p=0.015$).

Conclusion: Secondary enucleation after previous fSRT was mainly associated with (large) tumor height and optic disc involvement. Gain of chromosome 8q and high mitotic counts were frequent in the treatment failure group. Tumor biopsy prior to radiotherapy may not only be used to analyze the risk of metastasis but also the risk of secondary enucleation.

Introduction

Uveal melanoma (UM) is the most frequent intraocular malignancy among adults. Local therapy is based on achieving adequate tumor control with - if possible - conservation of the eye, vision and cosmetic appearance. Nowadays, most small- and medium-sized UMs are treated with one of the available eye-conserving therapies with excellent tumor control rates[1-2]. Brachytherapy and proton beam radiotherapy are frequently used techniques as well as stereotactic radiotherapy. However, proton beam radiotherapy is an expensive technique and therefore limited available across the world. Stereotactic radiotherapy is a suitable alternative to proton beam radiotherapy, providing comparable tumor control rates especially when administered in several treatment sessions as with fractionated stereotactic radiotherapy (fSRT)[3-5].

Eleven to twenty-four percent[2, 5-7] of patients treated with eye-conserving techniques has shown to develop complications, or has failure of local tumor control requiring secondary enucleation or re-irradiation[6, 8]. The choice between radiotherapy and enucleation in case of medium-to-large sized uveal melanomas may be easier made if the clinician has information on clinical, histopathological and genetic parameters that signify a higher risk of secondary enucleation.

The aims of this study therefore, were to evaluate what tumor characteristics – clinical, histopathological, and genetic – predispose to secondary enucleation after fSRT. Chromosomal analysis was conducted since the relation between secondary enucleation and cytogenetic aberrations of chromosomes 3 and 8 is still unknown.

Materials and methods

All patients diagnosed with choroidal or ciliary body melanoma and treated by fSRT between 1999 and 2009, were included in this retrospective study. Diagnosis of uveal melanoma was established by indirect ophthalmoscopy, fundus photography and ultrasonography. Standardized B-scan ocular ultrasonography was used for determination of largest basal tumor diameter and tumor height. According to the COMS-classification[9], there were 103 medium (87%) (diameter < 16 mm, height < 10 mm), and 15 large (13%) (diameter > 16 mm, height > 10 mm) size tumors present in this series. A total dose of 50 Gy in five fractions of 10 Gy was delivered on five consecutive days. The Rotterdam eye fixation system and the treatment techniques per se, have been described in detail in a previous publication[3]. The study was performed according to guidelines of the Declaration of Helsinki and informed consent was obtained from all patients prior to therapy. Clinical data such as gender, age at time of diagnosis, best corrected visual acuity, intraocular pressure, tumor characteristics involving size and location, and co-morbidities were collected and recorded at baseline.

All patients were evaluated six weeks after irradiation, at three months, and every three months thereafter for the first two years. After two years of follow-up, patients were evaluated every four months. During these visits, response to treatment was evaluated by ultrasound measurement of tumor dimensions, complications were recorded if present, and patients were screened for presence of metastases by liver enzyme blood tests. If blood tests showed abnormalities, abdominal ultrasonography or CT-scanning was conducted. If complications requiring therapy were found, these were treated accordingly.

Secondary enucleation was performed in patients with treatment failure due to progressive intraocular tumor growth or recurrence (hereafter referred to as treatment failure). Progressive tumor growth was determined if there was intraocular tumor growth of more than 25% on fundoscopic examination and ultrasonography at any time during follow-up. Tumor recurrence was determined if there was tumor growth after a period with no visible tumor tissue remaining. Secondary enucleation was also performed in patients who developed complications from fSRT such as severe ocular pain due to intractable neovascular glaucoma, other pain symptoms, or diffuse radiation retinopathy (hereafter referred to as complications from fSRT). For comparison, all secondarily enucleated patients were sub-grouped according to the reason for enucleation, either in the 'treatment failure' or 'complications' group. Baseline fSRT tumor characteristics of patients in each of these groups were analyzed and compared. Post-fSRT data from histopathological and genetic research on tumor tissue was obtained from secondarily enucleated eyes. The secondarily enucleated patients were also evaluated according to our standard follow-up program as mentioned earlier. Further follow-up data regarding development of metastasis and tumor-related death was obtained from medical records and by contacting the general physician. Metastatic development was recorded including time from diagnosis to metastasis (disease-free interval), and was analyzed for the total patient's group as well as sub-groups.

Pathologic research

Conventional histopathological examination was performed on all secondarily enucleated eyes and confirmed the origin and type of the tumor, as well as tumor dimensions. Cell-type was defined and recorded, as well as the presence of microvascular patterns (closed vascular loops), mitotic figures, necrosis, scleral invasion and optic nerve invasion. Mitotic figures were counted in an equivalent of 50 high power fields (HPF), and viewed under the microscope at 400X magnification with a single field view of 0.45 mm in diameter. This related to a total area of 7.95 mm^2. Additionally, neovascular membranes were recorded if present.

Cytogenetic research

Fresh tumor tissue and paraffin tumor sections were analyzed for presence of chromosomal alterations by fluorescence in situ hybridization (FISH) (chromosomes 1p, centromere 3, 3q, 6p, 6q, 8p, centromere 8, and 8q) as described by Naus et al[10] and Mensink et al[11]. Signals were counted in 300 interphase nuclei, according to the criteria of Hopman et al[12]. Cut-off threshold for deletion on fresh tumor tissue were: >15% of the nuclei with one signal, and for amplification: >10% of the nuclei with three or more signals, as described by Van Dekken, et al[13]. The cut-off threshold for deletion on paraffin sections (>25% of the nuclei with one signal) was adapted from our own research as a measure to correct for truncation and cutting-artifacts. The cut-off threshold of 10% for amplification was left unchanged as truncation and cutting-artifacts are not a major issue for cells showing more than two signals.

Statistical analysis

Univariate logistic regression was performed for the identification of significant clinical variables predicting secondary enucleation. With exact logistic regression, multivariate analysis was conducted of the variables that were significant in the univariate analysis. Correlations between pathological risk factors, genetic risk factors and sub-groups were analyzed by Fisher's exact tests with Bonferroni-Holm correction for multiple testing. Kaplan-Meier survival analysis was performed for the irradiated patients and secondarily enucleated patients. Statistical significance was evaluated by using the log-rank test and values were considered significant at a two-tailed p-value of <0.05. All analyses were performed using the statistical software SPSS Version 17.0 (SPSS Inc., Chicago, IL, USA).

Results

In total 118 patients had received fSRT, of which 19 patients (16%) had required secondary enucleation during follow-up. The median follow-up was 4.7 years, ranging to 11.0 years. Two patients were lost to follow-up before the end of our study. The first patient had been followed-up for 57 months before she was lost to follow-up at an age of 82 years old. The second patient had been followed-up for 81 months, she moved to an elderly home with unknown location at age 73. Both patients had not undergone secondary enucleation and were lost to follow-up without signs of metastasis at the last visit. Seventy percent of UMs were located within 3 mm of the optic disc or fovea, with 13 tumors (11%) involving the optic disc (Table 1). There were no cases with extra-ocular extension present in this series.

Exact logistic regression analysis showed that large tumor height (odds ratio [OR], 1.5; 95% confidence interval [CI], 1.2–1.9; $p<0.001$) and optic disc involvement (OR, 6.4; 95% CI, 1.2–37.7, $p=0.030$) significantly increased the risk of secondary enucleation (Table 2). There was no significant relation found between secondary enucleation and other known clinical risk factors such as male gender, old age, tumors with large basal diameter, or tumors that

Table 1. Demographics and clinical features patients of treated by fSRT (N = 118).

	Mean and median	Range
Age at diagnosis	62 and 62 years	28-84 yrs
Largest basal tumor diameter	12.0 and 11.9 mm	7.2-18.9 mm
Tumor height	6.20 and 6.20 mm	2.2-11.1 mm
	Number	**Percentage**
Gender		
Female	54	45.8
Male	64	54.2
Involved eye		
Left	73	61.9
Right	45	38.1
BCVA		
< 10/ 20	54	45.8
> 10/ 20	64	54.2
Involvement of ciliary body		
No	111	94.1
Yes	7	5.9
Anterior margin		
Post ora	81	68.6
Pre ora	37	31.4
Posterior margin		
< 3 mm from optic disc/ macula	82	69.5
> 3 mm from optic disc/ macula	36	30.5
Shape		
Dome	98	83.1
Mushroom	20	16.9
Coronal location		
Temporal	48	40.7
Nasal/ midline	70	59.3
Sagittal location		
Superior	49	41.5
Horizontal/ inferior	69	58.5
Optic disc involvement		
No	105	89.0
Yes	13	11.0
Vitreous hemorrhage		
No	113	95.8
Yes	5	4.2
Retinal detachment		
No	40	33.9
Yes	78	66.1
Intraocular pressure		
< 21 mmHg	115	97.4
> 21 mmHg	3	2.6
Diabetes/ poor general health		
No	114	96.6
Yes	4	3.4

BCVA = best corrected visual acuity

Table 2. Independent clinical risk factors predicting secondary enucleation identified by logistic regression of all 118 cases.

	p-value	Odds Ratio	95% Confidence interval	
Tumor height*	<0.001	1.482	1.172	1.930
Optic disc involvement	0.030	6.410	1.171	37.675

* per 1 mm increase

involved the ciliary body. Although a large tumor diameter was not correlated with high risk of secondary enucleation, it was confirmed as an independent prognostic factor for metastasis in this series (OR 1.5; 95% CI, 1.2-1.8; p<0.001) (results not shown).

Of the patients who required secondary enucleation, further analysis revealed progressive intraocular tumor growth to be the reason for enucleation in four out of 19 patients (21%), tumor recurrence in two out of 19 patients (11%), and painful eye due to neovascular glaucoma in 12 out of 19 patients (63%) and diffuse radiation retinopathy in one patient (5%) (Table 3). One of the eyes that had to be enucleated because of complications from fSRT was found to contain massive inflammation with necrotic cell debris and no vital tumor cells remaining (Table 3, case 19). Histopathological and cytogenetic research of tumor tissue from this eye was therefore not possible. The remaining 18 eyes all yielded sufficient tumor material for successful research of histopathological and genetic factors. In eleven cases FISH was successfully used on fresh tumor tissue and in seven cases tumor tissue sections had to be used additionally. In the 11 cases of neovascular glaucoma with tumor tissue available, the diagnosis could be confirmed by pathological research showing peripheral anterior synechiae, neovascular membrane along parts of the iris, and a closed angle (p=0.002, Table 4a). In the remaining case with radiation retinopathy, no anterior synechiae, or neovascular membrane, or closed angle were found. Mitotic figures (≥ 5 per 50 HPF) were not correlated with either the treatment failure group or the complications group (p=0.131).

Amplification of chromosome 8q (gain of chromosome 8q) was found in ten cases (53%) and in six out of ten cases there was no simultaneous gain of chromosome 8p, indicating that chromosome 8q aberrations were more frequent in this series than gain of a complete

Table 3. Clinical, histopathological and genetic features of patients who had undergone secondary enucleation (treatment failure: case 1 to 6, complications: case 7 to 19).

N	Sex	Age	Time to metastasis (yrs)	LTD/TH	Juxta papillary location	Pathology	Chromosomal aberrations	Ocular medical history	Co-morbidities during follow-up
1	f	73		15.0/ 11.1		Mixed/ epitheloid cell (M)	-3, -6q, +8p, +8c, +8q	Amblyopia other eye	Retinal detachment, progressive tumor growth
2	f	66		11.4/ 2.2		Spindle cell (S), Mitotic figures (MF)	+6p, +8q		Cataract surgery, diabetic retinopathy, tumor recurrence
3	m	80	2.19	18.9/ 8.4		M, Vascular loops (V), Ciliary body, MF	-1p, -3, -6q, +8p, +8c, +8q		Cataract surgery, progressive tumor growth
4	m	84	2.96	13.5/ 5.6		S, MF	-3, +6p, +6q, +8p, +8c, +8q		Progressive tumor growth
5	f	67		13.1/ 4.8		S, MF	1p n/a, -3, +6p, -6q, +8q	Scleral buckle surgery	Tumor recurrence
6	m	44		14.5/ 8.8	Yes	M, Optic nerve invasion	+8q		Vitrectomy (hemorrhage removal), cataract surgery, glaucoma medication, progressive tumor growth
7	m	67	4.73	11.6/ 9.9		M, Neovascular membrane (NV), MF	-1p, -3		Subretinal punction (retinal detachment), NVG
8	m	45	8.19	15.2/ 7.8		M, NV, Scleral invasion (SI)	+6p		Peripheral iridectomy, cataract surgery, NVG
9	m	51		10.7/ 6.9		M, V, NV, SI	-1p, -3, 6p n/a, 6q n/a, 8p n/a		Vitrectomy (hemorrhage removal), NVG
10	m	48		12.3/ 9.9		M, NV, SI	-3, 6q n/a, -8p, +8q		NVG, died due to cardiac arrest
11	m	62		10.2/ 10.5	Yes	M, NV	-1p, -3		Glaucoma medication, cataract surgery, NVG
12	m	28		12.3/ 7.3	Yes	M, NV	+1p, -3, +6p, +6q, +8p, +8c, +8q		Vitrectomy (removal traction membrane), NVG
13	m	65		8.8/ 5.9		S, NV	-1p		Cataract surgery, Vitrectomy (traction membrane), NVG
14	f	38		16.0/ 7.8		S	-6p, -6q		Unknown pain symptoms with radiation retinopathy

15	m	73		15.2/ 4.7	Yes	S, NV	+1p, +3, +6p, +6q, +8p, +8c, +8q	NVG
16	m	77		11.1/ 7.1		M, V, SI, NV	-1p, -3, +6p, -6q, +8q	Glaucoma medication, vitrectomy (ghost cell glaucoma
17	m	44		9.3/ 10.5		M, SI, NV	-6p	after anti-coagulant therapy), NVG
18	m	56		12.4/ 10.1		S, V, NV, SI	6p n/a, 6q n/a, 8p n/a, 8c n/a	Subretinal punction (retinal detachment), peripheral
								iridectomy, NVG
19	m	74	5.59	13.4/ 8.5		n/a	n/a	Vitrectomy (retinal detachment), cataract surgery,
								intravitreal antibiotic therapy (endophthalmitis), NVG

NVG = Neovascular glaucoma; Mitotic figures (MF): more than 5 mitosic figures per 50 HPF present

n/a = data not available

copy of chromosome 8. Gain of chromosome 8q was present in six out of six tumors (100%) from the treatment failure group, compared to four out of twelve tumors (33%) from the complications group (p=0.013, Table 4a). In the treatment failure group, gain of chromosome 8q was found in at least 20% of tumor cells analyzed from one tumor (Table 3, case 1), ranging to 83% of cells in the other five tumors (Table 3, case 3). In the complications group, four cases displayed gain of chromosome 8q: one tumor displayed gain of 8q in 18% of cells (paraffin tumor sections) where only 60 cells could be counted due to low quality of tumor cells (case 10). The second tumor had gain of 8q in 30% of nuclei as well as gain of chromosomes 1p, 6p, 6q, 8p, centromere 8, but with loss of chromosome 3 and therefore possibly represented a hypertriploid tumor with relative loss of chromosome 3 (case 12). The third tumor displayed gain of chromosome 8q in 85% of nuclei with chromosomal gain of all other probes tested, thereby possibly representing a hypertriploid case without relative loss of chromosome 3 (case 15). The remaining case from the complications group displayed gain of chromosome 8q in 18% of tumor cells (case 16) with the normal 300 nuclei counted. Although mitotic figures were not correlated to treatment failure, there was a significant correlation with the tumors that gained an extra copy of chromosome 8q (p=0.015, Table 4b). There were no additional extra copies of chromosome 8q found in this study other than one extra copy.

Loss of chromosome 3 was found in 10 tumors and simultaneous gain of chromosome 8q was present in six cases (Table 3) though no correlation was found between loss of chromosome 3 and either the treatment failure group or complications group. Although the tumor sizes from the complications and treatment failure group were comparable (Table 5), there were differences in tumor size when tumors from either group were compared with tumors

Table 4a. Correlation between histopathologic- and cytogenetic risk factors, and reason for secondary enucleation.

		Tumor failure	Complication	p-value
Histopathologic data				
Neovascular membrane	No	6	1	0.002
	Yes	0	11	
Cytogenetic data				
Gain of chromosome 8q	No	0	8	0.013
	Yes	6	4	

Table 4b. Correlation between mitotic figures and gain of chromosome 8q.

		Gain of 8q	No gain of 8q	p-value
Mitotic figures	< 5 / 50 HPF	2	7	0.015
	≥ 5 / 50 HPF	8	1	

HPF = high power field

Table 5. Comparison of mean tumor sizes of: the complications group with the treatment failure group, the complications group with the fSRT group*, and treatment failure group with the fSRT group†.

	Complications		Treatment failure		p-value	Successful fSRT treatment		p-value
Tumor diameter (range)	12.2 mm	(8.8-16.0 mm)	14.4 mm	(11.4-18.9 mm)	0.074	11.9 mm	(7.2-18.5 mm)	0.700* and **0.033†**
Tumor height (range)	8.2 mm	(4.7-10.5 mm)	6.8 mm	(2.2- 11.1 mm)	0.240	5.9 mm	(2.3- 10.9 mm)	**0.001*** and 0.355†
Tumor volume (range)	568.4 mm^3	(195.7-906.3 mm^3)	664.1 mm^3	(119.5-1307.7 mm^3)	0.645	434.9 mm^3	(62.2-1282.8 mm^3)	0.134* and 0.089†

Tumor volume measured by: π/ 6 x horizontal diameter x vertical diameter x height

from the successful fSRT treatment group: tumors from the complications group were greater in height (mean tumor height 8.2 mm vs 5.9 mm, p=0.001) and tumors from the treatment failure group were larger in diameter (mean tumor diameter 14.4 mm vs 11.9 mm, p=0.031) compared with the successful fSRT treatment group. There were no correlations found for post-fSRT tumor size and the sub-groups mentioned above.

Melanoma-related metastasis was eventually found in 25 out of the total of 118 patients (25.3%), with five patients from the group of 19 patients (26.3%) who had undergone secondary enucleation. Kaplan-Meier survival analysis revealed no difference in disease-specific survival of the patients who had required secondary enucleation and the patients who had not (Figure 1). Although tumors that metastasized had a large mean diameter at baseline (mean LTD 14.1 mm, p<0.001) (results not shown) and tumors from the treatment failure group were larger than the successfully treated tumors that did not require secondary enucleation (LTD 14.4 mm vs 11.9 mm, p=0.033), no difference in disease-specific survival could be found with the current follow-up and patients group. The two patients from the treatment failure group, who developed metastasis, had a disease-free interval of 2.2 and 2.9 years which is comparable to the median disease-free interval of 3.0 years in the fSRT group.

Discussion

Sixteen percent of our irradiated patients (N=19) required secondary enucleation. This is comparable to other studies, which report secondary enucleation rates of around 16-27% for fSRT[5, 14], 11-24% for proton beam radiotherapy[2, 15-18], and around 11% for conservative therapies combined[6] with 5 to 15 years of follow-up. However, it is difficult to compare studies because inclusion criteria for tumor size and location may differ, as well as follow-up time, therapeutic doses delivered, the number of fractions used and the strategy for managing painful neovascular glaucoma. Some physicians prefer to treat patients with neovascular glaucoma by analgesics and intravitreal injections, while others prefer enucleation.

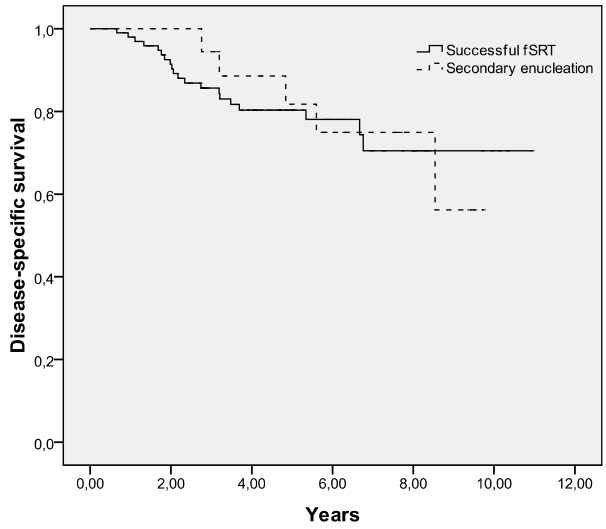

Figure 1. Kaplan-Meier survival curve of patients managed by stereotactic radiotherapy alone versus patients that underwent secondary enucleation after stereotactic radiotherapy.

Our results show that the overall risk of secondary enucleation due to fSRT failure as well as complications from fSRT, increased significantly if tumors were large in height or if they involved the optic disc. Large tumor height was reported as a prognostic factor for secondary enucleation in several other studies regarding eye-conserving treatment[6-7, 15, 19] as well, as to the involvement of the optic disc[6, 15-16], location close to the fovea[7, 15], large tumor diameter[6, 15-16] and poor baseline visual acuity[7]. In this study, large tumor diameter proves to be the most important clinical marker for metastasis underlining earlier publication[20].

Secondary enucleation enabled histopathological and cytogenetic examination of the previously irradiated tumors (which had not been biopsied prior to treatment). All cases with neovascular glaucoma were confirmed by the presence of peripheral synechiae, neovascular membranes and closed angle on pathologic examination. Painful neovascular glaucoma is a known cause for secondary enucleation after radiotherapy, which is associated with large tumor height in general[21-22] and a height greater than 5 mm[23-24] more specifically. Patients from the complications group all had tumors with a height greater than 5 mm, except for one patient who had a tumor measuring 4.7 mm in height (Table 3, case 15). The mean tumor height of 8.2 mm in patients from the complications group was the largest compared to the tumors of the treatment failure group or the successfully treated group (Table 5). Nearly all enucleated eyes and especially the cases from the complications group, also received extensive medical treatment to the affected eye before and after fSRT (Table 3).

These measures may also have increased the risk of other complications eventually resulting in secondary enucleation.

Gain of chromosome 8q was found in 10 out of the 18 (56%) secondarily enucleated eyes where cytogenetic markers could be analyzed. Gain of chromosome 8q is normally found in 40% of primary enucleated UMs[25-27]. All six tumors from the treatment failure group had gain of chromosome 8q, while four tumors from the complications group had gain of 8q (p=0.013) with one equivocal call for gain of 8q. In case 10 (Table 3), borderline amplification of chromosome 8q was found in 18% of tumor cells with only 60 cells counted due to low quality of tumor cells. The tumors from the treatment failure group were larger in diameter than tumors that had been treated successfully by fSRT (14.4 mm vs 11.9 mm, p=0.033). As gain of chromosome 8q is known to relate to larger tumor size[25, 28-29], this could partly explain the high frequency of 8q gain in the treatment failure group. However, there was no difference in tumor size for all tumors with and without gain of chromosome 8q in this study. There was a link between gain of chromosome 8q and high number of mitotic figures (≥5 per 50 HPF) in the tumor sections analyzed. Mitotic figures indicate the ability of tumor cells to reproduce resulting in active intraocular growth. The presence of mitotic figures has been linked to mortality before[30] indicating a high risk of metastasis in recurring tumors that were initially treated with proton beam radiotherapy[18, 31-32]. We hypothesized that tumors with gain of chromosome 8q were more aggressive in behavior due to their chromosomal alterations, thereby actively growing regardless of the radiation dose currently administered, which eventually resulted in secondary enucleation. It cannot be ruled out that the four tumors with gain of chromosome 8q from the complications group had been enucleated before recurrence could develop. The mean time to secondary enucleation in this group was 2.5 years (median 2.0 years) compared to 3.1 years in the treatment failure group (median 2.4 years). The tumors with gain of chromosome 8q and high mitotic numbers could thus potentially mark the sub-group of aggressive tumors. With the present follow-up, no increased risk of metastasis could be determined for patients having tumors with gain of chromosome 8q or progressive or recurring intraocular tumor growth after fSRT. Future biopsy prior to treatment would be valuable to determine the incidence of chromosomal alterations in the successfully irradiated eyes as well.

A total radiation dose of 50 Gray was found to be the optimal dose for killing the most radiosensitive cells, yet spare the critical structures[3]. Increasing the dose could potentially lower the number of cases with inadequate tumor control but could on the other hand increase the risk of neovascular glaucoma or radiation retinopathy and – thus – the number of secondary enucleations. Gain of chromosome 8q or isodisomy 8q was more frequent in this series than trisomy 8, suggesting the 8q-arm as interesting region for pinpointing of candidate genes involved in resistance to radiotherapeutic treatment. Until now, several regions on

chromosome 8q have been suggested to harbor candidate oncogenes involved in UM such as MYC[33] or DDEF1[34]. It is unknown whether these genes are involved in radiotherapeutic sensitivity as well. Fine mapping studies may further define smaller regions of interest on chromosome 8q and guide the search for candidate genes. Together with large tumor height, involvement of the optic disc by tumor, presence of mitotic figures, and gain of chromosome 8q could be important markers for individualizing dose fractionation schedules in the future. Further research evaluating these markers in larger studies is required.

One of the limitations of the current study is the low number of patients with tumor tissue available for research. Analysis of clinical factors was possible in all 118 cases and allowed for logistic regression analysis but this was not possible for histopathological and genetic factors as only 19 eyes were enucleated. Since the incidence of secondary enucleation after eye-conserving therapies is low, it is difficult to obtain data of a large group of secondarily enucleated patients. FISH analysis of irradiated tumor tissue is difficult due to necrotic cells but if sufficient DNA can be isolated from tumor cells, SNP-array analysis may provide data in these difficult cases. SNP-array would also be suitable for the analysis of chromosomal regions with loss of heterozygosity, as previously reported by Lake et al[35]. Another limitation is that histopathological and genetic factors could only be determined on tumors that had previously been irradiated, leading to uncertainty about the timing of the chromosome 8q alterations. These alterations may have been present before fSRT, induced by fSRT, or occurred while the UMs were actively growing regardless of the current dose of fSRT administered. As the cytogenetic alterations found in this study resemble the distinct chromosome 3 and chromosome 8 alterations frequently found in primary enucleated eyes containing UM[28, 36], it is less likely these were induced by fSRT. As several groups already reported fine-needle biopsy to be a safe and reliable technique yielding sufficient tumor tissue for cytogenetic analysis[10, 37-38], biopsies may be valuable for determining the timing of 8q alterations and guide future prognostication and planning for local therapy.

This is to our knowledge the first report with an analysis of cytogenetic risk factors next to clinical and histopathological risk factors for secondary enucleation after eye-conserving therapy and may thus serve as a starting point for further research. Future genetic research on biopsied tumor tissue may further define the relation between fSRT failure and chromosomal alterations, and differentiate non-responding tumors from responding tumors.

In summary, we have found that tumor thickness and location close to the optic disc indicate a high risk of secondary enucleation after fSRT. Certain genetic factors may be more frequent in patients who required secondary enucleation following fSRT. Tumor biopsy prior to radiotherapy may not only be used to analyze the risk of metastasis but also the risk of secondary

enucleation. This could help the clinician to better advice patients in the choice between primary enucleation and radiotherapy.

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Part 3.

Characterization of chromosomal aberrations in uveal melanoma

Chapter 4

MLPA equals FISH for the identification of patients at risk for metastatic disease in uveal melanoma

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Abstract

Aims: In uveal melanoma (UM) loss of chromosome 3 and gain of chromosome 8q are associated with high risk of metastasis. In this study, we validated the use of multiplex ligation-dependent probe amplification (MLPA) to detect patients at risk for metastatic disease in comparison to the predictive power of fluorescence in situ hybridization (FISH).

Methods: For 64 uveal melanoma samples, the MLPA results of chromosome 3 and 8 were compared with the results obtained by FISH. For seven samples, a single nucleotide polymorphism (SNP)-array was performed to clarify discrepancies. Clinical information together with the histopathology and chromosomal aberrations of chromosome 1, 3, 6 and 8 were evaluated for correlation with the patients' prognosis.

Results: Loss of chromosome 3, loss or gain of 8p and gain of 8q, found with MLPA, correlated with a significantly lower disease free survival ($p<0.001$). On the basis of the clinical outcome, 12 patients would have been classified incorrectly using MLPA results of chromosome 3 and 8. FISH results led to the same incorrect classification. Four patients with abnormalities of chromosome 3 and 8 in the tumor, detected with MLPA, are still alive without metastasis. Eight patients without concurrent aberrations of chromosome 3 and 8 in the tumors died due to metastasis. The sensitivity of MLPA to detect patients at risk for metastatic disease is higher than with the results obtained with FISH (0.795 vs. 0.692). The specificity is equal for both techniques (0.840).

Conclusion: MLPA is able to detect patients at risk for metastasis using the results for chromosome 3 and 8. There is no significant difference in the predictive power of MLPA compared with FISH.

Introduction

Uveal melanoma (UM) is the most common primary intra-ocular tumor with an incidence of 7 per million in the Western world. The treatment of primary UM consists of thermotherapy, radiotherapy or enucleation [1-2]. Approximately 45% of patients develop metastasis, mainly in the liver. As there is no effective treatment for metastatic UM, most patients will die shortly after metastatic spread [3]. Over the years, several prognostic factors have been identified, which can distinguish patients at risk for metastasis. Prognostic clinical and histological parameters include age at the time of diagnosis, tumor location, largest tumor diameter (LTD), the presence of epithelioid cells, a closed vascular loop pattern in the tumor and extra-scleral extension [4]. Extensive research has been performed to identify genetic prognostic factors. Loss of chromosome 3 and gain of chromosome 8q as detected by FISH are associated with metastatic related death [5-6], whereas gain of the chromosomal region 6p correlates with a favorable prognosis [7].

A large variety of molecular techniques is available to detect such chromosomal aberrations. Classical karyotyping can detect large chromosomal aberrations and balanced rearrangements of chromosomes. However, small abnormalities can be missed. Classical karyotyping is a labor-intensive and time consuming technique requiring specialized trained personnel.

Fluorescence in situ hybridization (FISH) has proven to be a faster and reliable technique for the detection of chromosomal aberrations in UM [8]. A drawback of this technique is that only a limited amount of loci can be tested in one single experiment and that it cannot be automated easily [9]. Multiplex ligation-dependent probe amplification (MLPA) is a PCR-based technique that allows the relative quantification of up to 45 loci in one single reaction.

The aim of this study was to test the efficacy of the Salsa P027 Uveal Melanoma MLPA-kit in identifying patients at risk for metastasis of UM in comparison with the predictive power of FISH. Aberrations on chromosomes 3 and 8 detected by MLPA were compared to the results obtained by FISH and/or chromosomal comparative genomic hybridization (cCGH). When differences in the results were observed, a SNP-array was performed to clarify these discrepancies.

Methods

Patients and tumor samples

UM samples were collected over a period of 10 years (1993-2003) from patients who underwent enucleation at the Erasmus University Medical Center or the Rotterdam Eye Hospital. Informed consent was obtained prior to enucleation and the study was performed according

to the tenets of the Declaration of Helsinki. Fresh tumor material was harvested within 1 hour after surgery and processed for FISH as described previously [8]. Part of the tumor was snap frozen and stored in liquid nitrogen. The remains of the eye were embedded in paraffin. Confirmation of the diagnosis UM was performed by histopathological examination. Only tumors located in the ciliary body and choroid were included in this study.

DNA isolation

Hematoxylin and eosin staining was conducted on a 5-µm section of snap-frozen tumor to survey tumor content. Depending on the size of the tumor, 10 to 15 sections of 20µm were used for DNA-isolation using QIAamp DNA-mini kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. DNA-concentration was measured using the NanoDrop® ND-1000 Spectrophotometer (NanoDrop technologies, Wilmington, Delaware, USA).

Multiplex ligation-dependent probe amplification

The Salsa MLPA-kit P027 Uveal Melanoma (MRC Holland, Amsterdam, the Netherlands) was used to identify the chromosomal abnormalities in 64 UM samples. This kit contains several probes that are located on the chromosomes frequently involved in UM: seven probes on chromosome 1p, 13 probes on chromosome 3 (nine probes located on 3p and four on 3q), six probes on chromosome 6 (four probes on 6p and two probes on 6q), and five probes on chromosome 8 (one probe on 8p and four probes on 8q). MLPA reactions were performed with 150ng of tumor DNA and 200ng of female or male reference DNA. As negative control 5µL of Milli-Q water was used in each experiment. The procedure was carried out as described by Schouten et al.[10]. MLPA reactions were carried out on a PCR thermocycler with a heated lid (Biometra Thermal Cycler, Westburg, the Netherlands). Ten microliters of highly deionized HI-DI-formamide (Applied Biosystems, Foster City, California, USA) was mixed with 0,15µl of an internal size standard (GeneScan LIZ-500) and 2µL of the PCR product. This mixture was analyzed by capillary electrophoresis on an ABI 3730 GeneScan system (Applied Biosystems).

Data analysis

The results obtained from the ABI 3730 GeneScan system were analyzed using GeneMarker 1.51 software (SoftGenetics, LLC, State College, Pennsylvania, USA). The peak areas and the sizes of the different MLPA-probes were determined. For each probe a Relative Quantity (RQ) value was calculated using population normalization and using the internal control probe normalization. Loss of DNA was defined as RQ value of less than or equal to 0.7 and gain as RQ value of more than or equal to 1.3. An RQ value between 0.7 and 0.8 was considered borderline loss, whereas an RQ value between 1.2 and 1.3 was considered as borderline gain. If more than 50% of the probes present on a chromosome arm showed

RQ values lower than 0.8 or higher than 1.2 that chromosome arm was considered to be aberrant. This implies that the RQ values of four probes for chromosome 1p must have an abnormal RQ value, and for five probes for chromosome 3p. The regions 3q, 6p and 8q must display two probes with aberrant RQ values, and for both 6q and 8p the RQ-value of one probe must be abnormal in order to consider that chromosome arm aberrant.

Fluorescence in situ hybridization

Dual-color FISH on non-cultured tumor cells was performed on 64 tumors as described [8]. If no sufficient fresh tumor material was available to test multiple loci, FISH was carried out on paraffin sections (n=25) of 4-5µm as described previously [11]. The following probes were used: RP11-48E9 (1p36), RP11-384L8 (3p22) or RP11-522N9 (3p13), Pα3.5 (centromere 3), YAC 827D3 (3q24), RP11-356B3 (6p22), RP11-787I22 (6q21), RP11-24P4 (8p21), D8Z2 (centromere 8) and RP11-88J22 (8q22). After optimization of the locations of the FISH-probes, probe YAC 827D3 (3q24) was replaced with probe RP11-64F6 (3q25). For tumor samples collected from December 2000 the probe located on 3q25 was used. Chromosome 5 is rarely involved in UM and therefore probe RP11-1059N10 (5q12) was used on paraffin sections as a control for cutting and truncation artifacts [11]. The concentration for centromere probes was 5 ng per slide, for all other BAC-probes 50-75 ng was used. After washing and staining, slides were counterstained with 4',6-diamidino-2-phenylindole and mounted in anti-fade solution (Dabco-Vectashield 1:1; Vector Laboratories, Burlingame, California, USA). Signals were counted in 200 interphase nuclei. Cut-off limits for loss (15% of the nuclei with one signal present) and gain (10% of the nuclei with more than two signals present) were adapted from the available literature [12]. For FISH on paraffin sections the cut-off limit for deletion (>25% of the nuclei with one signal present) was adapted from our own experiments [13].

Chromosomal comparative genomic hybridization

cCGH results were available for 21 samples. For all samples cCGH data was present in combination with FISH results. cCGH was carried out as described previously [14]. In brief, tumor and reference DNA were labeled in different colors, denatured, and hybridized to male metaphase spreads without chromosomal aberrations. Loss of DNA sequence was defined as chromosomal regions with the mean green (tumor DNA): red (reference DNA) ratio of less than 0.8; gain was defined by a ratio of more than 1.2.

Single nucleotide polymorphism array

For seven samples that showed discrepancies between results obtained with MLPA or FISH/cCGH, an SNP-array was performed using the Illumina Human CytoSNP12 Beadchip (Illumina San Diego, California, USA). UM DNA (200 ng) was processed according to the instructions of the manufacturer. Data were analyzed with Beadstudio software (Illumina

San Diego). Copy number alterations (represented by the log R ratio) and the B allele frequency were visualized. The normalized intensity ratio for each SNP in the tumor sample was compared with a 120 HapMap reference set. The log R ratio and B allele frequency data were analyzed with the software program Nexus Copy Number (Nexus BioDiscovery, El Segundo, California, USA). The results were compared with our own database of known copy number variations and a public copy number variations dataset containing approximately 3500 healthy controls (dataset of genomic variants).

Statistical analysis

The primary outcome for disease-free survival (DFS) was the time to development of metastatic disease. Kaplan-Meier curves were computed for the chromosome 3 and 8 aberrations found with MLPA and FISH. Correlation of single prognostic factors with the patients' survival were determined using the log-rank test (for categorical variables) and Cox proportional hazard analysis (for continuous variables). The following factors were evaluated: aberrations of the chromosomes 1p, 3, 6p, 6q, 8p and 8q, sex, age, tumor localization, tumor invasion of the ciliary body, cell type, LTD and the presence of closed vascular loops. For multivariate analysis, Cox proportional analysis was performed. A P value of 0.05 or less was considered significant. The statistical analyses were performed with SPSS 17.0 software (SPSS Inc., Chicago, Illinois, USA).

Results

All 64 tumors were confirmed as UM by histopathology. MLPA was successful in 64 tumor samples from 64 different patients. The characteristics of these tumors are listed in Table 1. Table 2 gives an overview of the MLPA results for the chromosomes 1, 3, 6 and 8 for all samples tested. MLPA showed loss of chromosome 1p in 18 tumors, loss of 3p in 36 tumors and loss of 3q was seen in 38 samples. Gain of 6p was observed in 28 tumors, loss of 6q in 10 tumors and 6q gain in seven tumors, loss of 8p in 14 tumors, whereas 14 other tumors showed gain of 8p. Gain of 8q was observed in 44 tumors. Numerous different combinations of chromosomal abnormalities were observed. Concurrent aberrations of the chromosomes 3 and 8q were most commonly seen (35 of 64). In six samples, no chromosomal aberrations were detected on chromosomes 1, 3, 6 and 8. The log-rank test showed significantly lower DFS in patients with tumors with a loss of chromosome 3, loss or gain of 8p, or gain of 8q ($P < 0.001$) detected with MLPA. Abnormalities of chromosome 1p, 6p and 6q did not reach significance ($P = 0.215$, 0.180, and p=0.170, respectively). Figure 2 shows the Kaplan-Meier survival plots for the abnormalities of chromosome 3 and 8 detected with MLPA and FISH.

Univariate analysis revealed other risk factors associated with a decreased DFS: age at the time of diagnosis ($P = 0.004$), LTD ($P = 0.010$), the presence of epithelioid cells ($P = 0.008$), tumors that invaded the ciliary body ($P = 0.024$) and the presence of a closed vascular loop

Table 1: Clinical and histopathological characteristics of the tumor samples tested

		P value	
Variable	Mean (range)	Univariate	Multivariate
Age at time of diagnosis, years	60.37 (21-85)	0.004 [a]	0.201
Largest tumor diameter (LTD), mm	13.44 (7-19)	0.010 [a]	0.681
Tumor thickness, mm	8.45 (3-20)	0.319 [a]	
	Number of patients		
Male sex	29/64	0.287 [b]	
Location			
Choroid	61/64		
Ciliary body	3/64		
Cell type			
Spindle cells	27/64		
Mixed cell type	26/64		
Epithelioid cells	11/64	0.008 [b]	0.608
Tumor invasion of the ciliary body	8/64	0.024 [b]	0.256
Presence of closed vascular loop pattern	31/64	0.001 [b]	0.018
	Number of patients	Mean (range) Follow up time (months)	
Patients alive without metastasis	25/64	124.12 (85.75-186.05)	
Patients with metastasis present or metastatic-related death	39/64	65.74 (5.17-135.69)	

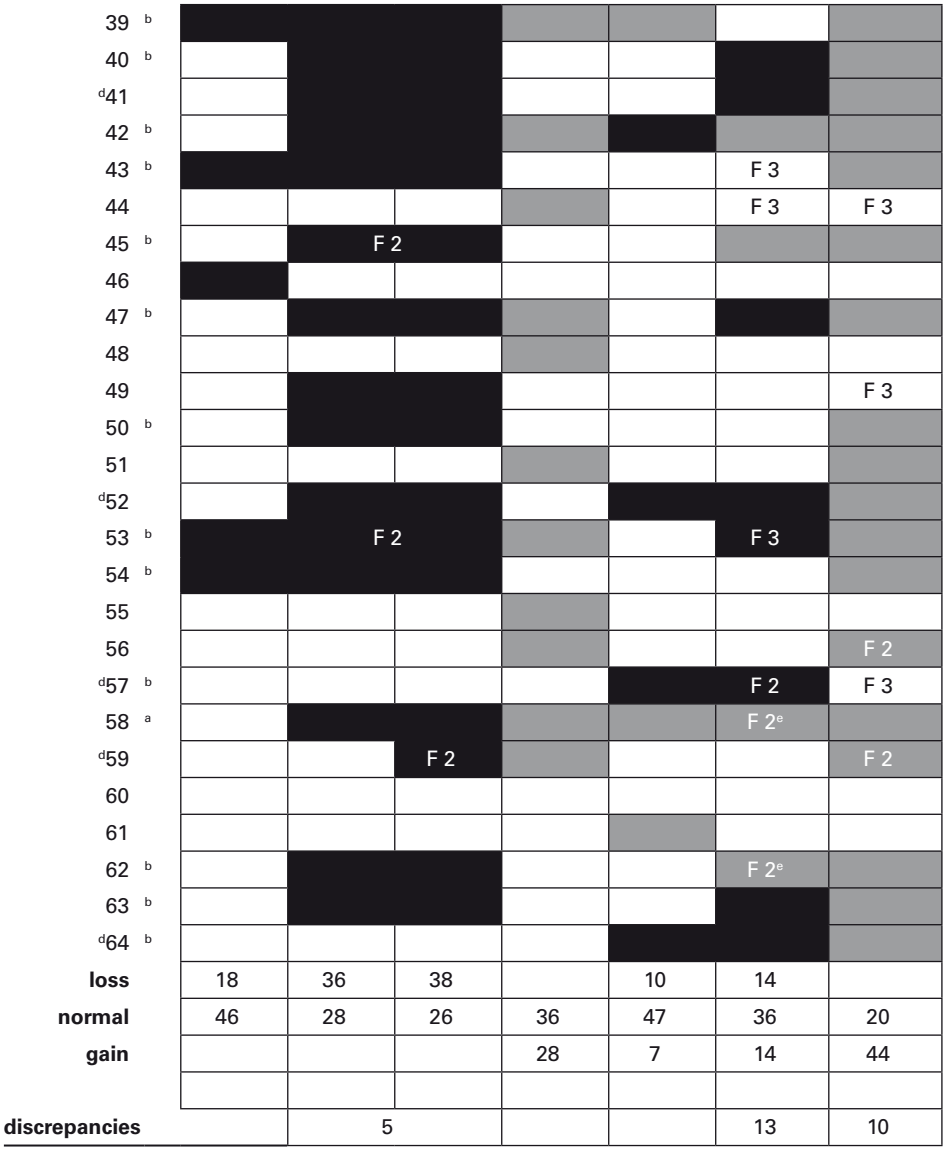
[a] Cox regression analysis
[b] Log-rank test

pattern ($P = 0.001$). Tumor thickness ($P = 0.319$) and sex ($P = 0.287$) did not influence the patients' clinical outcome (Table 1). Cox proportional hazard analysis was conducted to exclude a confounder effect. The presence of vascular loops ($P = 0.018$), chromosome 8p loss or gain ($P = 0.044$) and chromosome 8q gain ($P = 0.017$) were the only variables that remained significant after multivariate analysis.

On the account of the well-known predictive nature of abnormalities of chromosomes 3 and 8, only the MLPA results of these chromosomes were compared with FISH and cCGH results obtained earlier and will be discussed in this study. For seven samples in which differences

Table 2: An overview of the MLPA-results obtained for the chromosomes 1, 3, 6, and 8 for all samples tested.

	1p	3pc	3qc	6p	6q	8pc	8qc
***P* value**	**0.215**	**<0.001**		**0.180**	**0.170**	**<0.001**	**<0.001**
1 [b]							
2 [b]							
3							
4 [b]							
5							
[d]6 [b]							
7							
8 [b]						F 1	
9 [b]						F 2[e]	
10 [b]						F 1	
11							F 2
12 [b]							
13							
14 [b]		F 2				F 3	
15							
16 [b]							F 2
17							
18 [b]							
19							
20							
[d]21 [a]							F 2
22 [b]							
23 [a]							
[d]24 [b]							
25 [b]							
26 [a]			F 2				
[d]27 [b]							
[d]28 [b]						F 3	F 3
29							
30 [b]							
31							
32 [b]						F 2[e]	F 2[e]
33							
34 [b]						F 1	
35 [b]							
[d]36							
[d]37 [b]							
38 [b]							



loss	18	36	38		10	14	
normal	46	28	26	36	47	36	20
gain				28	7	14	44
discrepancies		5				13	10

The black boxes indicate loss, the gray boxes gain. The white boxes indicate that no gain of loss is found.

Tumors that showed discrepancies between FISH and MLPA-results are marked.

F:1 indicates that 1 copy was found using FISH, F:2 indicates two copies, and F:3 indicates three copies.

[a] represents patients with metastasis present.

[b] represents patients who died of metastatic disease.

[c] Represents the chromosomes which showed significant lower disease-free survival using the Log-Rank-test ($P < 0.001$).

[d] Tumors that would have been classified incorrectly using the MLPA results for chromosome 3 and 8.

[e] Tumors where consensus was found between MLPA and FISH results after using internal control probe normalization

Table 3: An overview of the discrepancies between multiplex ligation-dependent probe amplification and fluorescence in situ hybridization results for chromosomes 3 and 8

	3p	3q	8p	8q
8 [b]			F1; low percentage loss	
10 [b]			F1; low percentage loss	
11				F2; cCGH and
				add FISH confirm MLPA
14 [b]	F2; SNP-array hyperdiploid		F3; SNP-array hyperdiploid	
16 [b]				F2; add FISH confirms MLPA
[c]21 [a]				F2; add FISH and
				SNP-array confirm MLPA
26 [a]		F2; SNP-array hyperdiploid		
[c]28 [b]			F3; low percentage gain	F3; low percentage gain
34 [b]			F1; low percentage loss,	
			SNP-array confirms F1	
43 [b]			F3; low percentage gain	
44			F3; low percentage gain	F3; low percentage gain
45 [b]	F2; SNP-array hyperdiploid			
49				F3; low percentage gain
53 [b]	F2; SNP-array hyperdiploid		F3; SNP-array hyperdiploid	
56				F2; add FISH confirms MLPA
[c]57 [b]			F2; cCGH and	F3; cCGH confirms F3
			add FISH confirms F2	
[c]59		F2; SNP-array confirms loss		F2; cCGH, add FISH
				and SNP-array confirm MLPA

The black boxes indicate loss and the gray boxes show gain. The white boxes indicate that no loss or gain is found.

Tumors that showed discrepancies are marked. F:1 indicates that one copy was found using FISH, F:2 indicates two copies, and F:3 indicates three copies. Following the FISH results obtained, an explanation of the discrepancy is given. Add FISH indicates that additional FISH experiments were performed.

[a] Represents patients with metastasis present.

[b] Represents patients who died of metastatic disease.

[c] Tumors that would have been classified incorrectly using the MLPA results for chromosomes 3 and 8.

were found in the results, an SNP array was used to clarify these differences. In Table 2 and 3, differences in MLPA results compared with the data obtained from FISH experiments are listed. In five tumors, MLPA indicated (a partial) loss of chromosome 3, whereas FISH showed two copies using the probes Pα3.5 (centromere 3) and YAC 827D3 (3q24) or RP11-64F6 (3q25). Using internal control probe normalization, MLPA results for chromosome 3 remained unchanged. On performing FISH, four of these five tumors appeared to be near triploid or tetraploid with a relative loss of chromosome 3. For all four samples, this complex genotype was confirmed by an SNP-array (Figure 1). Interestingly, in one UM (tumor 59), the SNP-array did confirm the loss of the q-arm of chromosome 3 found with MLPA and therefore contradicts the results found with FISH.

Differences in chromosome 8p results were observed in 13 tumors. After using internal control probe normalization, nine discrepancies on chromosome 8p remained. For one tumor, MLPA showed 8p loss wheras FISH using probe Cos 105H8 (8p11) indicated two copies. In one tumor, MLPA indicated 8p loss, whereas FISH indicated gain in a small percentage of cells (15%) and two copies in the majority of cells (85%). This tumor displayed hyperdiploidy with relative loss of 8p, which was confirmed using an SNP-array. The FISH results of the other tumor were confirmed by cCGH and additional FISH experiments with the FISH-probe RP11-451O18 (8p12) flanking the location of the MLPA-probe. In three tumors, MLPA showed normal copy numbers of 8p and FISH results showed loss. In these three tumors, FISH showed a relative low percentage of loss of 8p (34, 54, and 56%). In one of these tumors, SNP-array confirmed the loss of chromosome 8p found with FISH. Four samples showed normal copy numbers for 8p, whereas FISH indicated gain. In three samples, 13, 56, and 56% of the tumor cells showed more than two signals for the FISH probe located on 8p. The FISH results of the other sample were confirmed by additional FISH experiments with probe RP11-451O18 (8p12) located near the MLPA-probe, and an SNP-array revealed a hyperdiploid nature.
The region 8q showed discrepancies between MLPA and FISH in 10 tumors. After using internal control probe normalization, nine discrepancies remained. On four occasions, MLPA indicated no copy number alterations for 8q, whereas FISH showed gain with the probe P1.164 (8q21). For one sample, the FISH result was confirmed by cCGH. For the other three tumors, a low percentage gain of 8q was found with FISH (15, 35, and 56%).

In five tumors, MLPA showed gain for the probes located on 8q24 and FISH using a probe on 8q21 did not show any copy number alterations. In all five tumors, additional FISH experiments with probe RP11-158K1 (8q24), flanking the region of the 8q24 MLPA-probes, confirmed the MLPA data. In three samples, cCGH and/or SNP-array also confirmed these additional FISH results.

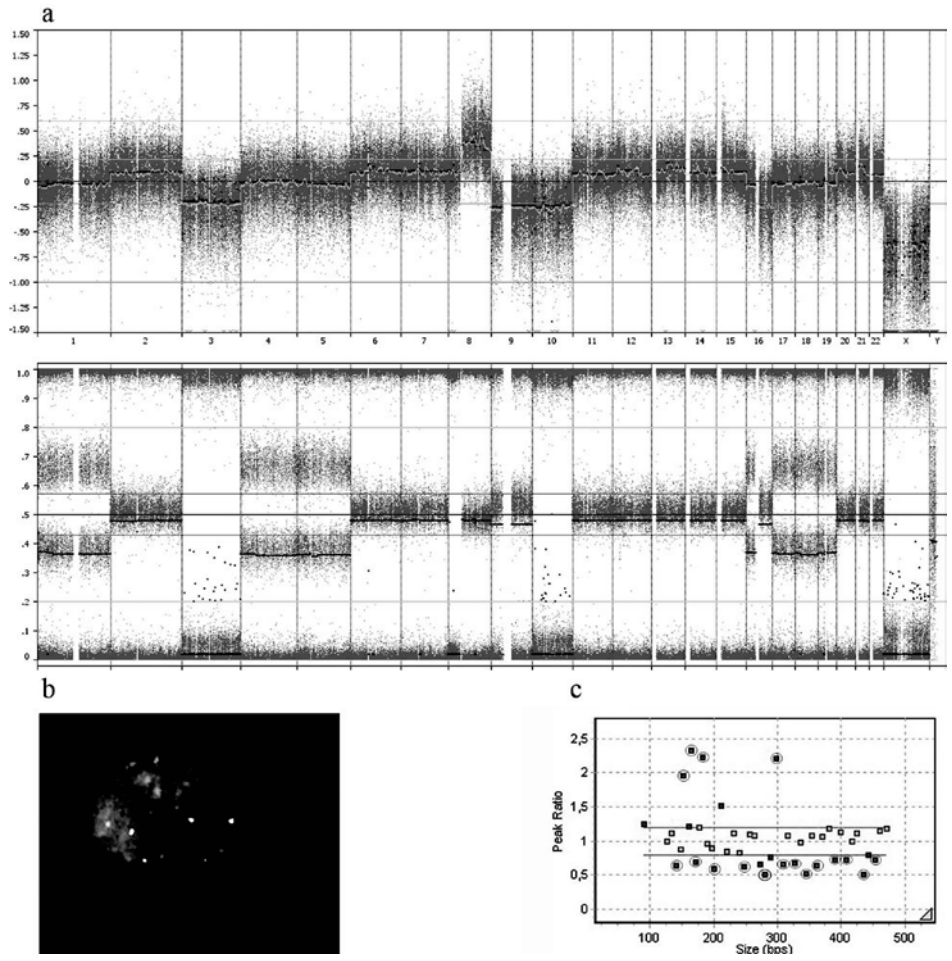


Figure 1: Example of a hyperdiploid tumor with relative loss of chromosome 3 tested with different techniques.
a: SNP-array results show hyperdiploidy with relative loss and LOH of chromosome 3. b: FISH analysis demonstrates three signals for the probe on chromosome 5q (red signals) and two signals of the probe on chromosome 3q (green signals). c: MLPA-results show loss of chromosome 3 and gain of 8q. The probes marked with a circle represent the probes located on chromosomes 3 and 8q. The peak ratio on the y-axis represents the RQ value. The x-axis represents the size of the different PCR products.

(color page 189)

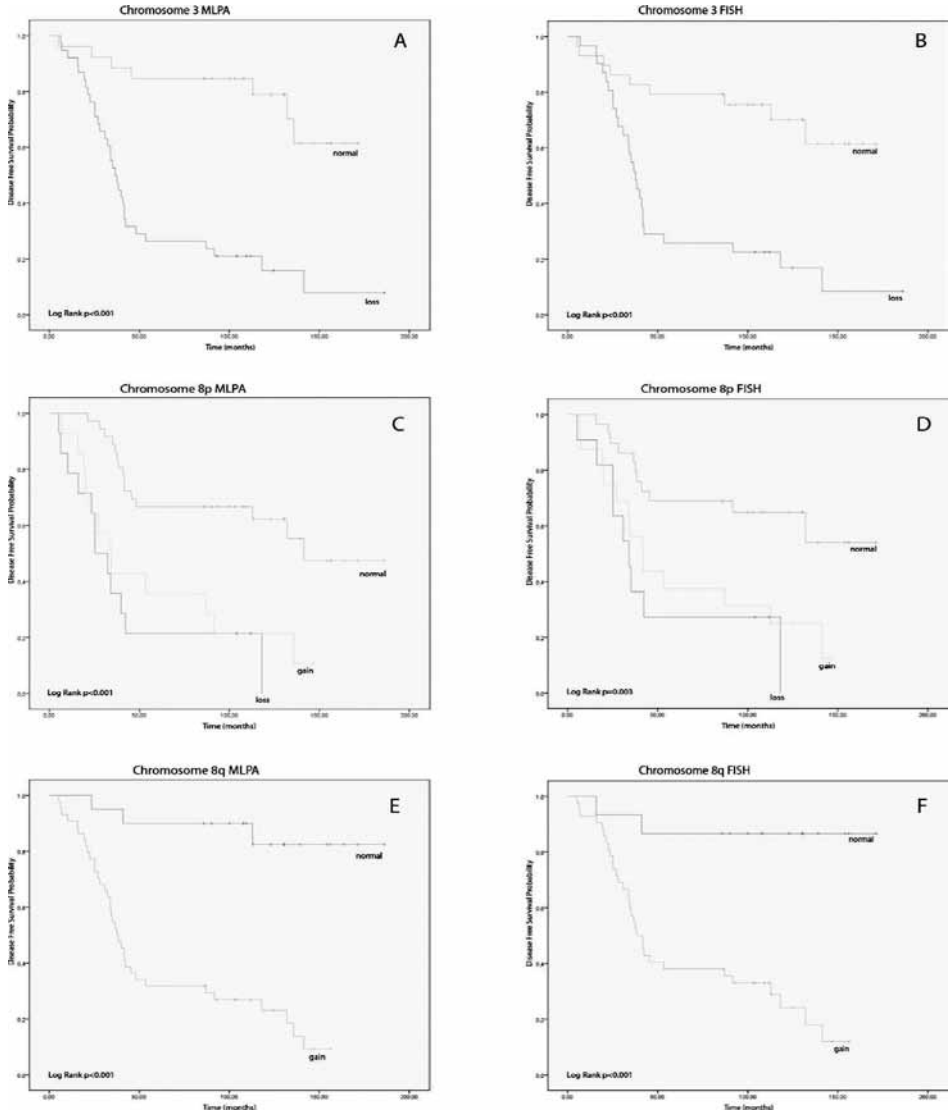


Figure 2: Kaplan-Meier survival plots for the abnormalities of chromosomes 3, 8p and 8q detected with MLPA and FISH.

A: loss versus normal chromosome 3 detected with MLPA (*P* <0.001) B: loss versus normal copy number of chromosome 3 detected with FISH (*P* <0.001) C: normal versus gain and loss of chromosome 8p detected with MLPA (*P* <0.001) D: normal versus gain and loss of chromosome 8p detected with FISH (*P* =0.003) E: normal versus gain of chromosome 8q detected with MLPA (*P* <0.001) F: normal versus gain of chromosome 8q detected with FISH (*P* <0.001)

In table 4, the sensitivity and specificity for the detection of patients at risk for metastatic disease of both MLPA and FISH, using the results for chromosomes 3 and 8, are listed. Using MLPA results, 31 of 39 patients with a poor prognosis were identified. FISH identified 27 of 39 patients with a poor clinical outcome. Both MLPA and FISH results classified 21 of 25 patients with a favorable prognosis correctly.

Discussion

In this study, the efficacy of MLPA in predicting UM patients at risk for metastatic disease was examined. We observed that MLPA is able to detect patients at risk with similar accuracy as FISH. Identification of high-risk patients enables clinicians to be aware of the increased risk for metastatic disease and, when adjuvant treatments become available, these can be offered on a selective basis.

The number of chromosomal aberrations observed using MLPA in our samples is in accordance with previous published data, indicating that we have a representative group of tumor samples [6,15]. We demonstrate that it is possible to identify patients with a reduced DFS using MLPA results for chromosomes 3 and 8. Aberrations on chromosomes 1 and 6 did not reach significance regarding clinical outcome. This in contrast to the data published by Damato et al.[16], who found a significant relation between chromosome 6 abnormalities and a favorable prognosis. A possible explanation for this discrepancy could be the difference in the group of tumors tested. The tumors used by Damato et al.[16] seemed to harbor more characteristics associated with metastasis than the tumors included in this study: epithelioid cell type (81 vs. 17%), presence of a closed vascular loop pattern (70 vs. 48%), and invasion of the ciliary body (42 vs. 13%). In addition, a difference in the categorization of the dosage quotients and RQ values can cause this discrepancy.

Other tumor characteristics such as invasion of the ciliary body, the presence of vascular patterns, and an LTD of more than or equal to 14mm also showed a significant correlation with a reduced DFS. Using Cox proportional hazard analysis, the presence of a closed vascular loop pattern ($P = 0.018$), chromosome 8p loss or gain ($P = 0.044$), and chromosome

Table 4: Sensitivity and specificity of MLPA and FISH to detect patients at risk for metastatic disease using concurrent chromosomes 3 and 8 results

	MLPA	FISH
Prevalence of metastatic disease	0.609 (39/64)	0.609 (39/64)
Sensitivity	0.795 (31/39)	0.692 (27/39)
Specificity	0.840 (21/25)	0.840 (21/25)
Positive predictive value	0.886 (31/35)	0.862 (25/29)
Negative predictive value	0.724 (21/29)	0.600 (21/35)

8q gain (P = 0.017) were the only variables that remained significant. Surprisingly, loss of chromosome 3 was not significantly correlated to a reduced DFS in this tumor group. A possible explanation could be that gain of 8q occurs in the majority of samples tested (n = 44) and nearly all the samples also show loss of chromosome 3 (n =35). Therefore, we can conclude that there is a correlation between the occurrence of loss of chromosome 3 and gain of 8q.

We found discrepancies between MLPA and FISH results 23 times in 17 different samples for chromosomes 3 and 8. Twenty discrepancies easily can be explained. Six cases can be explained by the presence of hyperdiploidy: four times MLPA indicated loss; FISH showed two signals, but these tumors displayed a near triploid or tetraploid baseline and therefore two signals have to be considered as relative loss. This also accounts for one case, in which MLPA showed loss, FISH showed gain in a small percentage of cells (15%), and two signals were found in majority of the cells (85%). In one tumor, MLPA indicated normal copy number; FISH showed three signals. This tumor appeared to be triploid and therefore three copies is considered to be normal. Nine differences can be explained by the low percentage of aberrant cells that was found with FISH. These samples (n = 3) showed loss in 34-56% and gain in 13-57% (n =6) of the counted nuclei. Studies showed that aberrations can only be detected using MLPA if they are present in at least 80% of the cells and that a mixed population of cells can result in some difficulties (unpublished data de Jong et al) [17,18,19]. Interestingly, in five tumors, MLPA revealed a partial gain of chromosome 8q. This partial gain of the end chromosome 8q has been described before and is often caused by translocations [20]. For this reason, it would be prudent to replace our FISH probe on 8q22 with a probe located on 8q24.

In two different samples, we found three discrepancies that could not be explained by the above-mentioned factors. In one sample, SNP array confirmed the partial loss of chromosome 3q that was found using MLPA and thereby contradicts the FISH results. In one sample, MLPA showed 8p-loss and FISH showed normal copy number in 100% of the counted nuclei. Both cCGH and additional FISH experiments with two probes flanking the MLPA probe region confirmed this earlier obtained FISH result. In that same tumor, MLPA showed normal copy number for chromosome 8q, whereas FISH indicated gain in 75% of the counted nuclei. This gain found with FISH was confirmed by cCGH. A possible explanation for these differences can be intratumor heterogeneity. Although UMs are generally homogeneous tumors, tumor heterogeneity has been described and may be the cause of this difference [11,21]. The tumor material obtained was divided into two parts during pathologic examination. One part was used to extract DNA to perform MLPA and the other part was used to obtain fixed cells for FISH. Both techniques were performed on different

parts of the same tumor specimen and intratumor differences could therefore not completely be excluded.

If the MLPA results for chromosome 3 and 8 are combined, 12 patients would be classified incorrectly on the basis of clinical outcome. The use of FISH results of chromosomes 3 and 8 also resulted in the incorrect classification of these 12 patients. Four patients with aberrations on chromosomes 3 and 8 are still alive without any symptoms of metastasis. The follow-up time of these patients ranges from 93 to 125 months. As a result of this long follow-up period, it is less likely that these patients will develop metastasis in the future. Although some cases have been reported where metastasis occurs after al long period of time, the peak of metastatic-related death occurs in the second to fourth year after diagnosis, and after 9 years, the majority of metastasis-related deaths would have occurred [3,22]. We do not have an explanation as to why these patients with aberrations of chromosomes 3 and 8 in the tumor are metastasis free after 9 years, and hence it would be very interesting to study these patients and tumors in more detail.

Eight patients died of metastasis, although MLPA did not show concurrent copy number alterations of chromosomes 3 and 8. The DFS of these patients ranges from 5 to 136 months. Six out of eight tumors only showed chromosome 8 aberrations and no loss of chromosome 3. One tumor only displayed loss of chromosome 3 and one tumor did not show aberrations of both chromosomes 3 and 8. A possible explanation as to why the seven tumors that did not show loss of chromosome 3 have a poor clinical outcome could be the presence of loss of heterozygosity on chromosome 3, resulting in two identical copies [23]. Another cause could be methylation that inactivates possible tumor suppressor genes located on chromosome 3 [24-25]. A possible candidate on chromosome 3 is the *BAP1* (BRCA1-associated protein-1) gene located on 3p21.1. This gene is found to be frequently mutated in metastasizing UMs [26]. It would be very interesting to test these seven tumors without loss of chromosome 3 for *BAP1* mutations. The tumor that only displayed loss of chromosome 3 and no aberrations of chromosome 8 could have small partial aberrations of chromosome 8, which are not detectable by MLPA and the FISH experiments [20]. Several oncogenes are located on chromosome 8q. A candidate in UM is *DDEF*1 (Development and Differentiation Enhancing Factor1) which is found to be overexpressed in high-grade UMs; hence, it would be very useful to look at the expression pattern of this particular tumor [27].

Comparing both sensitivity and specificity to detect patients at risk for metastasis between MLPA chromosomes 3 and 8 results with those obtained with FISH, we can conclude that MLPA yields slightly better results. In comparison with FISH, MLPA is very fast and easy to carry out. A small amount of tumor DNA is needed to perform this technique. A possible drawback of MLPA is that aberrations which are only present in a limited amount of cells are

difficult to detect. With FISH, individual cells can be examined, whereas with MLPA, only an overall estimation of chromosomal aberrations can be performed.

In conclusion, MLPA is able to identify patients at risk for metastasis using the results of chromosomes 3 and 8. There is no difference in this predictive power of MLPA compared with FISH.

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Chapter 5

Higher percentage of FISH-determined monosomy 3 and 8q amplification in uveal melanoma cells relate to poor patient prognosis

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Abstract

Aims: To investigate the relation between patient survival and incrementally increasing percentages of fluorescence in situ hybridization (FISH)-determined complete loss of chromosome 3 (monosomy 3) and gain of chromosome 8q in primary uveal melanoma (UM) cells.
Methods: Clinicopathological factors were related to disease-free survival. FISH was performed using probes on chromosomes 1, 3, 6, and 8. The percentages of UM cells with monosomy 3 or chromosome 8q gain were classified in groups with incrementally increasing percentages and related to disease-free survival. Correlations between clinical factors and cytogenetic aberrations were also analyzed.
Results: Two-hundred twenty choroidal and ciliary body melanomas were analyzed. The following proved to be significant predictors of survival in univariate analysis: older patient age ($P = 0.003$); large tumor diameter ($P < 0.001$); mixed cell type ($P = 0.001$); presence of closed microvascular loops ($P < 0.001$); loss of chromosome 1p ($P = 0.006$); monosomy 3 ($P < 0.001$); gain of 6p ($P < 0.001$); and gain of chromosome 8q ($P < 0.001$). Multivariate Cox analysis displayed monosomy 3 (Hazard ratio [HR] 2.83, $P = 0.002$) and gain of chromosome 8q (HR 3.13, $P = 0.002$) as the most important independent prognostic factors of poor survival, followed by older patient age (HR 1.02, $P = 0.017$). Increasing percentages of monosomy 3 and gain of chromosome 8q in tumor cells showed a correlation with worse prognosis (Log-rank test 49.9 and 40.4, both $P < 0.001$) and increased number of additional copies of 8q correlated with shorter disease-free interval (Log-rank test 45.7, $P < 0.001$).
Conclusions: A high percentage monosomy 3 and chromosome 8q gain in primary UM cells showed a strong relation with poor disease-free survival compared to low percentage aberrations.

Introduction

Uveal melanoma is the most common primary intraocular malignancy in adults with an annual incidence of 5-7 cases per million. Nearly half of all patients with UM eventually die of metastases which are most often late-appearing.[1-2] In search for prognostic factors, several clinical, pathological, and genetic parameters have been identified. Genetic factors have proven to be the most significant factors of all and can reliably indicate high risk of metastasis and poor survival in patients with UM.[3-4] Nonrandom chromosomal alterations are present in more than 80% of cases with most frequently a complete loss of chromosome 3 (monosomy 3) in 50% of cases. Monosomy 3 is the most important chromosomal factor relating to a 4-year overall survival of only 30%.[5-6] Other known but less frequently occurring alterations that also relate with prognosis are gain of chromosome 6p or 8q, loss of chromosome 1p or 6q, and co-occurrence of chromosome 1p and 3 loss.[5, 7-9]

Fluorescence in situ hybridization is a reliable technique for assessing chromosomal aberrations in UM; therefore many large referral centers routinely use FISH for the analysis of the chromosome 3 status of a tumor. FISH enables in situ analysis of chromosomal aberrations in tumor cells and by using a cutoff threshold for identification of loss, tumors are either classified as chromosome 3 disomic or monosomic. By classifying tumors in one of these groups using a cutoff threshold, information on exact percentages of aneuploidy and their possible relation to prognosis is disregarded. Using FISH analysis, this study assessed the percentages of tumor cells with loss of chromosome 3 or gain of chromosome 8q for each tumor separately and correlated these findings with patient prognosis. Tumors were classified according to the FISH counts in groups of incrementally increasing percentages of chromosome 3 or 8q aneuploidy: 15%-33% (10%-33% for gain of chromosome 8q), 33%-66%, and 66%-100%, and investigated whether a high percentage of aneuploidy in the tumor is related to a decreased survival. If so, this could provide a more precise prognosis for patients with low, intermediate and high percentages of chromosome 3 or 8q aneuploidy in their tumor and could be used for selecting patients eligible for adjuvant therapy.

Methods

Between July 1994 and November 2010, tumor material was collected from 248 patients who underwent enucleation for UM. Thirteen iris melanomas[10] and fifteen hyperaneuploid cases (Mensink, et al. submitted) were excluded from this study because of the differences in molecular behavior. Routine clinical systemic evaluation including blood liver function tests was conducted before enucleation was performed. Fresh tumor tissue was harvested within 1 hour after enucleation of the remaining 220 ciliary-body and choroidal melanomas, and was processed for histhopathologic and genetic research. Histopathologic examination was conducted according to standardized protocols and confirmed the origin of the tumor as well as tumor size, cell type, and presence of microvascular patterns (closed vascular loops).

Informed consent was obtained prior to enucleation and the study was performed according to guidelines of the Declaration of Helsinki. Clinical data and follow-up data regarding metastases and tumor-related death were obtained from medical records and by contacting the general physician. In total, three patients were lost to follow-up: the first patient was 57 years old when he was lost to follow-up after 28 months because he moved abroad with unknown destination. The second patient was 89 years old and was lost to follow-up after 69 months; the third patient was 93 years old and lost to follow-up after 18 months. These three patients had no sign of metastasis at the last follow-up moment.

Fluorescence in situ hybridization

FISH allows interphase cytogenetic analysis of fresh or archival tumor tissue by using differentially labeled fluorescent probes mapping to specific chromosomal regions. With this technique, copy number alterations can be determined in a large number of cells. Fresh tumor tissue from enucleated eyes containing UM was routinely used for direct interphase FISH (chromosome 1, 3, 6 and 8) as described previously.[11] The following probes were used: RP11-48E9 (1p36); RP11-384L8 (3p22) or RP11-522N9 (3p13); Pα3.5 (centromere 3); YAC 827D3 (3q24); RP11-356B3 (6p22); RP11-787I22 (6q21); RP11-24P4 (8p21); D8Z2 (centromere 8); and RP11-88J22 (8q22). After optimization, probe YAC827D3 (3q24) was replaced with probe RP11-64F6 (3q25) which was used for tumor samples collected from December 2000. If FISH on fresh tissue failed, due to insufficient availability of fresh tumor tissue or technical reasons, FISH was carried out on paraffin sections of the tumor (archival tissue) as described before.[12] In all cases, up to 300 cells were counted according to the criteria of Hopman.[13] Cutoff limits for FISH on fresh and archival tissue were adopted from available literature.[14-15] The cutoff limit used for amplification was 10% for FISH on fresh tissue and paraffin tumor sections. Cutoff limit for deletion with FISH on fresh tumor tissue was 15%; and for paraffin tumor sections, the cutoff limit was corrected for by a reference probe on chromosome 5[12] and defined as 25%. As cutoff limits for deletion differed by 10% for paraffin sections and fresh tumor tissue, paraffin section counts were adjusted by subtracting 10%, thereby allowing easy comparison.

After adjustment of the cutoff limit for deletion, the following groups were defined for tumors with monosomy 3: low percentage (15%-33%), intermediate percentage (33%-66%), and high percentage monosomy 3 (66%-100%). Due to the 10% cutoff limit for amplification, tumors with chromosome 8q amplification (gain) were classified in low percentage (10%-33%); intermediate percentage (33-66%); or high percentage gain of chromosome 8q (66%-100%).

Chromosome 3 and chromosome 8q counts were classified in groups of 33% as this resulted in the most reliable comparisons with the intermediate groups, which had a low number

of cases. Chromosome 3 status was assessed by a 3p probe, centromere 3 probe and 3q25 probe, to allow for assessment of monosomy of chromosome 3. If the chromosome 3 probes showed a difference in percentage of tumor cells with deletion, the highest percentage was used for further analysis. Chromosome 8q alterations were evaluated separately by one probe on 8q21 and no specific cutoff correction was made for using paraffin sections as truncation and cutting-artifacts are not a major issue for cells showing more than two signals.

Statistical analysis

The influence of single prognostic factors on metastasis-free survival was assessed by using univariate Log-rank analysis or Cox proportional hazards analysis. Cox regression multivariate analysis was performed to identify the independent value of the prognostic factors. Kaplan-Meier survival analyses were performed for estimation of survival probabilities with metastasis or death due to metastasis as primary clinical endpoint. Kaplan-Meier curves were computed for patients overall, and with incrementally increasing percentages of monosomy 3, and chromosome 8q gains. Mann-Whitney tests were used to assess correlations between different prognostic factors. All *P* values were two-sided and significance was set at $\alpha = 0.05$. The analyses were performed using the statistical software SPSS Version 17.0 (SPSS Inc., Chicago, IL).

Results

In total, 220 UMs were successfully analyzed with interphase FISH on fresh tumor tissue in 189 cases, and in 31 cases with additional FISH on paraffin tumor sections. Using both tissue types, chromosome 3 status was successfully assessed in all 220 cases and chromosome 8q status in 201 cases (91%). Monosomy 3 was present in 12 of 19 tumors where data on chromosome 8q status was missing. Co-occurrence of monosomy 3 and gain of chromosome 8q was present in 102 of 201 cases (51%); monosomy 3 without gain of 8q was present in 20 cases (10%); and gain of 8q without monosomy 3 was present in 32 cases (16%). Of the 220 patients recruited for this study, 121 were male. The mean age of all patients was 62 years (median 62 years, range 21-87 years). The mean duration of follow-up, from diagnosis to end of study, was 4.7 years (range 0.3-15.9 years), with metastases occurring at a mean follow-up of 3.1 years (range 0.3-11.0 years). Eighty-one patients died from metastatic disease and five were diagnosed with metastases at the time of evaluation.

Univariate analysis of the single prognostic risk factors showed a significantly decreased survival for patients with UM with the presence of epitheloid cells, closed vascular loops, loss of chromosome 1p, monosomy 3, and gain of chromosome 8q (Table 1). Large tumor diameter and older patient age were significantly related to poor survival as well. A gain of chromosome 6p related to a more favorable prognosis.

Table 1. Univariate analysis of prognostic markers on disease-free survival in 220 uveal melanomas

Variable	Mean, median (range)	No. of patients (%)	Missing data (%)	*P*value*†
Age at diagnosis	62 yrs, 62 (21-87)		-	0.003
Largest tumor diameter	12.8 mm, 13.0 (2-30)		-	<0.001
Tumor height	7.5 mm, 7.0 (1-22)		-	0.178
Male gender		121 (55)	-	0.218
Mixed/epitheloidcell type		154 (70)	-	0.001
Involvementciliary body		30 (14)	4 (2)	0.065
Closed vascular loops		90 (41)	15 (7)	<0.001
Loss of chromosome 1p		66 (30)	-	0.006
Monosomy 3		134 (61)	-	<0.001
Gain of chromosome 6p		93 (42)	26 (12)	<0.001
Loss of chromosome 6q		65 (30)	33 (15)	0.332
Gain of chromosome 8q		134 (61)	19 (9)	<0.001

* Cox-regression analysis, † Log-rank test.

Kaplan-Meier survival analysis displayed poor survival probabilities for patients having tumors with monosomy 3 (Log-rank test 36.5, $P < 0.001$) (Figure 1a). Survival probabilities were even worse if the patients' tumors had a high percentage of monosomy 3 in analyzed cells, and Kaplan-Meier survival analysis showed a significantly worse survival for patients with high percentage of monosomy 3 in their tumor compared to patients having tumors with low or intermediate percentage monosomy 3 (Log-rank test 49.9, $P < 0.001$) (Figure 1b). The disomy 3 group was analyzed next to medium and high percentage aneuploidy groups as well and displayed a more favorable prognosis than the higher percentage aneuploidy groups (Figure 1c). Presence of chromosome 8q gain in tumors also correlated with worsening patient survival (Log-rank test 31.6, $P < 0.001$) (Figure 1d). The high and intermediate percentage gain of chromosome 8q groups displayed comparable survival probabilities, which were worse than with low percentage gain (Log-rank test 40.4, $P < 0.001$) (Figure 1e). The disomy 8q group was analyzed next to higher percentage aneuploidy groups as well, displaying a more favorable prognosis (Figure 1f).

Cox proportional hazard analysis was performed with all factors that were significant after univariate analysis to exclude confounding variables and identify the independent prognostic value of chromosome 3 and 8q in this cohort. Older age, monosomy 3 and gain of 8q proved to be independent negative prognostic factors. If these factors were stratified for the different age groups and increasing percentages of monosomy 3 or gain of chromosome 8q, the highest age group and highest percentage tumor aneuploidy groups showed the strongest correlation with poor survival (Table 2). General prognostic factors such as tumor

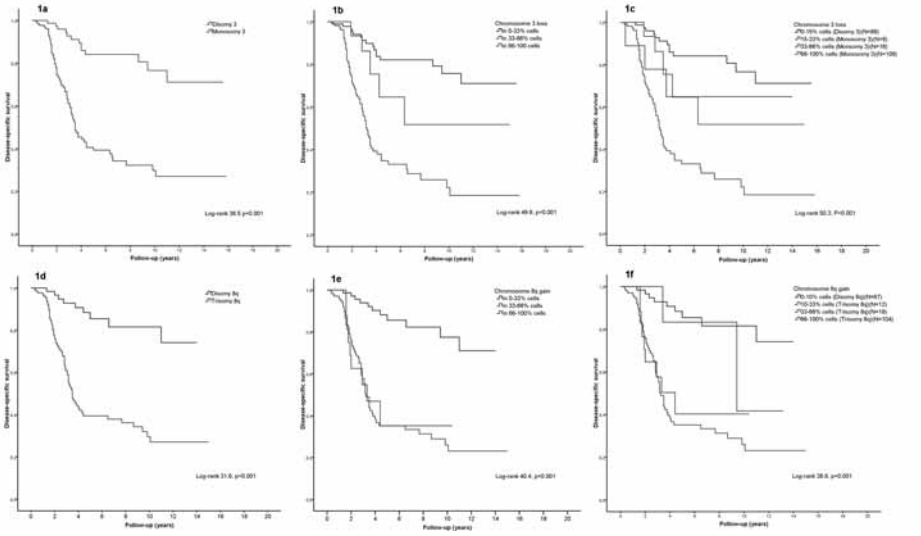

Figure 1. Kaplan-Meier survival curves. (A)Chromosome 3 status. (B) Chromosome 3 loss (illustrates incrementally increasing percentages). (C) Chromosome 3 status (illustrates increasing percentages). (D)Chromosome 8q status. (B) Chromosome 8q gain (illustrates incrementally increasing percentages). (F)Chromosome 8q status (illustrates increasing percentages).

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diameter, epitheliod cell type, presence of closed vascular loops, loss of chromosome 1p, and gain of 6p lost significance after multivariate analysis.

Tumors with high percentage monosomy 3 were larger in diameter than tumors with low percentage monosomy 3 ($P = 0.028$) (Table 3). The correlation between high percentage gain of chromosome 8q and large tumor diameter was slightly stronger than for monosomy 3 ($P = 0.024$). There was no direct correlation found between older patient age and larger tumor size. Tumors with monosomy 3 and high percentage of chromosome 8q gain frequently had additional copies of chromosome 8q present (Figure 2), which related to worse patient survival (Figure 3, Log-rank test 45.7, $P < 0.001$). Moreover, an increased number of additional copies of chromosome 8q related to a shorter disease-free interval.

Discussion

Study findings confirm the importance of monosomy 3 as an important prognostic cytogenetic factor for metastatic disease in UM. Additionally, gain of chromosome 8q and older patient age were classified as important independent prognostic factors in this study. Our FISH results show a gradually worsening patient prognosis for UMs with incrementally increasing percentages of monosomy 3. A higher percentage gain of chromosome 8q (more than 33% of tumor cells) correlates more with worsening survival than low percentage 8q gains (in 10%-33% of tumor cells), while the intermediate and high percentage 8q aneuploidy groups

Table 2. Multivariate analysis of prognostic markers on disease-free survival in 220 uveal melanoma patients.

Variable	Hazard Ratio	95% CI	*P* value
Age at diagnosis * ‡	**1.021**	**1.004-1.039**	**0.017**
< 40 years	1.507	0.607-3.743	0.377
40-50 years	1.636	0.928-2.882	0.089
50-60 years	1.450	0.918-2.290	0.111
60-70 years	1.201	0.759-1.901	0.434
> 70 years	3.064	1.387-6.771	0.006
Loss of chromosome 3†	**2.832**	**1.488-5.388**	**0.002**
Low percentage(15%-33% cells), *n* = 9	1.907	0.538-6.769	0.318
Intermediate percentage (33%-66% cells), *n* =16	2.004	0.688-5.841	0.203
High percentage (66%-100% cells), *n* =109	5.877	3.191-10.825	<0.001
Gain of chromosome 8q†	**3.131**	**1.538-6.375**	**0.002**
Low percentage (10%-33% cells), *n* =12	0.923	0.193-4.418	0.920
Intermediate percentage (33%-66% cells), *n* =18	5.720	2.190-14.941	<0.001
High percentag: (66%-100% cells), *n* =104	6.139	3.035-12.420	<0.001

* Cox proportional hazard analysis, † Likelihood ratio test, ‡ Per year increase, 95% CI = 95% Confidence Interval.

Table 3. Correlation between tumor size and abnormalities of chromosome 3 and 8q.

	Chromosome-3 loss			Chromosome-8q gain		
Clinical data	15%-33% cells	66%-100% cells	*P* value	10%-33% cells	66%-100% cells	*P* value
Clinical data						
Mean tumor diameter (mm)	12.2	13.4	0.028	11.8	13.8	0.024
Mean tumor thickness (mm)	7.5	8.0	0.341	6.5	7.9	0.154

display comparable patient survival (Figure 1). High percentage monosomy 3 in our study relates to a 4-year overall survival probability of 15-20% (Figure 1b,c). This is comparable to, or slightly worse than the reported 4-year overall survival of 30% for monosomy 3 tumors in general.[5-6] Also in the low percentage monosomy 3 group, patients had an increased risk of metastasis compared with the disomy 3 group (HR = 1.9) (Table 2); and three out of nine patients from the low percentage group died from metastasis after a mean follow-up of 2.1 years (range 0.4 – 3.8 years). With a longer follow-up, even more patients may develop metastases. Interestingly, these patients with metastases all had low percentage monosomy 3 next to high percentage gain of chromosome 8q. Figure 2 illustrates that a low percentage

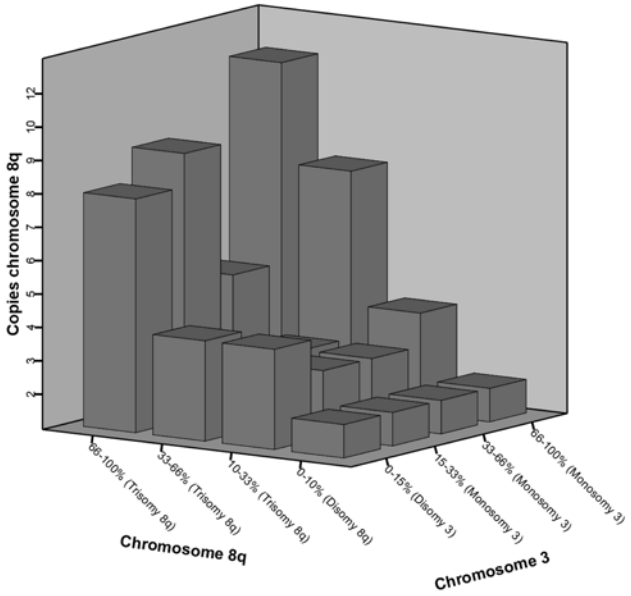

Figure 2. 3-D chart showing FISH counts of chromosome 3 and 8q according to chromosome 8q copy number. X-axis: classification according to the percentage of tumor cells with gain of chromosome 8q: 0%-10% (disomy 8q); 10%-33% (low percentage gain); 33%-66% (intermediate percentage gain); 66%-100% (high percentage gain). Y-axis: number of copies of chromosome 8q. Z- axis: classification according to the percentage of tumor cells with monosomy 3; 0%-15% (disomy 3); 15%-33% (low percentage monosomy); 33%-66% (intermediate percentage monosomy); 66%-100% (high percentage monosomy). **(color page 191)**

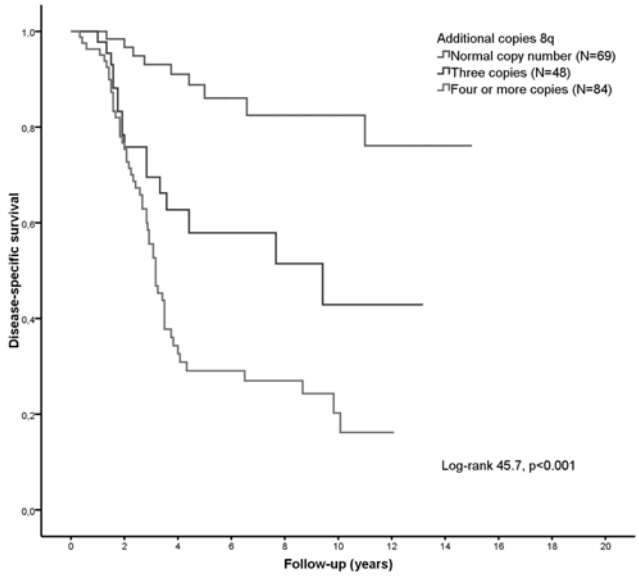

Figure 3. Kaplan-Meier survival curves according to copies of chromosome 8q present.

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of monosomy 3 may coincide not only with higher percentages of gain of chromosome 8q, but also additional copies of chromosome 8q in tumor cells. This supports previous results of Sisley et al.[16] where additional copies of chromosome 8q predicted a worse disease-free survival.

Considering polysomy 8q, low percentage gain showed borderline favorable prognosis after multivariate analysis (HR = 0.923), which is remarkable as all other aneuploidy groups are correlated with poor prognosis. This result could be due to the small number of patients in this group and may, therefore, need more cases to allow for a reliable estimation of the risk of metastasis for this group. At present, two out of 12 patients from this group of low percentage gain of chromosome 8q died from metastasis. Of the remaining patients within this group, 8 have a tumor with simultaneous loss of chromosome 3 and four patients have a follow-up of less than 2 years. Considering this, these patients might develop metastasis in the near future too.

Co-occurrence of chromosome 3 and 8 was reported before[16-17] and may be referred to as genetic imbalance, as stated by Patel et al.[18] Current study findings indicate the importance of determining chromosome 8q status when there is no monosomy 3 or only low percentage monosomy 3 in a tumor, as this may coincide with a high percentage gain of chromosome 8q (or increased copies of 8q) and lead to worsening patient survival. This hypothesis is also supported by Patel et al.[18] where a genetic imbalance (monosomy of chromosome 3, or gain of chromosome 8, or both) was associated with worsening survival. However, the cutoff limits in the study by Patel et al.[18] were 30% due to the sensitivity of the probes used. The authors report two patients with genetic imbalance in 20% of tumor cells who survived for over 100 months, but also hypothesized that these patients might develop metastases in the long run, and that minimal genetic imbalances (of 5%-10%) could lead to development of metastatic disease. Bronkhorst et al.[19] also report that tumors with 5% of monosomy 3 correlate to a high risk of metastatic disease using a centromere probe. However, in their study, they state that a threshold of 30% for monosomy 3 predicts high risk of metastasis more accurately. Even though the low and intermediate percentage aneuploidy groups in our study were small, still a significant number of patients with monosomy in less than 30% of tumor cells died due to metastasis. Therefore, using a threshold of 30% and higher for monosomy 3 will not lead to identification of these high-risk patients who would consequently be excluded from any adjuvant treatment.

In contrast to monosomy 3, chromosome 8 alterations are known to be a late event in UM development, relating to large tumor size.[20] This relation with tumor size was confirmed with the present study as high percentage chromosome 8q gain in tumor cells related to a larger tumor diameter than low percentage 8q gain (Table 3). The high percentage gain of chromosome 8q group also frequently showed increased copy numbers of 8q (Figure

2), which in turn correlated with reduced patient survival and shorter disease-free interval (Figure 3). This could indicate that when UMs grow larger, cytogenetic alterations accumulate in an increasing number of cells leading to additional copies of chromosome 8q and worsening survival. On the other hand, presence of cytogenetic alterations in tumor cells may give the tumor a growth advantage.

It remains a major issue whether actual percentages of aberrations found in analyzed tumor sections reflect the situation in all parts of the tumor. Several groups already reported intratumor heterogeneity to be present in UM[12, 21-22], and biopsy taking may therefore result in sampling error. However, discordance of chromosome 3 results was only found in a minority of cases analyzed by fine needle aspiration biopsy specimens and direct single-cell suspensions[11] and paraffin sections of different parts of the tumor.[12] This leads to misclassification in less than 1% of patients.[11-12] Dopierala et al.[22] analyzed 32 UMs by multiplex ligation-dependent probe amplification (MLPA) for different parts of the tumor and reported heterogeneity of chromosome 3 in 47% of cases, not leading to misclassification when compared with the whole tumor. The MLPA technique provides a relative quantification of monosomy 3 (and multiple other chromosomal regions) and cells with disomy 3 in the different tumor regions may dilute the obtained results. FISH, on the other hand, enables absolute quantification of monosomy 3 in single tumor cells. The problem of sampling error and misclassification is thought to be less important if larger enucleation specimens are used, as these are more representative of the tumor than biopsies.[21, 23] In this study, larger enucleation specimens were used from the patient tumors, minimizing the risk of misclassification.

Single nucleotide polymorphism (SNP)-array is a recent molecular genetic technique based on a series of DNA segments orderly arranged on a chip to which fluorescently labeled DNA can be hybridized. With this technique, rapid assessment of copy number alterations as well as zygosity changes on a genome-wide level with a high resolution is possible. MLPA allows for copy number analysis of up to 50 chromosomal regions in one experiment and is also less labor-intense than the FISH technique. Nevertheless, an important advantage of the FISH technique is that absolute copy numbers can be assessed and low mosaic cases (alterations in low percentage of cells) can be detected, which is more difficult with SNP-array and MLPA. This study demonstrated that even patients with low percentage aneuploidy of chromosomes 3 and 8q, who are at risk for developing metastasis, can be identified by FISH with absolute quantification of additional copies of chromosome 8q as well.

This study, based on current literature and data, the first to use incrementally increasing FISH counts of chromosome 3 losses and 8q gains, and evaluate its impact on disease-free survival. In total, 220 patients were studied and analyzed by FISH, providing first steps

towards a more individualized prognosis for UM patients. Future studies are needed in order to obtain more cases with low and intermediate percentages of chromosome 3 loss and chromosome 8q gain and enable an even more reliable comparison of these groups. There is a bias towards the larger tumors, since only enucleated eyes were included in this study. In the future, in-vivo biopsy of UMs treated by eye-sparing techniques may provide new information on the distribution of chromosome 3 and 8q alterations in the small and medium-sized tumors. The importance of chromosome 3 alterations in UM is recently further demonstrated by Harbour et al.[24], who reported on frequent somatic and one germline *BAP1*-mutation, located on chromosome 3p21.1, in class 2 metastasizing melanomas. In a previous study from this group, monosomy 3 was detected in four out of five class 2 tumors.[25] It would be interesting to assess whether patients with monosomy 3 tumors from this present study have a mutant *BAP1*-gene on the remaining allele as well. If so, then it is worthwhile to determine whether *BAP1* mutations have a better predictive value than monosomy 3.

In conclusion, patients having tumors with high percentage of monosomy 3 have a slightly worse 4-year overall survival probability than monosomy 3 tumors in general. The patients with an increased number of additional copies of chromosome 8q in their tumor are at risk of early metastasis. Since patients with high percentage monosomy 3, intermediate or high percentage gain of chromosome 8q, and additional copies of 8q in their tumor cells have a high risk of early metastasis, this group could be eligible for adjuvant treatment preventing development of metastasis. Therefore, results of adjuvant therapies may be observed much earlier within this group than with classic long-term studies.

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Chapter 6

Fine mapping of structural chromosome 3 deletions in uveal melanoma cell lines

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Manuscript in preparation

Abstract

Aims: A primary uveal melanoma (UM) and different metastatic cell lines from the same patient were used as a model system to analyze the role of chromosome 3 aberrations in UM tumorigenesis and metastasis.

Methods: The primary UM cell line Mel270, with a partial deletion on chromosome 3q, together with the four liver metastatic cell lines OMM2.2, OMM2.3, OMM2.5 and OMM2.6 were analyzed using SNP-array analysis. Chromosomal aberrations found in this genome-wide analysis were confirmed using polymorphic microsatellite markers. FISH was also used to determine in situ absolute copy number status in regions with loss of heterozygosity (LOH) and to confirm the results obtained by SNP-array.

Results: Regarding chromosome 3, a deleted region was found on chromosome 3q (3q21.2-q23) and the three regions with LOH on 3p (3p25.1-p25.2, 3p23-p24.2, and 3p14.3). These regions were further characterized in Mel270 and the metastatic cell lines.

Conclusion: The regions currently identified (3q21.2-q23, 3p25.1-p25.2, 3p23-p24.2, and 3p14.3) can be used for pinpointing of candidate genes involved in UM etiology and metastasis.

Introduction

Uveal melanoma (UM) is the most frequent intraocular malignancy among adults and is known for its tendency to develop metastasis[1-2]. Despite therapy, half of all patients eventually die of metastases which involve the liver in 90% of cases[3]. An important issue therefore is to identify patients at risk for metastasis. Cytogenetic alterations in the tumor such as complete loss of chromosome 3 (monosomy 3) or gain of chromosome 8q, correlate with a high risk of metastasis[4-5]. These same cytogenetic aberrations have been found in UM metastatic tissue as well[6], but only rarely in UMs that do not metastasize. Although partial loss of chromosome 3 is not common in UM[7-10], analysis of these cases could lead to identification of candidate genes involved in uveal melanoma metastasis. In a previous study[11], we analyzed primary UM cell lines using conventional comparative genomic hybridization (CGH) and reported the primary UM cell line Mel 270 to harbor a region of partial chromosomal loss ranging from 3q13-q21. However, the results obtained by using fluorescence in situ hybridization (FISH) and polymorphic microsatellite marker analysis on the Mel270 cell line and four of its liver metastatic cell lines were inconclusive. We therefore analyzed these same cell lines in the current study, by high-resolution Single Nucleotide Polymorphism (SNP)-array and additional FISH analyses. The aim of this study is to identify the chromosomal alterations present in both the primary tumor cell line as well as the metastatic cell lines, and thereby define chromosomal candidate regions involved in uveal melanoma metastasis.

Materials and methods

Uveal melanoma cell lines

The primary UM cell line Mel270, and metastatic liver cell lines OMM2.2, OMM2.3, OMM2.5, and OMM2.6, were established after harvesting of tumor tissue from a patient who was initially treated by local irradiation to the eye at the Bascom Palmer Eye Institute (Miami, FL)[12]. All cell lines, Mel270, OMM2.2, OMM2.3, OMM2.5, and OMM2.6, were a kind gift from Dr. Bruce Ksander (Schepens Eye Research Institute, Boston, MA). The earliest cell culture passage of each cell line available, was used for analysis; Mel270 (passage 27), OMM2.2 (passage 81), OMM2.3 (passage 13), OMM2.5 (passage 62), and OMM2.6 (passage 17). For Mel270, OMM2.2, OMM2.3, and OMM2.6, later passages were analyzed as well (supplementary figure 1).

SNP microarray analysis

DNA from the tumor cell cultures was isolated by using the QIAamp DNA mini-kit (Qiagen, Venlo, the Netherlands), and measured with the PicoGreen assay (Molecular Probes, Eugene, Oregon, USA), both according to the manufacturer's instructions. Two hundred nanograms of tumor DNA were used as input for whole genome analysis by SNP-array for each of the cell lines (Illumina 610Q BeadChip, Illumina, San Diego, California, USA).

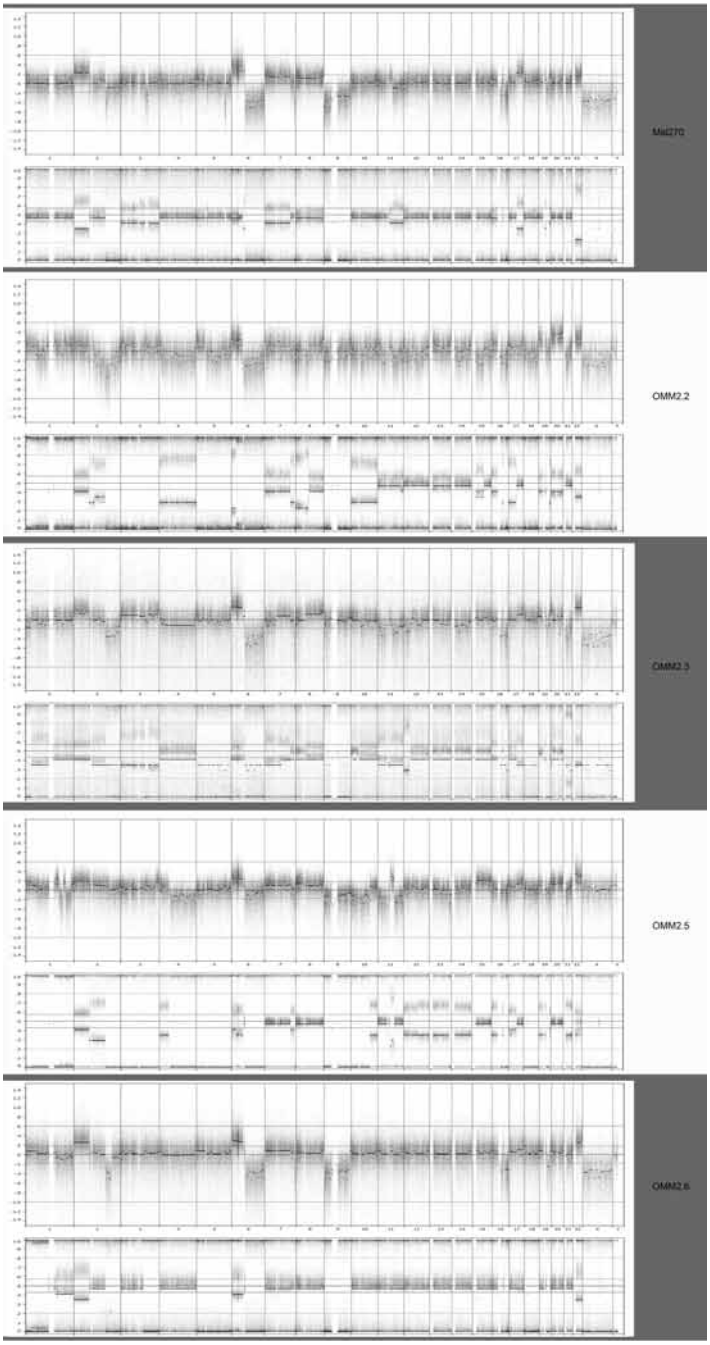


Figure 1. Whole genome SNP-array overview of all cell lines with the earliest passages analyzed The Log-R ratio is displayed in the upper panel for each cell line and the B-allele frequency in the lower panel for each cell line. **(color page 192)**

The SNP-array data were analyzed with the Nexus 6 software (Biodiscovery, El Segundo, California, USA).

Fluorescence in situ hybridization

Cell suspensions from the primary and metastatic tumor cell cultures were used for dual color FISH experiments as described previously[13]. The 34 FISH BAC probes used in this study are displayed in tables 1 and 2. For each probe, 300 interphase nuclei were counted, according to the criteria of Hopman et al[14]. In this case, the exact FISH counts were displayed for each single probe, without using a specific cut-off threshold.

Polymorphic microsatellite analysis

For determining loss of heterozygosity of chromosome 3, sixty different polymorphic microsatellites (Life Technologies, Breda, the Netherlands) were selected using the UCSC Genome Bioinformatics website (Human Genome build 36) (http://www.genome.cse.ucsc.edu).The PCR protocol primer sequences and locations are available upon request. Control DNA, e.g. DNA isolated from blood or normal tissue of the patient, was not available for research.

Mutational analysis of the BAP1, GNAQ and GNA11 genes

The genes *BAP1*, located on chromosome 3p21.1, *GNAQ,* located on 9q21.2, and *GNA11*, located on 19p13.3, were reported to be mutated in uveal melanoma before. Therefore, each of these genes was analyzed for presence of mutation in the current cell lines as well. The GNAQ and GNA11 genes were analyzed for the common mutations in exons 4 and 5 and for the BAP1 gene. All coding and non-coding exons and at least 50 bases of flanking intronic sequence of the candidate genes were examined by sequencing techniques as described before[15]. The primer sequences and PCR protocols are available from the authors upon request.

Results

Genome wide analysis by SNP-array

By using SNP-array, chromosomal alterations were found in all cell lines, mainly involving the chromosomes 2, 3, 6, 9, 16 and 22 (Figure 1). The most important common alterations present in all cell lines currently tested were: gain of chromosome 2p, loss of the telomeric part of 2q, gain of 6p with simultaneous loss of 6q, loss of chromosome 9 and 16, and gain of chromosome 22. A few alterations, such as loss of chromosome 1p and LOH of chromosome 5 were only observed in the metastatic cell lines. A detailed analysis of all karyotypes is currently in progress (manuscript in preparation). As alterations of chromosome 3 are frequently found in UM, and known prognostic factors in UM, these alterations were further examined by SNP-array, FISH experiments and polymorphic marker analysis.

Table 1. Results of **chromosome 3p** analysis of the Mel270 cell line, using FISH probes and microsatellite markers.

BAC	Position*	Copy number	Marker	Position*	Number of alleles
-			D3S3050	3p26.3	2
28P14	3p26.1	2	D3S1304	3p26.1	2
-			D3S3728	3p26.1	2
-			D3S3591	3p26.1	1
-			D3S1537	3p26.1	2
-			D3S4545	3p26.1	2
128A5	3p25.3	2	-		
-			D3S3691	3p25.3	2
-			D3S1597	3p25.3	2
481H17	3p25.3	2	-		
767C1	3p25.1	2	D3S3693	3p25.1	1
-			D3S3608	3p25.1	2
255O19	3p24.3	2	D3S1286	3p24.3	2
-			D3S1293	3p24.3	1
208G16	3p24.3	2	-		
41F5	3p24.3-24.2	2	-		
592A5	3p24.2	-	-		
-			D3S1266	3p24.1	1
11L6	3p24.1	2	-		
1005P19	3p24.1	2	-		
-			D3S3727	3p24.1	1
-			D3S1211	3p23	1
-			D3S2432	3p23-p22.3	2
-			D3S3518	3p22.3	2
-			D3S1619	3p22.3	2
209O16	3p22.2	2	-		
189H19	3p21.32-3p21.31	2	-		
78B21	3p14.3	2			
522N9	3p13	2	-		
-			D3S2406	3p13	2

BAC = Bacterial Artificial Chromosome (used as FISH probe)

* = position according to the UCSC Humane Genome Browser (March 2006)

Table 2. Results of **chromosome 3q** analysis of the Mel270 cell line, using FISH probes and microsatellite markers.

BAC	Position*	Copy number	Marker	Position*	Number of alleles
-			D3S3045	3q13.12	2
-			D3S3675	3q13.2	2
-			D3S1558	3q13.32	2
-			D3S3649	3q13.32	2
-			D3S1303	3q13.32	2
-			D3S3703	3q13.32	2
-			D3S3515	3q13.32	2
-			D3S3620	3q13.33	2
114A6	3q21.1	2	-		
217P4	3q21.1	2	D3S1267	3q21.1	2
95H16	3q21.2	2	D3S1269	3q21.2	2
205A6	3q21.2	1	D3S1589	3q21.2	1
59J16	3q21.3	1	D3S3606	3q21.3	1
-			D3S1587	3q22.1	1
-			D3S1292	3q22.1	1
-			D3S1273	3q22.1	1
220J13	3q22.2	1	D3S1615	3q22.2	1
219P10	3q22.3	1	D3S3528	3q22.3	1
162J10	3q22.3	1	D3S1576	3q22.3	1
166D18	3q23	1	D3S3554	3q23	1
-			D3S1309	3q23	2
-			D3S3694	3q23	ni
160A13	3q23	2	D3S3546	3q23	ni
165M11	3q24	2	D3S1569	3q24	2
72E23	3q24	2	D3S1557	3q24	2
-			D3S1593	3q24	2
88H10	3q24	2	D3S1608	3q24	2
-			D3S3627	3q24	2
-			D3S196	3q24	2
-			D3S2440	3q24	ni
-			D3S3618	3q24	2
-			D3S1306	3q24	2
-			D3S3626	3q24	2
229G6	3q24	2	-		
145F16	3q25.1	2	D3S1299	3q25.1	ni
64F6	3q25.1	2	D3S1279	3q25.1	2
65L11	3q25.1	2	-		
80I14	3q25.2	2	D3S1280	3q25.1-q25.2	2
-			D3S3702	3q26.1	2
-			D3S2421	3q26.31	ni
-			D3S1580	3q28	2
53D15	3q28	2	D3S1294	3q28	2
147L6	3q28	2	-		
-			D3S1601	3q28	2
-			D3S1272	3q29	ni

BAC = Bacterial Artificial Chromosome (used as FISH probe)

* = position according to the UCSC Humane Genome Browser (March 2006)

ni = noninformative marker

Chromosome 3p aberrations

The SNP-array results indicated several identical regions with allelic imbalance of chromosome 3p in Mel270, OMM2.3, and OMM2.6 (Figure 2). The first region found was spanning 3p25.2-p25.1 (Figure 3A). Using FISH probe *RP11-767C1*, located on 3p25.1, two allele signals were observed in 91% of tumor cells analyzed (Figure 3B). The corresponding polymorphic marker *D3S3693* displayed presence of one allele type only, thereby confirming the state of copy number neutral LOH (Figure 3B). Flanking markers *D3S1597* and *D3S3608*, and FISH probes *RP11-481H17* and *RP11-255O19* marked the telomeric-, and centromeric borders, respectively of this region.

A larger region with copy number neutral LOH was spanning 3p24.2-p23 (Figure 4A). A FISH probe, *RP11-11L6 (*3p24.1) displayed two allele signals whereas the corresponding markers *D3S1266* and *D3S1211*, mapping to 3p24.1 and 3p23, respectively, both displayed one allele type only (Figure 4B). Flanking markers mapping outside this region, indicated presence of two allele types (3p24.3 and 3p23-p22.3).

The smallest region with copy number neutral LOH on chromosome 3p comprised chromosomal band 3p14.3 (results not shown). The FISH probe *RP11-78B21*, mapping to this region, showed two signals, and polymorphic markers to determine whether this is a homozygous region are currently being tested.

In cell lines OMM2.2 and OMM2.5 copy number neutral LOH was determined along the complete chromosome 3 (Figure 2).

Chromosome 3q aberrations

A partial deletion was found spanning from 3q21.2-q23, in both the primary UM cell line Mel270 (passage 27) as well as the metastatic cell line OMM2.3 (Figure 2 and 5A).

FISH analysis of Mel270 confirmed deletion of the 3q21.2-q23 region by displaying loss in 41-45% of tumor cells for all loci tested within this region (Figure 5B). FISH probes *RP11-95H16* and *RP11-160A13*, with their corresponding markers *D3S1269* and *D3S1309*, mapping outside the deleted region, displayed a normal disomic state. In OMM2.3, FISH probe *RP11-59J16* displayed loss in 70% of tumor cells mapping to 3q21.3 (results not shown). *RP11-114A6,* mapping outside the deletion, displayed a normal disomic state in 91% of tumor cells analyzed (data not shown).

In addition to the cell lines OMM2.2 and OMM2.5 displaying copy number neutral LOH along the complete chromosome 3, copy number neutral LOH was also observed for a large part of the 3q-arm in cell line OMM2.6 (Figure 2).

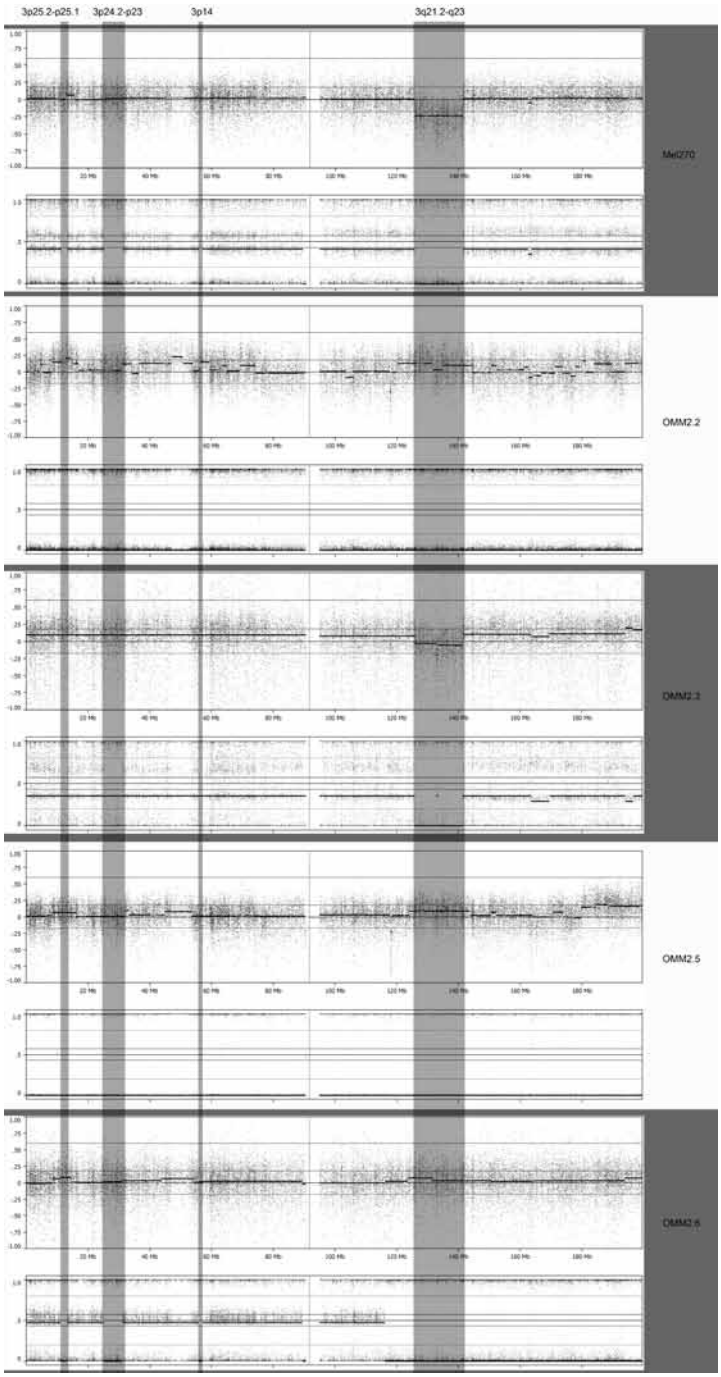
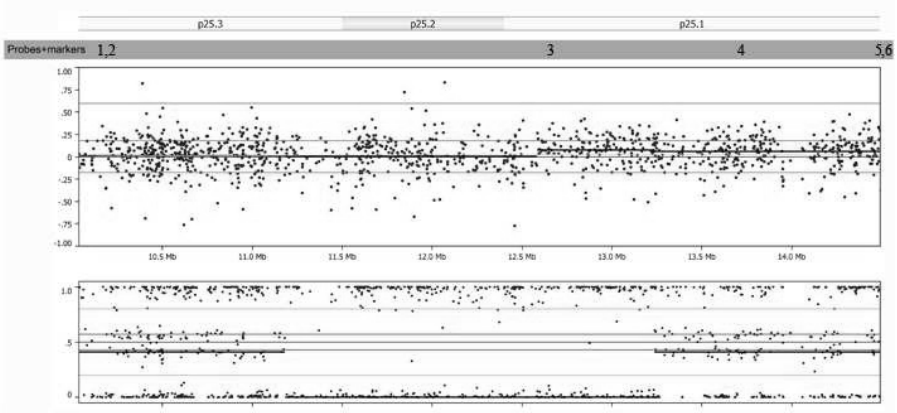


Figure 2: SNP-array overview of chromosome 3 of all cell lines with the earliest passages analyzed The Log-R ratio is displayed in the upper panel for each cell line and the B-allele frequency in the lower panel for each cell line. **(color page 193)**

A



B

Location probes + markers	BAC	Position*	Copy number	Largest clone (%)	Marker	Position*	Number of alleles
1,2	481H17	3p25.3	2	85%	D3S1597	3p25.3	2
3	767C1	3p25.1	2	91%	D3S3693	3p25.1	1
4	-				D3S3608	3p25.1	2
5,6	255O19	3p24.3	2	83%	D3S1286	3p24.3	2

C

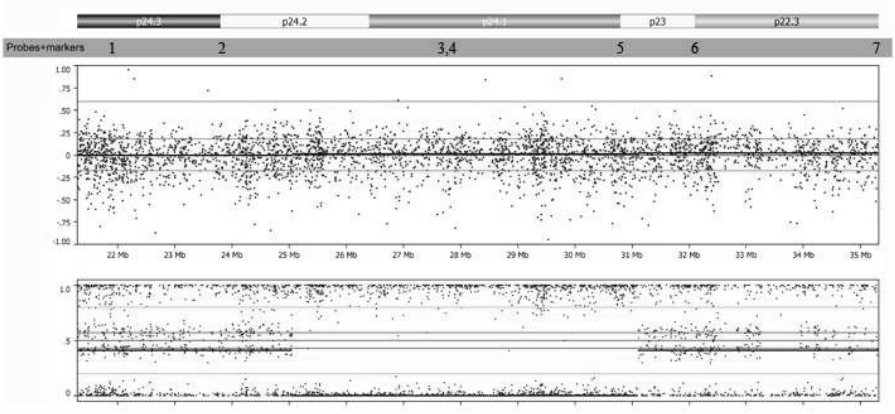
Position*	Candidate genes
3p25.3	*HRH1*
3p25.3-p25.2	ATG7
3p25.2	*VGLL4*, *SYN2*/ *TIMP4*, *C3orf31*
3p25.2-p25.1	*PPARγ*
3p25.1	*RAF1*, *TSEN2*, *MKRN2*, *IQSEC1*, *CAND2*, *RPL32*, *TMEM40*

Figure 3: Detail of the LOH region on 3p25.2-p25.1 with SNP-array results (A), FISH probe and microsatellite marker analysis (B), and candidate genes mapping to this region (C).
Figure A: Log-R ratio (upper panel) and B-allele frequency (lower panel) of SNP-array.
Figure B: Markers and probes mapping to the numbered locations displayed in figure A.
BAC = Bacterial Artificial Chromosome (used as FISH probe).
* = position according to the UCSC Humane Genome Browser (March 2006).
Figure C: Candidate genes mapping to the region identified by Endeavour prioritizing software.
(color page 194)

Increasing passage numbers

SNP-array data of the later passage of the Mel270 cell line with the more recent passage number (passage 38) (Figure 6C) displayed a smaller drop in Log-R signal within the 3q21.2-q23 region when compared with the earlier passage available (passage 27) (Figure 6A). This indicated that as passage numbers increased, copy numbers of the 3q21.2-q23 region in Mel270 restored to two copies in the majority of tumor cells though the LOH status remained. FISH analysis using probes *RP11-59J16* and *RP11-220J13* displayed two copies for the corresponding chromosomal regions in 89% and 87% of tumor cells, with only 11% and 13% of tumor cells displaying one FISH probe signal (Figure 6D). The regions with LOH on the p-arm of chromosome 3 remained as passage numbers of Mel270 and OMM2.3 increased, with no new altered regions found on chromosome 3. The SNP-array

A



B

Location probes + markers	BAC	Position*	Copy number	Largest clone (%)	Marker	Position*	Number of alleles
1	-				D3S1293	3p24.3	2
2	41F5	3p24.3-p24.2	2	90%	-		
3,4	11L6	3p24.1	2	92%	D3S1266	3p24.1	1
5	-				D3S1211	3p23	1
6	-				D3S2432	3p23-p22.3	2
7	209O16	3p22.2	2	93%	-		

C

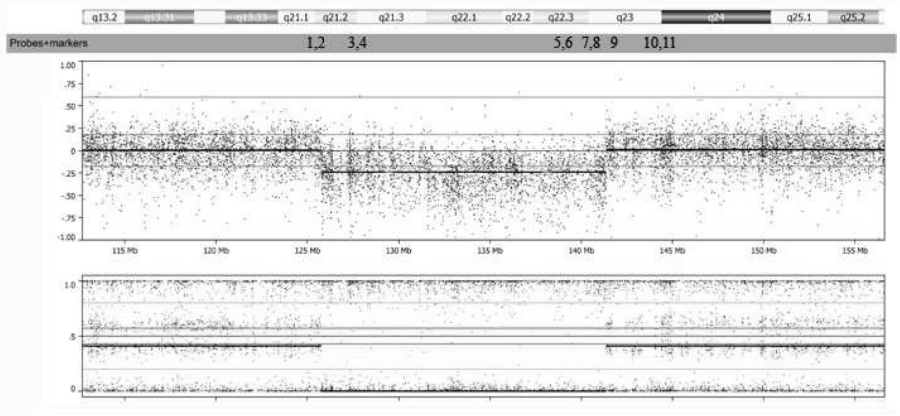
Position*	Candidate genes
3p24.2	*TOP2B*, *RARB*, *NGLY1*, *OXSM*
3p24.1	*TGFBR2*, *AZI2*, *SLC4A7*, *RBMS3*, *EOMES*, *LRRC3B*, *NEK10*, *ZCWPW2*
3p23	*GADL1*

Figure 4: Detail of the LOH region on 3p24.2-p23 with SNP-array results (A), FISH probe and microsatellite marker analysis (B), and candidate genes mapping to this region (C).
Figure A: Log-R ratio (upper panel) and B-allele frequency (lower panel) of SNP-array.
Figure B: Markers and probes mapping to the numbered locations displayed in figure A.
BAC = Bacterial Artificial Chromosome (used as FISH probe).
*** =** position according to the UCSC Humane Genome Browser (March 2006).
Figure C: Candidate genes mapping to the region identified by Endeavour prioritizing software.
(color page 195)

data for Mel270 (passage 27) showed the presence of two allele types of chromosome 3; one with the 3q21.2-q23 deletion and one without the deletion. Combining the Log-R data and B-allele frequency data indicated that the ratio of the first and second allele type was 2:3 with passage 27 of Mel270, which changed to 1:5 in the later passage 38. FISH confirmed the change in allele ratio by displaying loss in 41-45% of cells (Figure 6B) and in 87-89% (Figure 6D), respectively. An overview of the higher number passages for several cell lines is displayed in supplementary figure 1.

The resulting LOH in this region was also observed in all metastatic cell lines either as copy neutral LOH (OMM 2.2 and 2.5) or as a partial deletion of 3q14-qter (OMM2.6) (Figure 2). In contrast to the partial chromosome 3 deletions and regions with LOH in Mel270 and

A



B

Location probes + markers	BAC	Position*	Copy number	Largest clone (%)	Marker	Position*	Number of alleles
1,2	95H16	3q21.2	2	79%	D3S1269	3q21.2	2
3,4	205A6	3q21.2	1	45%	D3S1589	3q21.2	1
5,6	162J10	3q22.3	1	41%	D3S1576	3q22.3	1
7,8	166D18	3q23	1	42%	D3S3554	3q23	1
9	-				D3S1309	3q23	2
10,11	160A13	3q23	2	80%	D3S3546	3q23	ni

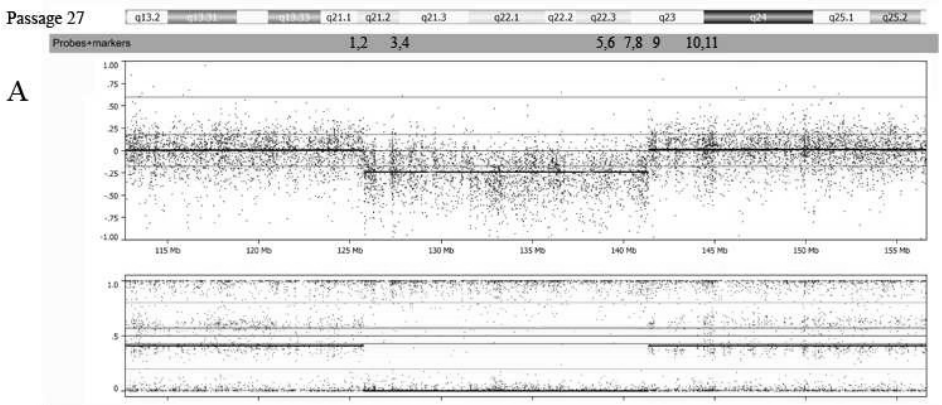
C

Position*	Candidate genes
3q21.2	*SLC12A8*, *ZNF148*
3q21.3	*MCM2*, *RUVBL1*, *MBD4*, *RHO*
3q22.1	*TOPBP1*, *RYK*, *NEK11*, *ATP2C1*
3q22.2	*PPP2R3A*
3q22.3	*PIK3CB*, *NCK1*, *STAG1*, *MRAS*

Figure 5: Detail of the deleted region 3q21.2-q23 with SNP-array results (A), FISH probe and microsatellite marker analysis (B), and candidate genes mapping to this region (C).
Figure A: Log-R ratio (upper panel) and B-allele frequency (lower panel) of SNP-array.
Figure B: Markers and probes mapping to the numbered locations displayed in figure A.
BAC = Bacterial Artificial Chromosome (used as FISH probe).
* = position according to the UCSC Humane Genome Browser (March 2006).
ni = noninformative marker
Figure C: Candidate genes mapping to the region identified by Endeavour prioritizing software.
(color page 196)

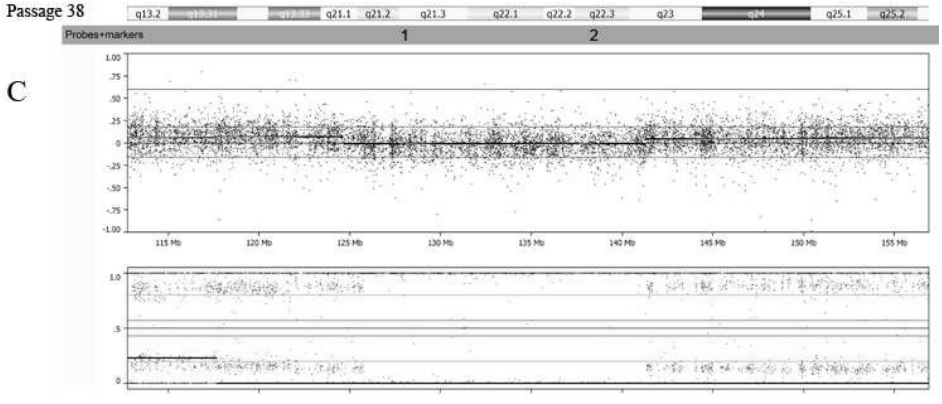
OMM2.3, metastatic cell lines OMM2.2 and OMM2.5 also displayed regions with amplification on chromosome 3. OMM2.2 displayed (five copies) of chromosome 3 in 50% of the tumor cells while OMM2.5 showed trisomy (three copies) of chromosome 3 in 50-90% of tumor cells, both with complete LOH. The 3q-telomere of OMM2.5, ranging from 3q26.33-q29, also displayed pentasomy and complete LOH. In OMM2.6, results indicated trisomic status in 30-75% of tumor cells (data not shown) with LOH ranging from 3q13.31 to the 3q telomere.

PCR sequencing experiments used for mutational analysis of *BAP1* are in progress, the results are due soon and will be included in the final manuscript. Although not located on



B

Location probes + markers	BAC	Position*	Copy number	Largest clone (%)	Marker	Position*	Number of alleles
1,2	95H16	3q21.2	2	79%	D3S1269	3q21.2	2
3,4	205A6	3q21.2	1	45%	D3S1589	3q21.2	1
5,6	162J10	3q22.3	1	41%	D3S1576	3q22.3	1
7,8	166D18	3q23	1	42%	D3S3554	3q23	1
9	-				D3S1309	3q23	2
10,11	160A13	3q23	2	80%	D3S3546	3q23	ni



D

Location probes + markers	BAC	Position*	Copy number	Largest clone (%)	Marker	Position*	Number of alleles
1	59J16	3q21.3	2	89%			
2	220J13	3q22.2	2	87%			

Figure 6: Detail of the deleted region 3q21.2-q23 for the earlier passage of Mel270 (passage 27) and the later passage of Mel270 (passage 38).
SNP-array results for passage 27 (A), passage 38 (C), and FISH probe and microsatellite marker analysis for passage 27 (B), and FISH analysis for passage 38 (D).
Figure A: Log-R ratio (upper panel) and B-allele frequency (lower panel) of SNP-array.
Figure B: Markers and probes mapping to the numbered locations displayed in figure A.
BAC = Bacterial Artificial Chromosome (used as FISH probe).
* = position according to the UCSC Humane Genome Browser (March 2006).
ni = noninformative marker
Figure C: Candidate genes mapping to the region identified by Endeavour prioritizing software.
Figure D: Markers and probes mapping to the numbered locations displayed in figure C.

(color page 197)

chromosome 3 but on chromosome 9q21.2, a *GNAQ* point mutation was found within exon 5 in all cell lines, whereas no wild-type allele was found to be present. This confirmed the observation in the SNP-array where we identified LOH of chromosome 9 in all cell lines (Table 1). There was no mutation found in the *GNA11* gene.

Discussion

Only a few genes involved in UM pathology or progression to metastasis have been identified until now. Recently, *GNAQ/GNA11* mutations were reported in the majority of UM specimens analyzed[16-17]. These *GNAQ/GNA11* mutations appeared to be early events, not related to outcome and the risk of progression to metastasis[18-19]. Mutations of *BAP1*, located on chromosome 3p21.1, were reported in metastasizing UMs[20] and other malignancies such as lung carcinoma and meningioma as well[21-22]. Although not unique to UM, *BAP1* is a potential target for gene targeted therapy preventing metastasis in patients with UM, and clinical trials are currently underway. As chromosome 3 is frequently involved in metastasizing UMs, it is possible that more candidate (metastasis) suppressor genes are located on chromosome 3 and their identification may lead the way for further research. The same accounts for putative tumor suppressor or oncogenes on chromosome 1p36 and 8q, since these regions are also frequently deleted and amplified, respectively, in metastasizing UMs[23]. Van Gils et al reported[24] that expression of *APITD1*, a gene located on 1p36, was not related to deletion of chromosome 1p36 and concluded that *APITD1* is not one of the candidate genes involved in UM metastasis. *MYC* and *DDEF1* have been observed in UMs before, but its role in tumor metastasis remains unclear[25-26].

In the current study several different copy number alterations were found in the cell lines, including gain of 5p in OMM2.5, and deletion of chromosome 9, which is remarkable as both are seldom found to be altered in UM. For chromosome 3, three regions with LOH located on 3p25.1-p25.2, 3p23-p24.2, and 3p14.3, and one region of partial deletion on 3q21.2-q23 were found in the primary UM cell line Mel270 and the liver metastatic cell lines OMM2.3 and OMM2.6. Parrella et al.[10] reported one of the regions with LOH mapping to chromosome 3p25.1-p25.2 in a set of primary UMs. In uveal melanomas that metastasized, two regions of LOH mapping to 3p25 and 3q24-q26, were observed by Tschentscher et al.[9] Both regions were therefore suggested to harbor tumor suppressor genes. In the present study, the 3p25.1-p25.2 LOH region was identified by SNP-array and confirmed with polymorphic marker *D3S1293*. The 3p25.1-p25.2 LOH region in cell lines Mel270, OMM2.3 and OMM2.6 resemble the LOH region reported by Parrella et al[10] and therefore seems to be a promising region for pinpointing of candidate genes involved in metastatic development. Specific genes mapping to this 3p25.1-p25.2 region were identified by using Endeavour gene prioritizing software (http://homes.esat.kuleuven.be/~bioiuser/endeavour/tool/endeavourweb.php) with a training set of genes related to UM-, and cutaneous melanoma

pathogenesis. With this technique, the following candidate genes were identified: *RAF1*, *PPARγ*, *TSEN2*, *HRH1*, *MKRN2*, *VGLL4*, *IQSEC1*, *CAND2*, *SYN2*, *C3orf31*, *RPL32*, *ATG7*, *TMEM40*, *and TIMP4* (Figure 3C).*RAF1*, also known as *CRAF*, is an interesting candidate gene as it is known to be involved in the RAS-RAF-MEK-ERK pathway interacting with many known genes such as *BRAF*, *RAS*, *PTEN*, *GNAQ*, *GNA11* (recently reviewed by van den Bosch et al 2011[27]). The *PPARγ* gene had previously been reported by Lake et al[28] who studied 34 early metastasizing UMs by MLPA and SNP-array, and suggested *PPARγ* (together with *RBM5* and *ROBO1*) as keys to early metastatic progression. *PPARγ* had been implicated in several solid malignancies as well, such as breast-, prostate-, and colorectal cancer[29-31] and is known to interact with β-Catenin and the Wnt signaling pathway. Decreased levels β-Catenin and *PPARγ* were found in liver metastasis of colorectal cancer[32]. Whether these genes play a similar role in UM remains to be resolved as an increased expression of β-Catenin and Wnt5a showed an association with poor survival in UM[33].

The region with copy neutral LOH spanning from 3p23-p24.2 does not overlap with the smallest region of overlap defined by Tschentscher et al and Parrella et al. Gene analysis of the 3p23-p24.2 region indicated the following candidate genes involved in UM pathology: *TGFBR2*, *TOP2B*, *AZI2*, *SLC4A7*, *RARB*, *RBMS3*, *NGLY1*, *EOMES*, *LRRC3B*, *NEK10*, *GADL1*, *OXSM*, *ZCWPW2* (Figure 4C). The candidate tumor suppressor gene *Retinoic Acid Receptor β2* (*RARβ2*), located on 3p24.2, is highly ranked in the prioritization analysis. Decreased levels of this receptor are associated with malignancies, such as breast tumors, lung cancer and squamous cell cancer of head and neck[34]. Additional genes involved in the 3p24 locus are *NEK10* and *SLC4A7*, which both lie in a small region of LOH previously reported in breast cancer[35]. The genes *AZI2*, *RBMS3*, *LRRC3*B, *GADL1* and *ZCWPW2* had been reported by Lake et al[28] to be deleted exclusively in in a group of metastasizing UMs, analyzed by SNP-array.

The smallest LOH region found on chromosome 3, 3p14.3, is partly comparable to another region of loss reported by Parrella et al[10]: 3p12-14. Genes mapping to this 3p14.3 location are: *ARHGEF3*, *C3orf63*, *ERC2*, *CCDC66*. The nearby region of 3p14.2 is a frequently rearranged region known to contain the candidate gene *FHIT* which spans the FRA3B fragile site[10].

As far as we know, tumor suppressor gene mapping to 3q21.3-q23 has not been identified to date. This region was found to be deleted in the Mel270 cell line as well as the liver metastatic cell line OMM2.3 (Figure 2 and 5), and could thus also be an important region involved in the development of metastases. The present deleted region does not overlap with the LOH region on 3q previously reported by Tschentscher[9] (3q24-q26). The difference in reported region could be due to the fact that Tschentscher et al used a low-resolution maker set. Furthermore, our data corroborate with a study by Dahlenfors et al[36] pointing to

the 3q23 region as a possible tumor suppressor gene location, based on a rearrangement found in one UM. Gene analysis of the 3q21.3-q23 region with Endeavour prioritization software, indicated the following candidate genes: *PIK3CB, MCM2, TOPBP1*, *NCK1*, *RUVBL1, PPP2R3A, PIK3R4, RYK, STAG1, NEK11, MBD4, MRAS, ATP2C1, RHO, SLC12A8*, and *ZNF148* (Figure 5C). *NEK11* had also been reported before in the study by Lake et al[28] as candidate metastasis suppressor gene in the series of early metastasizing UMs.

While conventional CGH analysis previously revealed a deletion from 3q13-q21[11], our current fine-mapping studies pointed to a deletion ranging from 3q21.2-q23. This discrepancy can be explained by the fact that the resolution of conventional CGH on metaphases is limited[37]. Increased passage numbers of Mel270 (from 27 to 38) resulted in an acquired second allele in the majority of tumor cells (from 55-59% to 87-89%, Figure 6) for the previously deleted region 3q21.2-q23 but with retention of LOH. This could indicate a growth advantage of tumor cells that regained a second allele, even though regaining of the second allele resulted in a copy number neutral region of LOH. Allelic loss, in this case presenting as LOH, could lead to UM metastasis through inactivation of the second allele by gene deletion, point mutations or promoter hypermethylation. Using MLPA and SNP-array, Lake et al[28] identified frequently deletions of the regions 3p25.3, 3p25.1, 3p14.2 and 3p12.2 and regions of LOH on chromosome 3 in early metastasizing UMs, which were previously determined as disomy 3 by FISH. This indicates that more FISH-determined disomy 3 UM cases may have small copy number alterations or regions of LOH and might explain the disomy 3 cases that have metastasized.

The 3p21.1-p21.31 region could not be reliably assessed for copy number alterations and zygosity changes as the used SNP-array BeadChip had low SNP coverage within this region (low intensity of Log-R calls and B-allele frequency call). Therefore unfortunately no conclusions can be drawn for copy number status of the *BAP1* gene. Mutational analysis data of *BAP1* will follow soon. A *GNAQ* mutation of exon 5 (*GNAQ* located on 9q21.2) was found in all cell lines and there was no remaining wild type allele.

The unusual appearance of loss of chromosome 9 and partial chromosome 3q loss and regions of LOH on chromosome 3p, might be a result of previous radiotherapy. However, the current regions were found both in both the primary cell line and the metastatic cell lines and similar regions have been reported before by others[9-10]. This could indicate that these regions are involved in UM metastasis, through mutation of one or more genes within these regions or other mechanisms. The regions of LOH along chromosome 3p, could also be germ line alterations as these were present in all cell lines analyzed (OMM2.2 and OMM2.5 had complete LOH of chromosome 3). There was unfortunately no normal tissue available to test for germ line alterations.

One of the difficulties studying UM cell lines is the possibility of continuing genetic instability and development of sub-clones. This especially accounts for high passage number cell lines. However, cell lines guarantee an unlimited supply of material, which is very helpful in the search for putative tumor suppressor genes. Extensive fine-mapping research as we did in the present study would not be possible without using cell lines; most uncultured primary UMs do not yield sufficient tumor tissue for all these genetic tools to be used in combination. We analyzed earlier and late passages of each cell line and found only Mel270 to have changed in karyotype. In this case we found a regression to a more normal situation with regaining of a second allele for the previously deleted region 3q21.2-q23 (passage 38) in Mel270 but with retention of LOH.

Only few melanomas with structural abnormalities of chromosome 3 or translocations involving chromosome 3 have been reported up till now, which complicates mapping of putative tumor suppressor genes. To our knowledge this is the first report of a combined cytogenetic and molecular genetic evaluation of a primary UM- and liver metastatic cell lines with partial loss of chromosome 3 found. We found that the in-situ results of copy number- and zygosity status obtained by earlier FISH and polymorphic microsatellite marker analysis corroborate and complement the information gained by SNP-array. The SNP-array technique combines analysis of allelic imbalances and copy number variations in a rapid, reliable and genome-wide manner. This is convenient as microsatellite analysis is not always a reliable technique for discriminating loss of one allele from lack of heterozygosity of the used microsatellite[38] in the case only one allele is observed. For in-situ absolute copy number assessment by using SNP-array, validation should take place using another technique such as FISH. In the future, expression analysis could be performed to assess what effect the copy number variations have on the transcriptional level. The currently identified candidate regions and genes require further research before their involvement in UM metastasis can be definitely determined.

Conclusion

In the present study, cell lines were used a model system for UM metastasis. Several regions with copy number neutral LOH (3p25.1-p25.2, 3p23-p24,2 3p14.3) and one deleted region (3q21.2-q23) had been found in the primary UM cell line Mel270 and several metastatic liver cell lines from the same patient. The genes located within the mentioned regions might be candidate genes involved in UM metastasis and require further research in the future.

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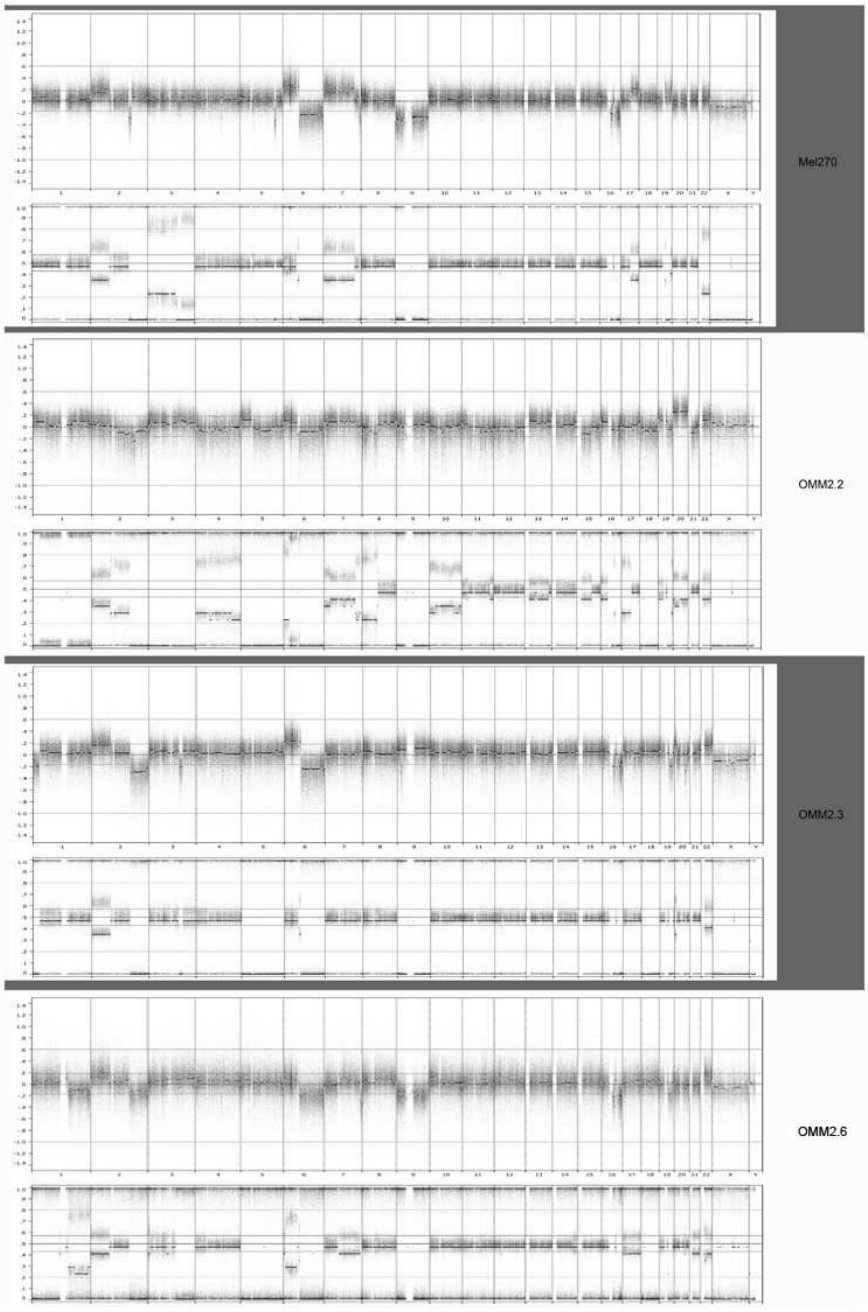

Supplementary figure 1: Whole genome SNP-array overview of cell lines with the highest passage numbers analyzed (OMM2.5 not available).
The Log-R ratio is displayed in the upper panel for each cell line (except OMM2.5), and the B-allele frequency in the lower panel for each cell line. **(color page 198)**

Chapter 7

Histopathologic, immunohistochemical, ultrastructural, and cytogenetic analysis of oncocytic uveal melanoma

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*Both authors contributed equally to this paper.

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Introduction

The histological findings of malignant melanoma may be highly variable, and the tumor can mimic many other neoplasms.[1] Oncocytic change is defined histologically by abundant, eosinophilic, finely granular cytoplasm due to densely packed mitochondria. Oncocytic change has rarely been described in dermal nevi,[2] meningeal melanocytoma,[3] cutaneous melanoma,[4, 5] or metastatic melanoma.[6] To our knowledge, we give the first description of exclusively oncocytic uveal melanoma.

Report of a Case

A 73-year-old male visited the outpatient department of ophthalmology with signs of a paracentral scotoma, decreased vision and metamorphopsia in his left eye for 1 month. Best corrected visual acuity was 40/40 OD and 20/40 OS. On dilated fundoscopic and ultrasonographic examination of the left eye, a mushroom-shaped hypopigmented subretinal mass was seen superior and temporal to the fovea with a thickness of 7.1 mm, a diameter of 11.4 mm, and medium to low internal reflectivity (Figure, A). No atypical cutaneous pigmented lesions were observed. Systemic radiologic evaluation revealed no metastatic lesions. The patient opted for enucleation. After a follow-up of 24 months there were no signs of metastases.

Sections of the eye confirmed a mushroom-shaped tumor (Figure, B), exclusively composed of a trabecular arrangement of epithelioid cells with abundant, finely granular, eosinophilic cytoplasm (Figure, C). Mitotic figures were present at 2 per 10 high-power fields. Intracytoplasmatic brown pigment stained positive with Masson-Fontana stain. The cytoplasm stained positive periodic acid-Schiff stain with resistance to diastase treatment. Vascular mimicry with a closed loop pattern was present. The tumor did not show extrascleral extension. Tumor cells stained positive for Melan-A, HMB-45 (Figure, D), and tyrosinase, confirming melanocytic lineage. They stained negative for keratin A1/A3, CD56, chromogranin, and synaptofysin, excluding epithelial (neuroendocrine) metastasis.

Ultrastructural studies on formalin-fixed, paraffin-embedded tumor tissue that was deparaffinized, postfixated with osmiumtetraoxide, and re-embedded in Epon (Hexion Specialty Chemicals, Inc, Danbury, Connecticut) showed cytoplasm densely packed with mitochondria and sparse melanosomes (Figure, E). No epithelial features were observed.

Fluorescence in situ hybridization experiments on tumor tissue indicated 2 copies of chromosomes 1, 3, 6, and 8. Multiplex ligation-dependent probe amplification testing with the salsa P027 uveal melanoma kit (MRC-Holland, Amsterdam, the Netherlands) confirmed this normal disomic state for a total of 31 different regions tested on multiple chromosomes. Single-nucleotide polymorphism array analysis revealed no copy number alterations or

regions of heterozygosity on any of the chromosomes. These investigations have been carried out according to tenets of the declaration of Helsinki.

Comment

The many histologic faces of melanoma include primary and metastatic carcinoma, neuroendocrine tumors, sarcoma, leukemia and germ cell tumors.[1] Intraocular oncocytoma has been considered in the differential diagnosis of mesectodermal leiomyoma of the ciliary body.[7] A granular cell tumor of the iris and ciliary body has been described.[8] The diagnosis of choroidal melanoma and exclusion of other cancers was based on the tumor's characteristic mushroom shape, positive immunohistochemical staining for HMB-45, Melan-A, and tyrosinase, and a 2-year follow-up without evidence of another primary cancer.

The prognostic significance of oncocytic change in uveal melanoma is not clear. Our case displayed unfavorable histological prognostic parameters in tumor size, epithelioid cell type and vascular pattern. This was not corroborated with cytonuclear negative parameters as no cytogenetic aberrations were present. Earlier, it was reported that cytogenetic aberrations were detected in 80% (59 of 74 cases) of a series of uveal melanoma.[9] In cutaneous melanoma, no prognostic significance could be determined.[5] Oncocytic change is generally proposed to be a reactive degenerative adaptation[10]; however, the fact that no cytogenetic changes were observed in this tumor poses the possibility of a distinct tumor variant as opposed to a degenerative change.

In conclusion, to our knowledge we present the first description of an oncocytic uveal melanoma that is not to be mistaken histologically for other tumors, including metastatic carcinoma.

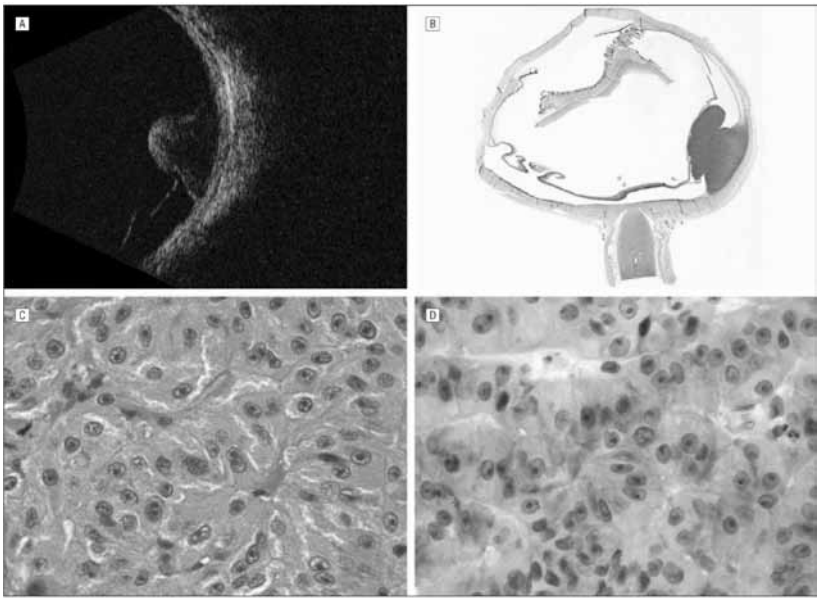

Figure ABCD: Ultrasonographic, whole-mount, histologic, and immunohistochemical appearance of the tumor. A B-scan of the tumor (A) and a whole-mount hematoxylin-eosin-stained section (B) of the left eye show a mushroom-shaped subretinal mass in the posterior pole. C, The tumor was exclusively composed of a nested and trabecular pattern of polygonal epithelioid cells with distinct borders and a granular eosinophilic cytoplasm that sometimes contained brown pigment. The nests and trabeculae were surrounded by a delicate capillary network. Nuclei were enlarged with coarse open chromatin and prominent irregular nucleoli (hematoxylin-eosin, original magnification x400). D, The cells stained positive for the melanocytic marker HMB-45 (original magnification x400). **(color page 199)**

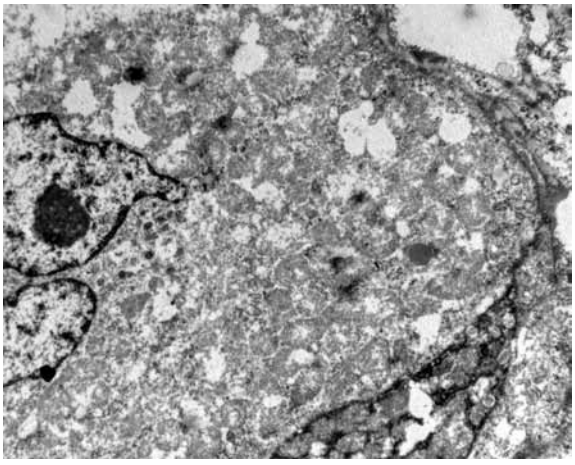

Figure E: Ultrastructural appearance of the tumor. Ultrastructural examination of formalin-fixed, deparaffinized, plastic re-embedded tumor tissue shows early and late melanosomes in cytoplasm, densely packed with structures that can be identified as mitochondria. Due to improper fixation many artefacts can be seen. **(color page 199)**

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Part 4.

Candidate genes associated with uveal melanoma progression

Chapter 8

PARK2 copy number variations and mutations are not present in uveal melanoma

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Article submitted for publication

Abstract

Background: Uveal Melanoma (UM) is the most common primary intraocular malignancy in adults. Loss of the long arm and gain of the short arm of chromosome 6 are frequently observed chromosomal aberrations in UM, together with loss of chromosome 1p36, loss of chromosome 3 and gain of chromosome 8. This suggests the presence of one or more oncogenes on 6p and tumor-suppressor genes on 6q that are involved in UM development.
Methods: DNA of 10 UM samples with loss of chromosome 6q and 10 UM samples with normal copy numbers of chromosome 6 was isolated and used for the detection of loss of the 12 exons of *PARK2* using multiplex ligation-dependent probe amplification (MLPA). To detect smaller mutations all exons of *PARK2* were re-sequenced using Sanger sequencing.
Results: Loss of chromosome 6q as determined by routine FISH was confirmed with MLPA and no small deletions involving single exons were observed.
Conclusion: Our results indicate that *PARK2* is not instrumental as a tumor suppressor gene in UM development metastasis. However rearrangements of this 6q26 region have been observed in a special set of uveal melanomas and it will be interesting to determine (Copy number variations) CNV or mutations of the *PARK2* locus in these specific tumor samples.

Introduction

Uveal melanoma (UM) is the most common primary eye malignancy with an incidence of about 7 per million people every year in the western world[1]. Several prognostic parameters are available to identify patients at risk of developing metastases. Among these are the cytogenetic parameters: loss of chromosome 1p36, loss of chromosome 3, gain of chromosome 8 and abnormalities on chromosome 6[2]. Abnormalities on chromosome 6 have also been described in other types of tumors. Gain of the short arm of chromosome 6 is a frequently occurring event in many other neoplasms, including lymphoid tumors, sarcomas, retinoblastoma and cutaneous melanoma[3-7]. This suggests the presence of a common oncogene in this chromosomal region. Correlation of gain of 6p with decreased survival was found in certain types of sarcoma and cutaneous melanoma[3 8-9]. In UM, aberrations of chromosome 6p also occur frequently and a correlation with favorable survival has been described[10]. Deletion of the long arm of chromosome 6 also occurs in many neoplasms, including carcinomas of the prostate and breast, and melanomas[11-13]. In several studies on different types of tumors the relation of chromosome 6q loss with survival was investigated. In cutaneous melanoma and acute lymphoblastic leukemia, loss of 6q is correlated with a poor clinical signature[14-15]. Partial deletions of chromosome 6q have been observed without abnormalities of chromosome 1, 3 or 8[16]. Aalto et al. showed that loss of chromosome 6q is associated with decreased survival in UM patients[17]. This suggests the presence of a tumor-suppressor gene, or genes at 6q. Although the chromosome 6p findings are contradictory and a possible relation between abnormalities on chromosome 6 and prognosis is difficult to determine, it seems very likely that there are genes on regions on both arms of chromosome 6 involved in tumor development and progression. In UM, the deletion of 6q appears to be a late event resulting from tumor progression[18]. One of the candidate tumor suppressor genes on chromosome 6q is the *PARK2* gene, one of the Parkinson genes. Although malignant melanoma and Parkinson's disease are very different diseases there is clearly a relation. The incidence of melanoma is twice as high among Parkinson's patients[19] and melanoma patients have a 50% increased risk of developing Parkinson's disease[20].

Homozygous *PARK2* mutations, known to cause early-onset Parkinson's disease[21] were recently identified as mutated tumor suppressor genes in solid malignancies[22]. Copy number variations (CNV) of *PARK2* exons were found in glioblastoma and colon cancers as well as somatic intragenic mutations which were reported for the first time in human malignancy.

PARK2 is located on chromosome 6q26 and mutation of *PARK2* could thus be an important feature connecting both diseases. Uveal melanomas harbor frequently a deletion of chromosome 6q, suggesting *PARK2* to be a candidate tumor suppressor gene involved in progression to metastasis. We therefore searched for CNV and somatic mutations of the *PARK2* gene in primary UMs with and without loss of one copy of chromosome 6q. We assessed whether

deletion of 6q occurred simultaneously with mutation of *PARK2* on the remaining allele, and like in glioblastomas, if homozygous deletions were present.

Materials and methods

Patients and tumor samples

UM samples were collected over a period of 10 years (1993-2003) from enucleated patients of the Erasmus University Medical Center and the Rotterdam Eye Hospital. Informed consent was obtained prior to enucleation and the study was performed according to the tenets of the Declaration of Helsinki. Fresh tumor material was harvested within 1 hour after surgery and processed for FISH as described previously[23]. Part of the tumor was snap-frozen and stored in liquid nitrogen. The remains of the eye were embedded in paraffin. Confirmation of the diagnosis UM was performed by histopathological examination. Only tumors located in the ciliary body and choroid were included in this study.

Directly fixated tumor cells or paraffin fixed tissue slides were assayed as part of the routine FISH analysis for loss of chromosome 6q using the BAC probe RP11-787I22, located on 6q21[24]. 10 UM tumors with deletion of the chromosome 6q-arm and 10 tumors without deletion of 6q were selected. None of the analyzed patients had a history of Parkinson's disease.

DNA-isolation

H&E staining was conducted on a 5 µm section of snap-frozen tumor to survey tumor content. Depending on the size of the tumor, 10 to 15 sections of 20 µm were used for DNA-isolation using QIAamp DNA-mini kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. DNA-concentration was measured using the NanoDrop® ND-1000 Spectrophotometer (NanoDrop technologies, Wilmington, Delaware USA).

Multiplex ligation-dependent probe amplification (MLPA)

All tumors were analyzed for CNV of the *PARK2*-gene by MLPA using the Salsa MLPA Parkinson probe kit p051 (MRC Holland, Amsterdam, the Netherlands). This kit contains several probes that are located on chromosomes 6q and contains probes for the 12 exons of the *PARK2* gene. MLPA reactions were performed with 150 ng of tumor DNA and 200 ng of female or male reference DNA. As negative control 5 µL of Milli-Q water was used in each experiment. The procedure was carried out as described[25-26]. MLPA reactions were carried out on a PCR thermocycler with heated lid (Biometra Thermal Cycler, Westburg, the Netherlands). 10 µl of highly deionised HI-DI-formamide (Applied Biosystems) was mixed with 0,15 µl of an internal size standard (GeneScan LIZ-500) and 2 µL of the PCR product. This mixture was analyzed by capillary electrophoresis on an ABI 3730 GeneScan system

(Applied Biosystems) and analyzed using GeneMarker 1.51 software (SoftGenetics, LLC, State College, PA, USA). The peak areas and the sizes of the different MLPA-probes were determined. For each probe a Relative Quantity (RQ) value was calculated using population normalization. Loss of DNA was defined as RQ ≤ 0.7, gain as RQ ≥ 1.3. A RQ-value between 0.7 and 0.8 was considered borderline loss, whereas an RQ-value between 1.2 and 1.3 was considered as borderline gain.

PARK2 Sequence analysis

The 20 tumors were further analyzed for mutations of *PARK2* by using Sanger sequencing as reported by Breedveld et al[27]. In brief all 12 coding and non-coding exons and at least 50 bases of flanking intronic sequence of *PARK2* were sequenced using PCR primers designed by Primer3 software. PCR products were purified and sequenced using BigDye Terminator chemistry v3.1 on an ABI Prism 3130xl genetic analyzer (Applied Biosystems). Sequences were aligned and compared with consensus sequences (Ensemble ENST00000366898) obtained from the human genome databases (SeqScape v2.5 software, Applied Biosystems).

Results

From our large collection of UM specimens we selected 10 tumor samples with FISH verified loss of chromosome 6q and 10 samples with normal copy number FISH results using the BAC probe RP11-787I22, located on chromosome 6q21. With MLPA, we confirmed the deletion of chromosome 6q in the 6q26 region of the *PARK2* gene (Table 1). In seven out of the ten samples with FISH-verified 6q loss, we confirmed a heterozygous deletion of *PARK2* (EOM # 57, 207, 246, 270, 480, 486, 508). In one case, loss close to the detection level was found (EOM 159), and in another case we observed a lack of signal of exon 7 suggestive for a homozygous deletion of this exon (EOM 502). No amplifications indicating further rearrangements of the *PARK2* locus were observed. Of the cases that were determined as disomic for chromosome 6q by FISH, none of the samples showed a complete deletion of all 12 *PARK2* exons. There were three cases with deletion of exon 4 (EOM 193, 198 (homozygous deletion) and 208) and one case (EOM 461) with deletion of exons 8 and 12. Case EOM 193 and EOM 208 displayed duplication of exon 8 and exon 1, respectively.

Using PCR sequencing, we observed single nucleotide changes at two base pair locations within exon 8 of *PARK2* in five cases with a FISH-verified deletion of 6q. In two cases (EOM 207 and 486) there was deletion of exons 1 till 12 occurring simultaneously with a base pair change on the other allele. By using the NCBI database (www.ncbi.nlm.nih.gov) for genetic variations, the currently found changes were identified as known variants or Single Nucleotide Polymorphisms (SNPs); rs3765475 and rs3765474**)** and therefore do not qualify as true mutations. Of these SNPs, the alleles were present in the normal population with a frequency of 0.67(G)/0.32(C) (rs3765475) and 0.72(T)/0.27(C) (rs3765474), respectively. In

Table 1. Cases with FISH-determined loss of chromosome 6q, MLPA and sequence analysis results.

	FISH		MLPA													
		Percentage	Exons:	Exon												
EOM	6q status	of cells 2N	gain(A)/ deleted(D)	1	2	3	4	5	6	7	8	9	10	11	12	UTR
57	deletion	74%	D:1-12	n	n	n	n	n	n	n	n	n	n	n	n	n
157	deletion	72%	N	n	n	n	n	n	n	n	P[a]	n	n	n	n	n
159	deletion	22%	D:1-12	n	n	n	n	n	n	n	P[a]	n	n	n	n	n
207	deletion	81%	D:1-12	n	n	n	n	n	n	n	P[a]	n	n	n	n	n
246	deletion	44%	D:1-12	n	n	n	n	n	n	n	n	n	n	n	n	n
270	deletion	80%	D:1-12	n	n	n	n	n	n	n	n	n	n	n	n	n
480	deletion	93%	D:1-12	n	n	n	n	n	n	n	n	n	n	n	n	n
486	deletion	93%	D:1-12	n	n	n	n	n	n	n	P[a]	n	n	n	n	n
502	deletion	68%	D:7	n	n	n	n	n	n	n	P[a]	n	n	n	n	n
508	deletion	85%	D:1-12	n	n	n	n	n	n	n	n	n	n	P[b]	n	n
123	disomic	96%	N	n	n	n	n	n	n	n	n	n	n	n	n	n
131	disomic	97%	N	n	n	n	n	n	n	n	n	n	n	n	n	n
168	disomic	92%	N	n	n	n	n	n	n	n	n	n	n	P[b]	n	n
171	disomic	100%	N	n	n	n	n	n	n	n	n	n	P[c]	n	n	n
193	disomic	78%	N; A:8; D:4	n	n	n	n	n	n	n	h	n	P[c]	n	n	n
198	disomic	83%	D:4	n	n	n	n	n	n	n	n	N	n	n	n	n
208	disomic	86%	A:1; D:4	h	n	n	n	n	n	n	n	n	n	n	n	n
461	disomic	100%	D:8,12	n	n	n	n	n	n	n	n	n	n	n	n	n
494	disomic	99%	N	n	n	n	P[d]	n	n	n	n	n	n	n	n	n
513	disomic	100%	N	n	n	n	n	n	n	n	n	n	n	n	n	n

FISH: 2N: percentage of cells with two FISH probe signals present
MLPA: A:duplicated exon; D:deleted exon; N: no gain or deletions observed; black box: homozygous loss?; gray boxes: deletion (dark) or borderline deletion (light gray); filled: gain of copy number; UTR: untranslated region of the *PARK*2-gene.
Sequence: n: normal reference sequence; P: polymorphism or known SNP.
Known SNPs:[a] rs3765475 and rs3765474, [b] rs1801334, [c] rs1801582, [d] rs1801474

the 10 cases with FISH-verified disomy of 6q, three known variants (SNPs) were found with a remaining normal allele (rs1801334, rs1801582, and rs1801474) (Table 1).

Discussion

Veeriah et al[22] reported copy number loss of the *PARK2* gene in 53 out of 216 (25%) glioblastoma samples and 24 out of 98 (24%) colon cancer samples analyzed. Of the 62 glioblastoma samples with loss on 6q, 53 (85%) had loss of *PARK2* within the area of CNV. This was the case in 24 out of 24 (100%) colon cancer samples. In our cohort of UM

samples, a deletion of chromosome 6q was found in 73 out of 214 cases (34%) analyzed by interphase FISH. Our results also indicate that loss of chromosome 6q frequently involves loss of the *PARK2* locus. However, not in all cases we found loss of all exons of *PARK2*. For example, in tumor EOM 159 we detected loss of exons 1-4 and exon 9. An explanation could be that the 6q and hence *PARK2* loss is below detection levels as the 22 % observed FISH loss suggests. In EOM 502 no loss of exon 1-12 was observed despite a reported 68% chromosome 6q loss with FISH. Remarkably, this sample did show loss of exon 7 with an extremely low RQ value. In our series there were two samples with a RQ of 0.000 (exon 7 in EOM 502 and exon 4 in EOM 198), which could be classified as homozygous deletions of *PARK2* although a malfunction of one of the MLPA probes cannot be ruled out. This also accounts for the three cases without loss of 6q that displayed deletion of exon 4 of *PARK2* (EOM 193, 198, 208). Amplifications were found in two UMs: EOM 208 (exon 1) and EOM 193 (exon 8). These aberrant MLPA results have to be validated by additional Q-PCR or high density SNP-array to confirm these findings or to explain the discrepancies between FISH and MLPA.

Currently there were no mutations found in the 20 UM samples tested by sequencing so far, only base pair changes associated with known SNPs of exon 4, 8, 10 and 11. It therefore seems that in contrary to other solid malignancies, *PARK2* is not inactived by small indels or basepair changes in uveal melanoma but mainly by heterozygous deletions of the whole q-arm of chromosome 6 and hereby encompassing the *PARK2* locus at chromosome 6q26. Partial deletions involving the terminal part of chromosome 6 could result from breaks in one of the most active fragile sites in the human genome, FRA6E. This fragile site is located within the *PARK2* locus and results in frequent rearrangements of *PARK2* leading to Parkinson's disease[28]. Rearrangement of this 6q region have been observed in a special set of uveal melanomas[29] and it will be interesting to determine CNV or mutations of the *PARK2* locus in these specific tumor samples.

Veeriah et al[22] reported both heterozygous and homozygous mutations in glioblastoma (22%, and 2%, respectively) and colon carcinoma (18%, and 6%, respectively). If *PARK2* is a classic tumor-, or metastasis- suppressor gene, then homozygous deletions or heterozygous deletions with mutation of *PARK2* on the remaining allele, could lead to progression to metastatic disease. However, as small homozygous deletions or mutations involving the *PARK2*-gene were found in the majority of cases, Veeriah et al[22] suggested that inactivation of a single copy of *PARK2* would be sufficient to create a clonal growth advantage during tumor development. In our series of UM tumors there were no such small deletions of *PARK2*, or only heterozygous deletions which seemed to result from larger deletions spanning the complete long arm of chromosome 6. There were also no mutations or indels found of *PARK2* in our UM tumors which indicate that *PARK2* is not instrumental as a

tumor suppressor gene in UM metastasis. However, *PARK2* might be involved through other mechanisms than CNVs and mutations, such as loss of haplo-insufficiency of the gene, or epigenetic silencing (of the gene itself or promoter regions). The present results corroborate the previous view of a role of chromosome 6q in the process of metastasis as several studies have demonstrated that loss of chromosome 6q appeared more often in primary tumors and their metastases than in non-metastasizing melanoma[30-31].

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Chapter 9

General discussion

The diagnosis and treatment of posterior uveal malignant melanoma and the associated chromosomal alterations and genetic mutations in this tumor have been discussed extensively in the introduction of this thesis (**chapters 1 & 2**). However, a few items deserve further debate and are presented here.

Therapy

Eye-conserving therapies for ocular melanoma have proven to be effective in controlling intraocular tumor growth and conserving the eye and vision. Since the tumor control rates are comparable to enucleation, the eye-conserving therapies are at present the first choice of treatment for small- and medium- sized uveal melanomas[1-2]. However, in approximately 5% of patients local tumor growth is not adequately controlled by radiotherapeutic eye-conserving therapy[3], necessitating additional treatment and in several cases secondary enucleation. In **chapter 3**, we discuss the risk factors for secondary enucleation in stereotactically irradiated eyes with uveal melanoma. The results provide the first move towards prediction of radiotherapy failure in patients and emphasize the need for biopsies in combination with innovative detection methods to optimize the treatment strategy.

Biopsy

Several studies have shown that fine-needle aspiration biopsies in uveal melanoma provide sufficient material for genetic testing in a safe and reliable manner, even in smaller tumors [4-5] [6-7]. Shields et al[8-9] reported melanomas with an elevation of just 1.0 mm to be capable of metastasizing, hence there is a need to have prognostic markers that predict the chance of metastatic disease in small uveal melanomas. Early biopsy may thus not only be used for differentiating uveal melanomas from simulating lesions, but also for identification of pathological and genetic prognostic factors at an early stage. These factors could in their turn, serve as targets for future systemic therapies aimed at interfering in the process of micro-metastasis rather than macro-metastasis. In another study, Shields et al[7] assessed chromosome 3 alterations using micro-satellite analysis on biopsied tumor material before plaque-radiotherapy had been administered. They demonstrated that loss of a complete copy of chromosome 3 as well as partial losses were present in the small- and medium-sized uveal melanomas, both correlating with poor survival. In our stereotactically irradiated tumors (**chapter 3**), it would have been interesting to analyze chromosome 8 (and 3) alterations (previous to local therapy) in all irradiated tumors and not only the secondary enucleation cases, because gain of chromosome 8q was frequently found in the secondarily enucleated patients. This would allow for determination of the incidence of chromosome 8 alterations in the successfully irradiated uveal melanomas and the significance of the chromosome 8 alterations in the secondary enucleation cases.

It is important to realize the risk of sampling error if fine-needle aspiration biopsy samples of uveal melanomas are used, due to the possibly heterogeneous distribution of chromosome abnormalities within uveal melanoma[7, 10-11]. However, several groups reported that tumor heterogeneity leads to misclassification in only up to 1% of cases[12-13]. To reduce a possible sampling error multiple biopsies can be taken from each tumor, although this may prove technically difficult in small melanomas.

Chromosomal alterations

Despite the effectiveness of both eye-sparing and eye-removing therapies for uveal melanoma to locally control tumor growth, metastatic disease cannot be prevented at this point in time, leading to metastatic death in about 50% of patients[14]. This has been explained by the hematogenous spread of clinically undetectable micro-metastases to other parts of the body prior to treatment of the primary ocular melanoma. Only months to years later, these metastases develop into frank tumor and become clinically apparent. This underlines the need for early diagnosis and treatment and determination of (more) accurate prognostic markers.

Several prognostic factors have been identified up till now, including an older patient age at diagnosis (60 years or older), a large tumor size, a tumor localization in the ciliary body or localization near the optic nerve or an extra-ocular extension of tumor. Histopathologic markers include presence of epitheloid tumor cells and micro vascular patterns. Genetic markers have been shown to be the most important predictive markers of high risk of metastasis and these include specific gene expression profiles or cytogenetic alterations such as: loss of a complete copy of chromosome 3 (monosomy 3)[16-18], or monosomy 3 combined with gain of chromosome 8q. Furthermore, additional copies of chromosome 8q have been shown to correlate with a worse prognosis[19]. Monosomy 3 combined with loss of 1p36[20] correlates with a worse prognosis as well as loss of chromosome 6q, and gain of 6p correlates with a more favorable prognosis.

In the clinical genetics field a way to determine chromosomal alterations in a rapid manner with high resolution and reliability is important. Karyotyping and FISH are classic, relatively labor-intense cytogenetic techniques, while the more recent techniques such as MLPA allow for copy number analysis of multiple chromosomal regions (up to 50)[21]. SNP-array enables high-resolution assessment of genome-wide copy number alterations, including analysis of allelic imbalances. Though these new techniques show interesting results, standardization of techniques is necessary before they can be used in standard clinical practice. In **chapter 4** we compared MLPA and FISH for determining prognosis in uveal melanoma patients and found no difference in predictive power between the two techniques with regard to the analysis of the most important prognostic chromosomal alterations. These results provide

first steps towards reliable and reproducible genetic testing in a comparative manner. MLPA thus leads to a more rapid analysis of chromosomal alterations and prognosis in patients with uveal melanoma than FISH. Nevertheless, FISH still is a valuable analytic tool in specific situations such as when determination of absolute copy number status on the single tumor cell level is required. Furthermore, FISH allows for detection of low mosaic cases (chromosomal alterations in a low percentage of tumor cells). In **chapter 5** we described that a more individualized prognosis could be determined by using FISH counts of chromosome 3 and 8 probes on uveal melanoma tissue in 220 patients. Higher percentages of monosomy 3 or gain of chromosome 8q in tumor cells related to a worse patients' survival and additional copies of 8q indicated the patients at risk for early metastatic disease. The ability of the new high density SNP-arrays to detect low mosaic anomalies, and in the future targeted deep sequencing techniques are new techniques which are currently validated to replace MLPA and FISH.

New techniques and developments

Although the present techniques have not yet resulted in improved survival for uveal melanoma patients, future advances such as regular biopsy taking, next generation sequencing and new adjuvant systemic therapies or gene targeted therapy could be valuable tools for improving survival by effectively diagnosing, classifying and systemically treating patients with micro-metastatic disease in a more patient-tailored manner.

SNP-array analysis

In our opinion, SNP-array would be the most suitable tool at present for analyzing genetic changes in biopsy material from uveal melanomas. With SNP-array, only 200 ng of isolated tumor DNA is required for genetic testing, which is convenient if there is low yield of tumor tissue from biopsy. With FISH, vital tumor cells have to be harvested that need to be fixated and at least 300 cells are necessary for two chromosomal FISH probes to be analyzed. Tumor DNA can easily be isolated from tumor cells and if no sufficient DNA is present for SNP-array analysis, the DNA can be amplified by PCR reactions. MLPA may also be suitable just as SNP-array but does not allow for analysis of allelic imbalances, therefore requiring additional techniques such as micro-satellite analysis. Fresh (frozen) tumor tissue is required for SNP-array analysis as it is still difficult to analyze paraffin embedded uveal melanoma tissue, although promising results have been obtained by others[22].

Next generation sequencing

It could be possible that with deep sequencing, such as next generation sequencing techniques, small genetic alterations will be determined in the uveal melanomas that were previously classified as having no chromosomal alterations. The *BAP*1 and *GNAQ*/ *GNA*11 mutations were identified by sequencing techniques and this technique provides a bright

future for determining other candidate genes involved in uveal melanoma carcinogenesis and progression to metastasis. Next generation sequencing techniques also require isolated DNA for input and may thus readily be combined with biopsy, leading to analysis of chromosomal alterations up to the base pair level. However, for copy number analysis, additional quantitative PCR (qPCR) or SNP-array would still be necessary.

Metastatic disease

The presence of early micro-metastasis in uveal melanoma patients is evident[23] and the identified markers might predict early conversion to clinically detectable metastasis in patients. It could also be that micro-metastasis occurs in all patients and that certain (combination of) chromosomal alterations such as gains, deletions or mutations are drivers for early metastatic dissemination or activation of micro-metastases. This could explain the peak incidence of 25% for metastasis-related death around two to four years after diagnosis in patients with monosomy 3[7]. Lake et al[22] analyzed primary uveal melanomas with SNP-array and reported several genes on chromosome 3 with loss of heterozygosity in metastasizing tumors, indicating that allelic imbalances of chromosome 3 may be sufficient for metastases to develop. In our own uveal melanoma cohort, there are patients with histopathologically proven metastases from uveal melanoma after 10 years, who only have loss of chromosome 6q and gain of 6p. This could indicate micro-metastases remaining dormant or inactive for longer periods of time due to absence of chromosome 3 and 8 alterations.

Using primary and metastatic cell lines of one patient as a model system (**chapter 6)** for uveal melanoma metastasis with consequent SNP-array analysis, displays some of the typical chromosome 3 alterations: deletion of 3q21.2-q23, and loss of heterozygosity of the regions 3p25.1-p25.2 (also reported by Parrella et al[24]), 3p23-p24.2 and 3p14.3 in the primary cell line as well as several metastatic cell lines. These regions are therefore theorized to harbor genes involved in uveal melanoma carcinogenesis or progression to metastasis. As uveal melanoma is known to disseminate by hematogenous route to the liver most frequently[25], it would be interesting to analyze blood for circulating tumor cells with these distinct chromosomal alterations.

Genes in uveal melanoma and indications for therapy

Identification of candidate chromosome regions, genes, and pathways involved in uveal melanoma are important for increasing our understanding of uveal melanoma carcinogenesis and progression to metastatic disease, thereby also providing potential targets for treatment. Up till now, several candidate genes are reported in uveal melanoma with most of these genes not being universal in uveal melanoma patients or specific for the tumor. In **chapter 8** a candidate tumor suppressor gene recently reported in several solid tumors[26] and located on chromosome 6q, was analyzed in our uveal melanoma patients. Classic PCR

sequencing and MLPA copy number analysis was used in 10 uveal melanoma samples with FISH-determined loss of one copy of chromosome 6q and 10 cases without loss of chromosome 6q. No small indels or base pair changes of *PARK2* were found, only heterozygous deletions which seemed to result from larger deletions spanning the complete long arm of chromosome 6. This indicates that *PARK2* is not instrumental as a tumor suppressor gene in uveal melanoma metastasis. Other more promising candidate genes are the recently reported *GNAQ/ GNA11* genes and *BAP1*-gene.

GNAQ/ GNA11

Somatic *GNAQ* or *GNA11* mutations have been reported to occur with high frequency in primary uveal melanomas and appear to be exclusive to melanocytic tumors[27-28]. These are important characteristics for future therapy, enabling highly specific targeting of mutant cells only. However, the *GNAQ/ GNA11* mutations have been suggested to occur early in melanomagenesis as there is no association with disease-free survival[29] or with other clinical, pathological, immunohistochemical or genetic factors associated with progressive uveal melanoma[30]. Therapeutic targeting of mutant *GNAQ/ GNA11* or its up- or downstream effectors could therefore lead to overtreatment of patients with a favorable prognosis and it is uncertain whether this treatment may improve survival in uveal melanoma patients at all. However, in the event that all patients with uveal melanoma develop micro-metastases, it might still be valuable to target these early occurring *GNAQ/ GNA11* mutations or other members from the involved RAF-RAS-MEK-ERK pathway. Whether this will lead to an effective strategy for improving patients' survival is yet to be determined. Therapeutic trials targeting several downstream effectors are currently underway and should provide us with more knowledge about the effectiveness of targeted therapy in the near future.

BAP1-gene

Somatic *BAP1* mutations were reported to be frequent in gene expression class 2 (high-risk of metastasis) uveal melanomas[31], thereby marking the *BAP1*-gene as potential 'metastasis-gene' in uveal melanoma. Recent reports showed presence of *BAP1* germ line mutations in uveal melanoma as well as in other malignancies[31-34]. This would indicate *BAP1* as a classic tumor suppressor gene, where a somatic mutation next to a germ line mutation gives rise to uveal melanoma or metastasis from uveal melanoma. *BAP1* mutations are not unique to uveal melanoma and future therapy targeting the mutated *BAP1*-gene will therefore theoretically be less specific for the total group of patients with uveal melanoma. However, this is the first and long-awaited gene involved in metastatic development in uveal melanoma and could still be a suitable target for gene therapy through modulation of its down- or upstream effectors. Further research regarding this gene is therefore valuable and required for designing gene therapy trials effectively preventing metastasis. One of the challenges is to design agents that are able to restore one or more functions of *BAP1* following its

inactivation in uveal melanoma. At present, clinical trials are planned involving histone deacetylase (HDAC) inhibitors facilitating the deubiquitinating function of *BAP1*, but also its interaction with DNA repair through PARP inhibition. Continuing the research on other candidate genes will be valuable as well, as it is unlikely that *BAP1* is the only gene involved in metastasis.

Follow-up after local therapy

Even in the present setting, with absence of effective treatment for widespread metastatic disease, frequent systemic investigations in patients with uveal melanoma may be valuable. Early detection of metastasis confined to the liver can lead to extended survival if the lesions are treatable by local resection or hepatic chemoembolization. However, survival is only marginally extended in most cases and there is a need for more effective treatment of metastases. With accurate and reliable prognosis in UM patients, we are able to differentiate patients requiring intense or less intense follow-up programs. The low-risk patients can be reassured of their small chance of clinically significant metastatic disease and can be monitored less often. With close monitoring of the high-risk patients, intervention can be initiated earlier probably improving survival. Frequent examinations during the first 2 to 4 years of follow-up (i.e. the period of time with the peak incidence of metastasis) would be important in the subgroup of high-risk patients. In spite of the local therapies, it is likely that adjuvant systemic therapies are required for preventing early macro-metastatic disease and improvement of survival in these high-risk patients[35]. There are adjuvant systemic trials currently underway which look promising, and with the genetic background of uveal melanomas and metastasizing uveal melanomas emerging, this provides new therapeutic targets and possibilities.

Dendritic cell therapy

Current chemotherapeutic drug treatments have not resulted in a significant improvement in survival and are known for their related toxicity. Following the promising results of an immune vaccine trial in cutaneous melanoma patients[36], we have recently started an immune vaccine trial in uveal melanoma patients known as the Dendritic Cell Therapy used as immunologic adjuvant therapy next to local therapy in uveal melanoma patients (collaboration of The Rotterdam Eye Hospital and The Radboud University Hospital Nijmegen in the Netherlands with cooperation of the majority of academic hospitals in the Netherlands as well as Moorfields Eye Hospital in London, England). In this trial, high-risk patients are immunized by their own dendritic cells, trained to detect remaining (circulating) tumor cells and metastases after local therapy and which induce a specific and long lasting immunity to protect against recurrent disease.

Currently, high-risk uveal melanoma patients eligible for Dendritic Cell Therapy are identified by both FISH and SNP-array determined chromosome 3 changes. FISH analysis on chromosome 3 is used for absolute copy number assessment (monosomy) and detection of monosomy of chromosome 3 in low mosaic cases. SNP-array is used for confirmation of the FISH data and could determine chromosome 3 loss of heterozygosity, thereby identifying the potential false negative cases that were determined as disomy 3 by using FISH. In **chapter 5** we described that FISH-determined chromosome 8 alterations are independent prognostic markers for poor survival as well as monosomy 3, and that additional copies of chromosome 8q correlate with early metastasis. Further studies are needed to definitely confirm chromosome 8 as independent prognostic marker, but if this is the case, we should consider using chromosome 8 alterations as inclusion criterion for the Dendritic Cell Therapy. The same applies for hyperdiploid uveal melanoma cases (Mensink et al, submitted), the tumors with hyperaneuploid genomes and relative loss of a complete copy of chromosome 3, which have been found to be prognostic markers of poor survival as well. Fine-needle aspiration biopsy could be used in our patients with small- or medium-sized uveal melanomas eligible for stereotactic radiotherapy to determine presence of prognostic chromosomal alterations and inclusion in the Dendritic Cell Therapy.

Conclusion

Uveal melanoma is an aggressive tumor with a 50% risk of metastasis, resulting in premature death. Genetic analysis of tumors provides us with valuable prognostic information although effective therapies are lacking at this moment. The current experience and new genetic techniques help to increase our understanding of uveal melanoma and discover patterns of growth and metastatic spread. This may result in precise identification of high-risk patients and targets for future adjuvant systemic treatments preventing metastatic disease. The technique of gathering tumor tissue by fine-needle aspiration biopsy and new genetic techniques such as SNP-array and next generation sequencing will likely be crucial tools for improving diagnosis and therapy in the future.

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Summary

Uveal melanoma is a rare but aggressive type of intra-ocular malignancy, eventually leading to metastatic dissemination in half of all patients. When metastases develop, the remaining therapeutic options and the patient' survival are limited. Several clinical, histological and genetic markers have been identified which are capable of predicting prognosis in uveal melanoma patients. Genetic markers such as chromosome 3 loss or the expression of a specific set of genes have proven to be the most significant prognostic markers. Recent genetic research and DNA sequencing techniques resulted in new genetic markers that could not only lead to a more accurate prognosis in patients but could also serve as targets for future (gene) targeted treatments. Although current local therapies (eye-conserving radiotherapy and enucleation) are effective in controlling intra-ocular tumor growth, there is a need for (systemic) treatments preventing metastatic disease in uveal melanoma patients.

The current clinical practice in uveal melanoma regarding diagnosis, treatment, and the use of chromosomal alterations and genetic mutations as prognostic markers is described in **chapter 1**. Recent genetic research techniques are discussed along with their contribution to our current understanding of uveal melanoma pathogenesis. In **chapter 2** we describe the patient and tumor characteristics of cutaneous melanoma compared to uveal melanoma. Both types of pigmented malignancies have features in common though there are several dissimilarities, including the chromosomal alterations and genetic mutations. Uveal melanomas are more sensitive to therapeutic irradiation than cutaneous melanomas and eye-conserving radiotherapy is at present the first choice of treatment for small- and medium-sized uveal melanomas. **Chapter 3** focuses on the risk factors for failure of eye-conserving radiotherapy in a series of 118 uveal melanoma patients. Initial large tumor thickness and location close to the optic disc were correlated with a high risk of secondary enucleation due to treatment failure or complications. Gain of chromosome 8q was frequently present in tumors from secondarily enucleated eyes and also correlated with high number of mitotic figures, thereby possibly indicating a subset of more aggressive uveal melanomas. These results may serve as first steps towards more accurate prediction of therapy failure in patients and aid the physician in the decision of treatment type.

In **chapter 4**, we evaluate whether MLPA can replace FISH by comparing the predictive value of each technique in 64 uveal melanoma patients. Considering the known prognostic alterations on chromosomes 1, 3, 6 and 8, MLPA has a predictive value similar to FISH but is less labor-intensive and requires less time to provide the data. MLPA could thus be preferred over FISH in most cases, although FISH would still be the most suitable genetic tool in certain specific cases such as low mosaic, and determination of absolute copy number.

In **Chapter 5**, FISH data of 220 uveal melanomas was reviewed to determine whether absolute copy numbers of chromosome 3 and 8q, or total percentage of tumor cells with chromosomal alterations correlate with patients' survival. Our study shows that a high percentage of tumor cells with monosomy 3 or gain of chromosome 8q correlates with a worse patient's prognosis, and increasing numbers of additional copies of chromosome 8q predict a shorter disease-free survival. By using FISH in this manner, a more individualized prognosis can be established for uveal melanoma patients.

In **chapter 6**, primary and metastatic cell lines of one patient are used as a model system for uveal melanoma metastasis. Using SNP-array, FISH and microsatellite marker analysis, we describe a deletion of 3q21.2-q23, and loss of heterozygosity of the regions 3p25.1-p25.2, 3p23-p24.2 and 3p14.3 in the primary cell line as well as several metastatic cell lines. As these regions are identical in the different cell lines, we hypothesize that these regions may harbor candidate genes involved in uveal melanoma carcinogenesis or progression to metastatic disease. Indications for furhter research are also the chromosomal aberrations which are unique to the metastatic cell lines.

In **chapter 7**, a rare case of uveal melanoma with specific histopathological changes is presented. A combined molecular- cytogenetic analysis using FISH, MLPA and SNP-array shows no presence of chromosomal alterations thereby indicating a favorable prognosis. Further follow-up in this patient is required to evaluate whether metastasis develops in this genetically determined low-risk patient.

In **chapter 8**, classic PCR sequencing and MLPA copy number analysis are used to detect mutations and copy number alterations of the *PARK*2-gene in uveal melanoma samples. This candidate tumor suppressor gene located on chromosome 6q, was recently reported to be mutated in other solid tumors. In our study no small indels or base pair changes of the *PARK*2-gene were found, indicating that *PARK*2 is not instrumental as a tumor suppressor gene in uveal melanoma.

The major results and implications of the results described in this thesis are discussed in **chapter 9**. Future genetic techniques as well as the use of biopsy in uveal melanoma may be valuable for new therapeutic strategies preventing metastatic disease in uveal melanoma patients.

Samenvatting

Het uveamelanoom is een zeldzame maar agressieve vorm van kanker in het oog, uitgaande van de gepigmenteerde cellen (melanocyten) rondom het vaatvlies (uvea) in het oog. Deze aandoening leidt bij uiteindelijk de helft van alle patiënten tot uitzaaiingen, meestal in de lever. Als er eenmaal uitzaaiingen ontstaan zijn, dan is de mogelijkheid tot behandeling en ook de overleving van de patiënt beperkt. Er is een aantal factoren bekend die de prognose kunnen voorspellen zoals de klinische-, pathohistologische-, en genetische factoren. De meest betrouwbare voorspellende factoren vormen de genetische factoren, en dan met name verlies van een kopie van chromosoom 3 (monosomie 3) of de expressie van een bepaalde groep genen in het tumorweefsel. Nieuwe ontwikkelingen in het genetisch onderzoek, waaronder het snel bepalen van een verandering in de nucleotidevolgorde in het tumor DNA (DNA sequencing), kunnen leiden tot een accurater voorspelling van de prognose, maar ook tot aangrijpingspunten voor toekomstige (gen)gerichte behandelingen. De huidig beschikbare behandelingen zoals oogsparende bestraling en chirurgische verwijdering van het oog (enucleatie) zijn beide effectief in het afremmen dan wel stoppen van de tumorcelgroei in het oog maar voorkomen niet de uitzaaiingen. Er is dus een grote behoefte aan (systemische) behandelingen die uitzaaiingen voorkomen bij patiënten met een uveamelanoom.

De huidige klinische praktijk met betrekking tot de diagnose, behandeling, en prognose op basis van de chromosomale afwijkingen en genmutaties in het tumorweefsel is beschreven in **hoofdstuk 1**. Recente genetische onderzoekstechnieken evenals de bijdrage ervan aan ons actuele inzicht in het ziekteproces van uveamelanomen en huidmelanomen worden besproken in **hoofdstuk 2**. Beide vormen van gepigmenteerde kanker kennen overeenkomsten, al zijn er ook verschillen waaronder de aanwezigheid van specifieke chromosomale afwijkingen en genmutaties. Uveamelanomen zijn bijvoorbeeld gevoeliger voor bestralingstherapie dan huidmelanomen en mede daarom is oogsparende bestralingstherapie op dit moment de voorkeursbehandeling voor de kleine en middelgrote uveamelanomen. **Hoofdstuk 3** richt zich op de risicofactoren voor het mislukken van oogsparende bestralingstherapie in een groep van 118 patiënten met uveamelanoom. Grotere tumordikte en een tumorlocatie nabij de oogzenuw geven een verhoogd risico op secundaire enuclatie na eerdere bestralingstherapie. Winst van de lange arm van chromosoom 8 (8q) was frequent aanwezig in tumoren die secundair geënucleëerd waren en ook werden in deze tumoren een relatief hoog aantal celdelingen aangetroffen. Deze tumoren vormen mogelijk een subgroep van meer agressief groeiende uveamelanomen. De resultaten kunnen als eerste stappen dienen richting een accurater voorspelling van het succespercentage van oogsparende bestralingstherapie, en mogelijk ook de arts ondersteunen in het selecteren van de meest geschikte behandeling voor de individuele patiënt. In **hoofdstuk 4** evalueren we of de multiplex ligation-dependent

probe amplification (MLPA)-techniek de fluorescence in situ hybridization (FISH)- techniek kan vervangen voor wat betreft de prognosebepaling door middel van vergelijking van de voorspellende waarde van beide technieken voor de overleving van 64 patiënten. Als er wordt gekeken naar afwijkingen van de chromosomen 1, 3, 6 en 8, dan heeft MLPA een voorspellende waarde die vergelijkbaar is met FISH en tevens minder arbeidsintensief is en op kortere termijn resultaten oplevert. Dit betekent dat MLPA in de meeste gevallen de voorkeurstechniek is ten opzichte van FISH, hoewel FISH nog steeds de meest geschikte techniek is in specifieke omstandigheden met laag mozaïcisme van de tumorcellen en wanneer men het absolute aantal aanwezige kopieën van een chromosoom wil bepalen. In **hoofdstuk 5** zijn 220 uveamelanomen onderzocht op het absolute aantal aanwezige kopieën van de chromosomen 3 en 8q maar ook het totale percentage tumorcellen met chromosomale afwijkingen. Deze resultaten werden gerelateerd aan de overlevingscijfers van de patiënten, en daar bleek dat een hoog percentage chromosomale afwijkingen in de tumorcellen samenhangt met een hoog risico op uitzaaiingen. Een hoger aantal kopieën van chromosoom 8q resulteerde vaak in een kortere overlevingsduur voor de patiënt. Als FISH op deze manier toegepast wordt op het tumorweefsel van patiënten, kan dit tot een accurater en meer individuele prognose leiden voor de patiënt.

In **hoofdstuk 6** worden gekweekte tumorcellen van de primaire en uitgezaaide tumoren van één patiënt gebruikt als modelsysteem voor het uitzaaiingsproces van uveamelanomen. Met behulp van SNP-array, FISH en microsatelliet marker analyse, beschrijven we een deletiegebied op chromosoom 3 wat zich uitstrekt van 3q21.2-q23 en aanwezig is in zowel de primaire cellijn als een uitgezaaide cellijn. In meerdere cellijnen worden gebieden met verlies van heterozygotie (LOH) gevonden en beschreven welke zich bevinden tussen 3p25.1-p25.2, 3p23-p24.2, en 3p14.3. Omdat de gevonden regio's zich op het voor uveamelanomen bekende chromosoom 3 bevinden in zowel de primaire tumor als de uitzaaiingen in de lever, stellen we de hypothese dat deze regio's kandidaat-genen bevatten die betrokken zijn bij het ziekteproces dan wel uitzaaiingsproces van uveamelanomen. Ingangen voor verder onderzoek vormen ook de chromosomale afwijkingen welke uniek zijn voor de uitgezaaide cellijnen.

In **hoofdstuk 7** wordt een zeldzaam geval van een uveamelanoom gepresenteerd zonder chromosomale- of moleculair cytogenetische afwijkingen maar met specifieke histopathologische veranderingen. De afwezigheid van chromosomale- en moleculair cytogenetische afwijkingen (met behulp van FISH, MLPA, en SNP-array getest) indiceert een gunstiger prognose voor de patiënt maar verdere opvolging van het beloop is nodig om vast te stellen of er niet alsnog uitzaaiingen ontstaan.

In **hoofdstuk 8** wordt een klassieke PCR sequencing- techniek samen met MLPA gebruikt om genmutaties en chromosomale afwijkingen van het *PARK2*-gen op te sporen in uveamelanomen. Recent werd beschreven dat het *PARK2*-gen, gelegen op chromosoom 6q, vaker gemuteerd voorkomt bij verschillende vormen van solide kanker. In onze studie werden echter geen inserties, deleties, of basepaar veranderingen gevonden in het *PARK2*-gen in uveamelanomen. Dit suggereert dat *PARK2* geen rol speelt als tumor suppressor gen in uveamelanomen.

De belangrijkste bevindingen en implicaties van de resultaten in dit proefschrift worden in **hoofdstuk 9** becommentarieerd. Toekomstige genetische technieken en ook het afnemen van biopten van uveamelanomen kunnen waardevol zijn voor het ontwikkelen van nieuwe effectieve behandelingsstrategieën om uitzaaiingen te voorkomen bij patiënten.

List of abbreviations

BAC	Bacterial artificial chromosome
BAF	B- allele frequency
BAP1	BRCA-associated protein-1
BCVA	Best corrected visual acuity
cCGH	chromosomal comparative genomic hybridization
CGH	Comparative genomic hybridization
CI	Confidence interval
CM	Cutaneous melanoma
CNA	Copy number alteration
CNV	Copy number variation
COMS	Collaborative Ocular Melanoma Study
CT	Computed tomography
DCT	Dendritic cell therapy
DFS	Disease-free survival
DNA	Deoxyribonucleic acid
FFPE	Fresh-frozen paraffin -embedded
FISH	Fluorescence in situ hybridization
FNAB	Fine needle aspiration biopsy
fSRT	fractionated stereotactic radiotherapy
Gy	Gray
HapMap	Haplotype map
H&E	Haematoxylin & eosin
HMB-45	Human melanoma black-45
HPF	High power field
HR	Hazard ratio
Indels	insertions or deletions
LRR	Log-R ratio
LOH	Loss of heterozygosity
LTD	Largest tumor diameter
MAPK	Mitogen-activated protein kinase
MLPA	Multiplex ligation-dependent probe amplification
mRNA	messenger ribonucleic acid
miRNA	micro ribonucleic acid
MSI	Microsatellite instability analysis.
NGS	Next generation sequencing
NVG	Neovascular glaucoma
OR	Odds ratio

PAS	Periodic-acid Schiff
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RQ	Relative quantity
siRNA	small interfering ribonucleic acid
SKY	Spectral karyotyping
SNP	Single nucleotide polymorphism
SRT	Stereotactic radiotherapy
STS	Sequence-tagged site
TSG	Tumor suppressor gene
TNM	Staging system for malignant tumors
UM	Uveal melanoma
q-PCR	quantitative polymerase chain reaction

Curriculum vitae

The author of this thesis was born on the 1st of April 1983 in Rhenen. He graduated from VWO (gymnasium) at the "Christelijk Lyceum Veenendaal" in 2001. Before he started his medical study at the University of Utrecht in 2002, he obtained his propaedeutic degree in Psychology at the University of Utrecht in the year 2001/2002. During his medical study, he worked as a medical student and research nurse at the Pediatric Surgery department of the Wilhelmina Children's Hospital in Utrecht, the pharmacy, and a clinical pharmacological unit. He was also member in several councils of the faculty's student organization. In 2009 the author obtained his medical degree and subsequently started his research on uveal melanoma at the departments of Ophthalmology and Clinical Genetics of the Erasmus Medical Center Rotterdam and Rotterdam Eye Hospital (dr. A. de Klein, dr. D. Paridaens and prof. dr. van Rij). The results of the PhD study are described in this thesis, and the author presented the data at several national and international meetings. In January 2012, the author commenced his residency in Ophthalmology at the Rotterdam Eye Hospital (prof. dr. J.C. van Meurs).

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PhD Portfolio Summary

Summary of PhD training and teaching activities

Name PhD student:	Thomas van den Bosch
Erasmus MC Departments:	Clinical Genetics and Ophthalmology (collaboration with Rotterdam Eye Hospital)
PhD period:	July 2009 – December 2011
Promotor:	prof. dr. G. van Rij
Supervisors:	dr. A. de Klein and dr. D. Paridaens

1. PhD training

	Year	Workload (Hours/ECTS)
General academic skills		
- Biomedical English Writing and Communication	2011	4 ects
Specific courses (e.g. Research school, Medical Training)		
- Biomedical Research Techniques (MolMed)	2009	1.5 ects
- Integration of Cytogenetics, Microarrays and Massive Sequencing in Biomedical and Clinical Research (EGF)	2009	40 hrs
- Basic and Translational Oncology (MolMed)	2009	1.8 ects
- Epigenetic Regulation (MGC)	2010	16 hrs
- Techniques in Cancer Genomics (NKI-VUMC)	2010	8 hrs
- Nexus Training Course (MolMed)	2010	1 ects
- Molecular Medicine (MolMed)	2010	0.7 ects
- Basic Introduction Course on SPSS (MolMed)	2010	0.8 ects
- Introductory Course Statistics & Survival Analysis (MolMed)	2010	0.5 ects
- SNP's and Human Diseases (MolMed)	2010	2 ects
- Next Generation Sequencing (MGC)	2010	32 hrs
- Veilig werken in het laboratorium (MGC)	2010	6 hrs
Seminars and workshops		
- Immunology in Ophthalmology	2010	8 hrs
- PhD day Erasmus MC	2010	8 hrs
- Scientific seminars weekly Dept. of Clinical Genetics	2009-2011	60 hrs
Presentations and (inter)national conferences		
- NOG annual meeting (poster contribution)	2009	10 hrs
- NOG annual meeting (oral presentation)	2010-2012	36 hrs
- ARVO-NED annual meeting (oral presentation)	2009, 2010	24 hrs
- Ophthalmology weekly meetings Erasmus MC (oral presentation)	2010, 2011	20 hrs
- EWCMST annual meeting (poster contribution)	2010	14 hrs
- Clinical Genetics Erasmus MC weekly meetings (oral presentation)	2010, 2011	24 hrs
- EVER annual meeting (oral presentation)	2010, 2011	28 hrs
- ARVO annual meeting (poster contribution)	2011	12 hrs
- Science day Rotterdam Eye Hospital (oral presentation)	2011	12 hrs
2. Teaching activities		
- Supervision of MBO laboratory students	2009-2011	24 hrs
- Supervision of Medical Student	2010	6 hrs

List of publications

T. van den Bosch, J. van Beek, E. Kiliç, N.C. Naus, D. Paridaens, A. de Klein. *Genetics of uveal melanoma*. Advances in Malignant Melanoma - Clinical and Research Perspectives, Armstrong AW (Ed.), ISBN: 978-953-307-575-4, InTech, online open access.

T. van den Bosch, E. Kiliç, D. Paridaens, A. de Klein. *Genetics of uveal melanoma and cutaneous melanoma: two of a kind?* Dermatol Res Pract. 2010;2010:360136. Epub 2010 Jun 6.

T. van den Bosch, J. Vaarwater, R.M. Verdijk, K. Muller, E. Kiliç, D. Paridaens, A. de Klein, N.C. Naus. *Risk factors associated with secondary enucleation after fractionated stereotactic radiotherapy in uveal melanoma*. Article submitted for publication.

J. Vaarwater, T. van den Bosch, H.W. Mensink, C. van Kempen, R.M. Verdijk, N.C. Naus, D. Paridaens, H.T. Brüggenwirth, E. Kiliç, A. de Klein. *MLPA equals FISH for the identification of patients at risk for metastatic disease in uveal melanoma*. Melanoma Res. 2012;22(1):30-7.

T. van den Bosch, J.G.M. van Beek, J. Vaarwater, R.M. Verdijk, N.C. Naus, D. Paridaens, A. de Klein, E. Kiliç. *Higher percentage of FISH-determined monosomy 3 and 8q amplification in uveal melanoma cells relate to poor patient prognosis*. Invest Ophthalmol Vis Sci. 2012 Mar 16. [Epub ahead of print]

T. van den Bosch, E. Brosens, A. Koopmans, D. Mooijman, J. Vaarwater, M.M. Verbiest, W. van Gils, H.T. Brüggenwirth, E. Kiliç, D. Paridaens, A. de Klein. *Fine mapping of structural chromosome 3 deletions in uveal melanoma cell lines*. Article submitted for publication.

R.M. Verdijk, T. van den Bosch, N.C. Naus, D. Paridaens, C.M. Mooy, A. de Klein. *Histopathologic, immunohistochemical, ultrastructural, and cytogenetic analysis of oncocytic uveal melanoma*. Arch Ophthalmol. 2011 Nov;129(11):1501-2.

T. van den Bosch, J. Vaarwater, H. Douben, G.J. Breedveld, D. Paridaens, A. de Klein. *PARK2 copy number variations and mutations are not present in uveal melanoma*. Manuscript in preparation.

Dankwoord

Veel mensen hebben direct of indirect bijgedragen aan het tot stand komen van dit proefschrift. In dit hoofdstuk wil ik al deze mensen danken voor hun hulp en ondersteuning, en een aantal mensen in het bijzonder.

Allereerst bedank ik mijn co-promotoren. Annelies, ik heb veel kunnen leren van jouw expertise op het gebied van moleculaire cytogenetica en je recht-door-zee mentaliteit. Bedankt voor je dagelijkse begeleiding en betrokkenheid de afgelopen jaren. Dion, jij weet als geen ander mensen te enthousiasmeren door je kennis en interesse voor zowel de oogheelkunde als het wetenschappelijk werk. Bedankt voor je vertrouwen vanaf het eerste moment dat ik in Rotterdam kwam, ik hoop nog jaren samen te werken in het Oogziekenhuis.

Mijn promotor, professor van Rij, ik waardeer het zeer dat u na uw emeritaat betrokken wilde blijven bij dit project, u was altijd geïnteresseerd in de voortgang en de plannen voor verder onderzoek.

De leden van de kleine commissie, prof. dr. J.C. van Meurs, prof. dr. E.C. Zwarthoff, en prof. dr. R.J.W. De Keizer bedank ik hartelijk voor de snelle beoordeling van het manuscript.

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werkten meestal samen via de mail en ik dank je voor al je adviezen. Ook de collega's uit Nijmegen waaronder Carl, Erik, Jolanda, Kalijn en Winette bedank ik voor de prettige samenwerking binnen het dendritische celtherapie-onderzoek evenals alle collega's van de Rotterdam Ocular Melanoma Study group.

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Kleur katern

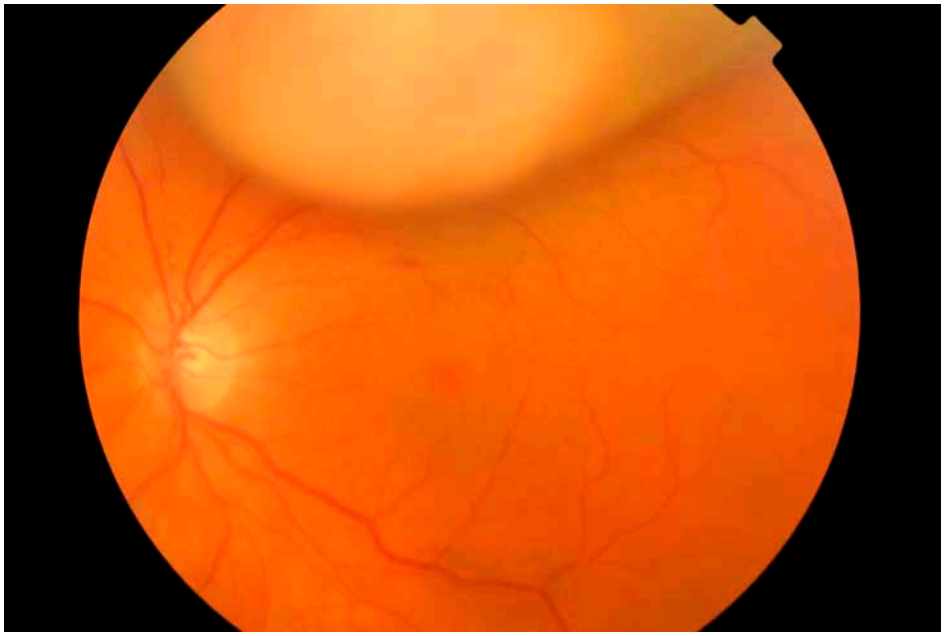


Figure 1: Fundus photography showing a superiorly located uveal melanoma of the left eye
(page 14)

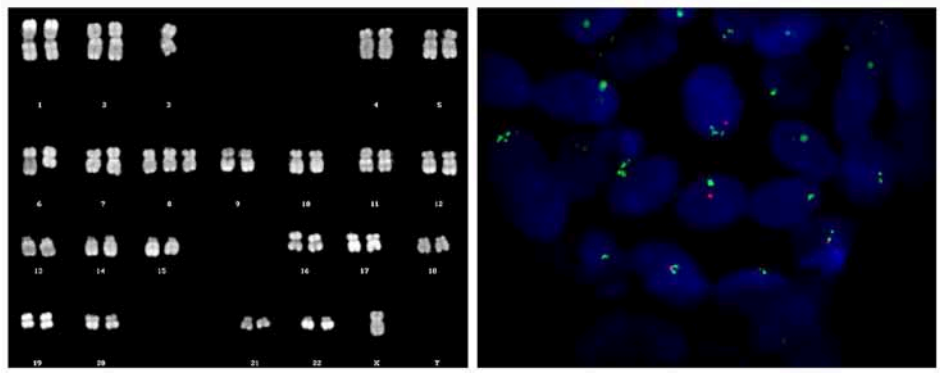


Figure 2: Karyogram showing loss of chromosome 3, isodisomy of 6p, and gain of chromosome 8 (left), FISH nuclei showing one signal for chromosome 3p (red) and centromere 3 (green) (right)
(Page 19)

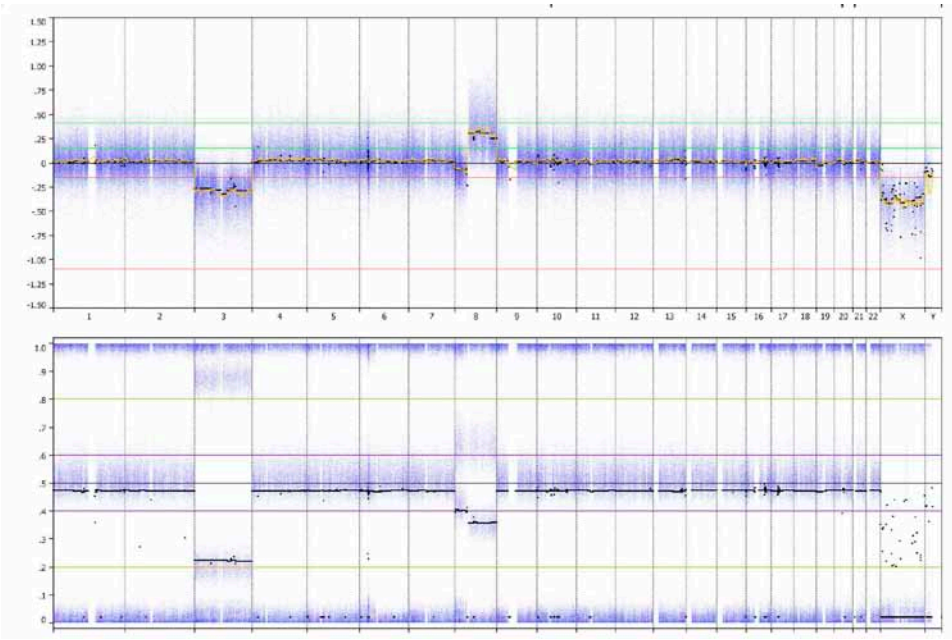


Figure 4: SNP-array results of a male patient with uveal melanoma, showing monosomy 3, loss of 8p, and gain of chromosome 8q (Log-R ratio upper panel). The lower panel represents the B- allele frequency showing allelic imbalance of chromosome 3 and 8 **(page 21)**

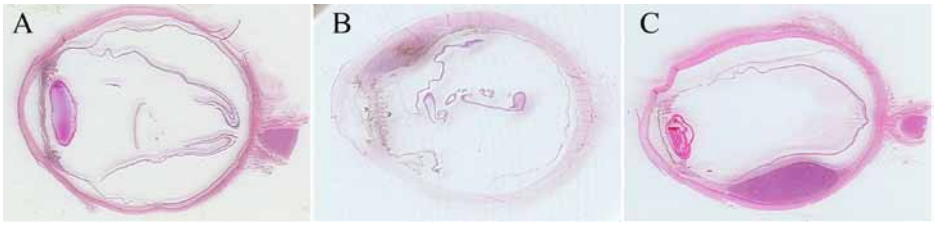


Figure 1: Uveal melanoma located in: iris (A), ciliary body (B), choroid (C) **(page 39)**

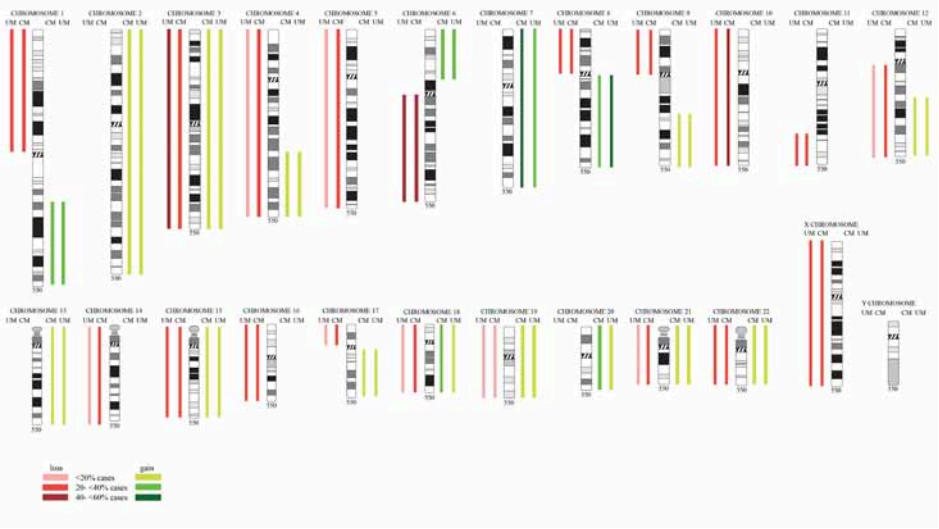


Figure 2: Chromosomal aberrations in cutaneous melanoma (CM) and uveal melanoma (UM): based on all cases in the Mitelman Database of Chromosomal Aberrations in cancer, by Höglund [42]. **(page 43)**

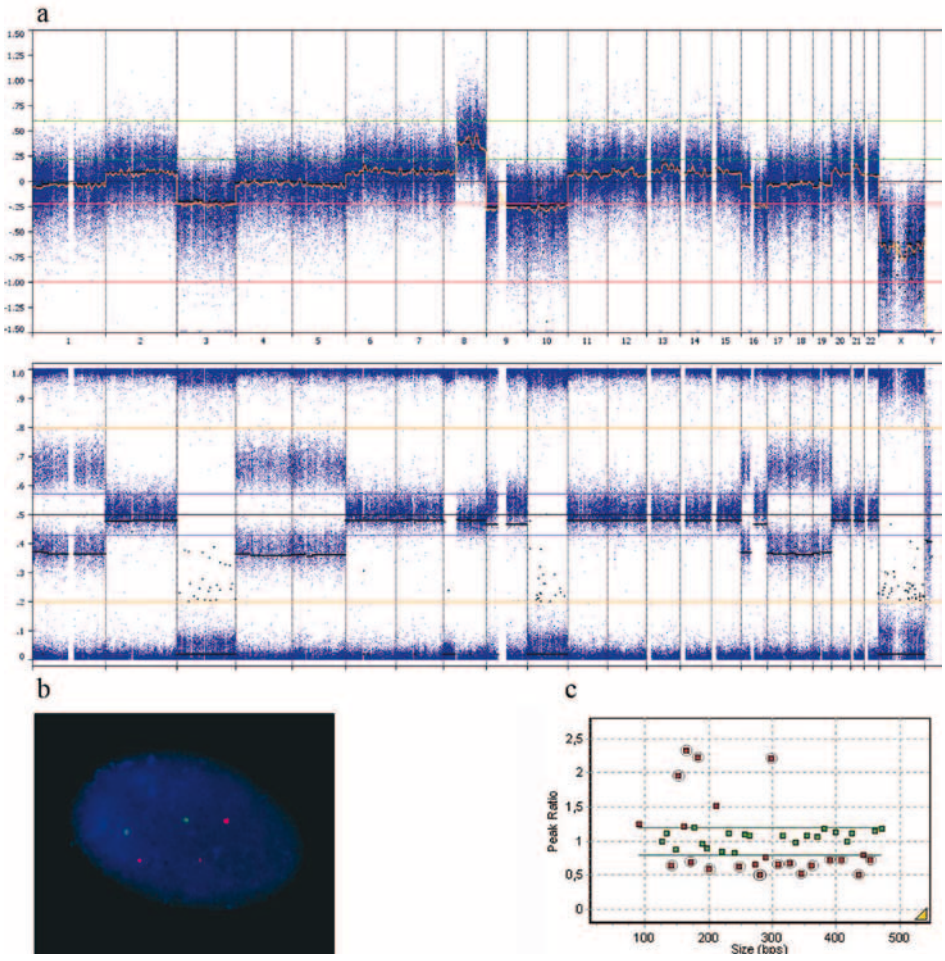


Figure 1: Example of a hyperdiploid tumor with relative loss of chromosome 3 tested with different techniques.
a: SNP-array results show hyperdiploidy with relative loss and LOH of chromosome 3. b: FISH analysis demonstrates three signals for the probe on chromosome 5q (red signals) and two signals of the probe on chromosome 3q (green signals). c: MLPA-results show loss of chromosome 3 and gain of 8q. The probes marked with a circle represent the probes located on chromosomes 3 and 8q. The peak ratio on the y-axis represents the RQ value. The x-axis represents the size of the different PCR products.**(page 92)**

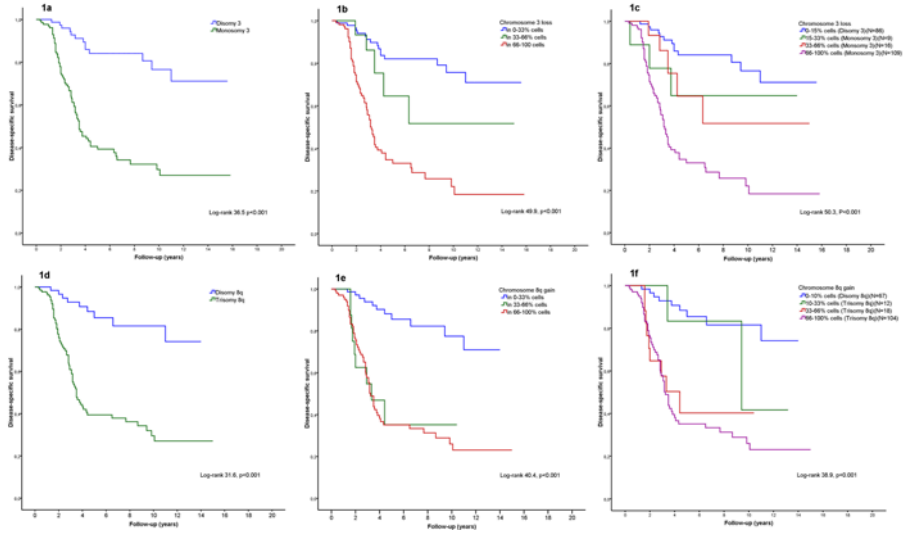


Figure 1. Kaplan-Meier survival curves. (A)Chromosome 3 status. (B) Chromosome 3 loss (illustrates incrementally increasing percentages). (C) Chromosome 3 status (illustrates increasing percentages). (D)Chromosome 8q status. (B) Chromosome 8q gain (illustrates incrementally increasing percentages). (F)Chromosome 8q status (illustrates increasing percentages).**(page 107)**

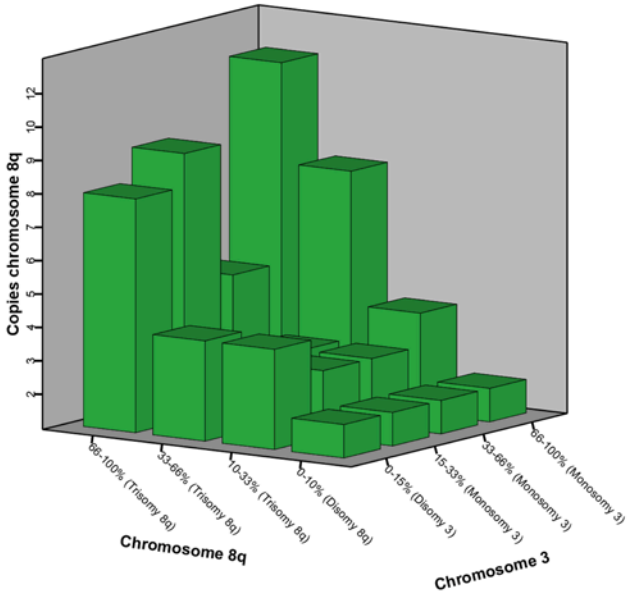


Figure 2. 3-D chart showing FISH counts of chromosome 3 and 8q according to chromosome 8q copy number. X-axis: classification according to the percentage of tumor cells with gain of chromosome 8q: 0%-10% (disomy 8q); 10%-33% (low percentage gain); 33%-66% (intermediate percentage gain); 66%-100% (high percentage gain). Y-axis: number of copies of chromosome 8q. Z- axis: classification according to the percentage of tumor cells with monosomy 3; 0%-15% (disomy 3); 15%-33% (low percentage monosomy); 33%-66% (intermediate percentage monosomy); 66%-100% (high percentage monosomy). **(page 109)**

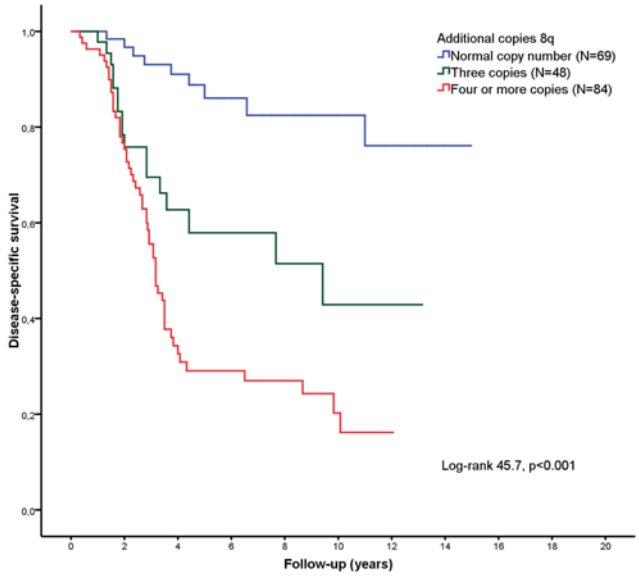


Figure 3. Kaplan-Meier survival curves according to copies of chromosome 8q present. **(page 109)**

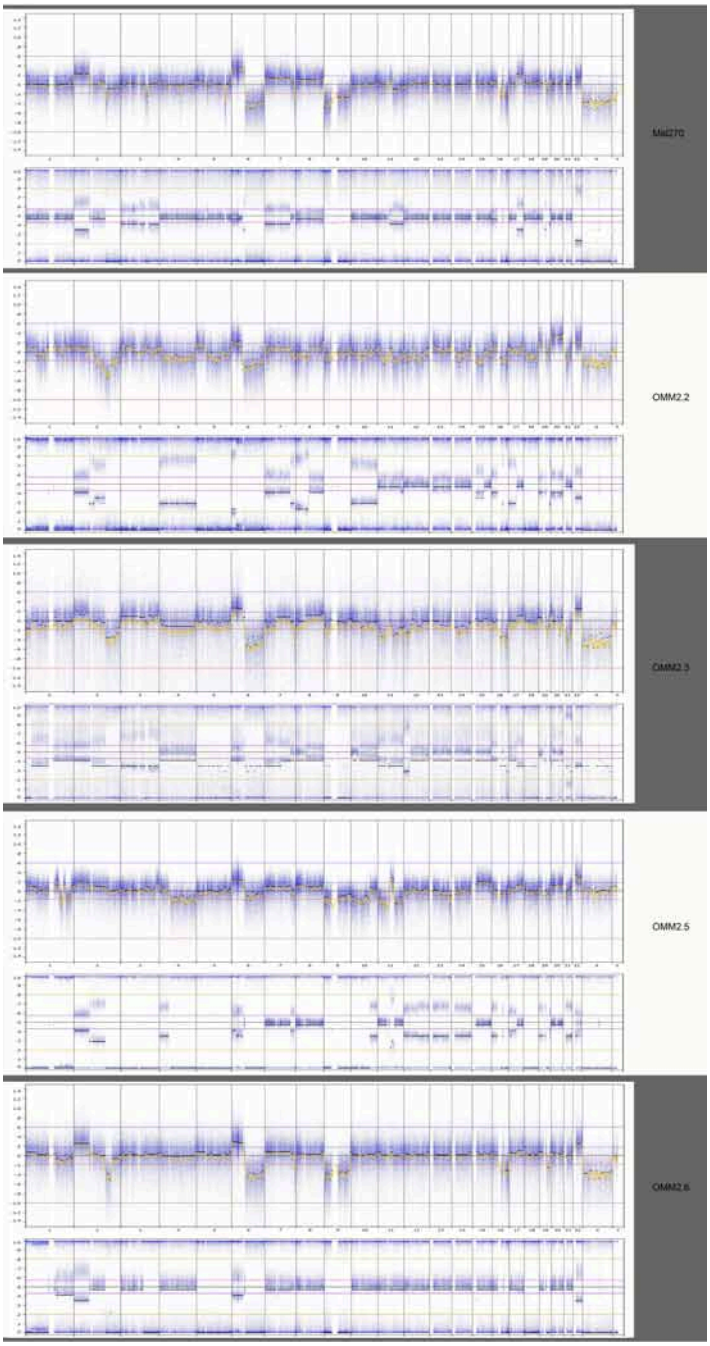


Figure 1. Whole genome SNP-array overview of all cell lines with the earliest passages analyzed The Log-R ratio is displayed in the upper panel for each cell line and the B-allele frequency in the lower panel for each cell line. **(page 118)**

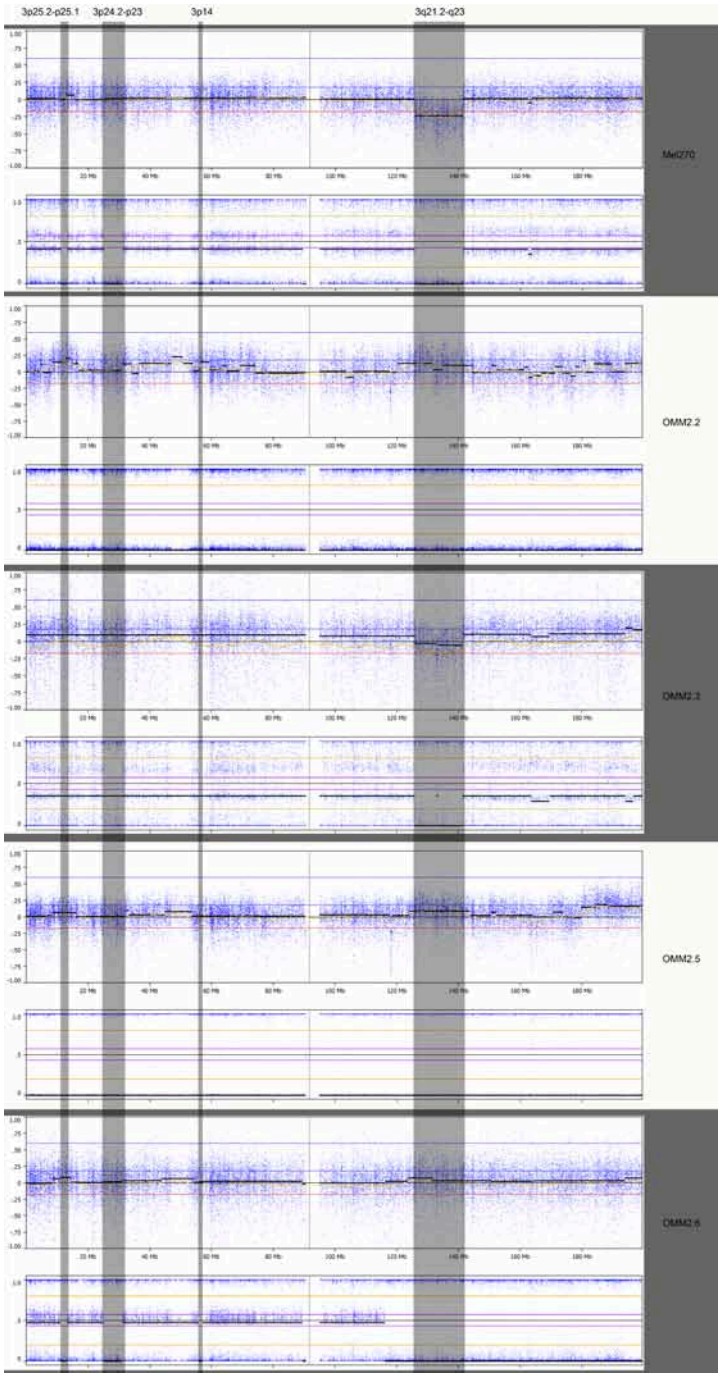
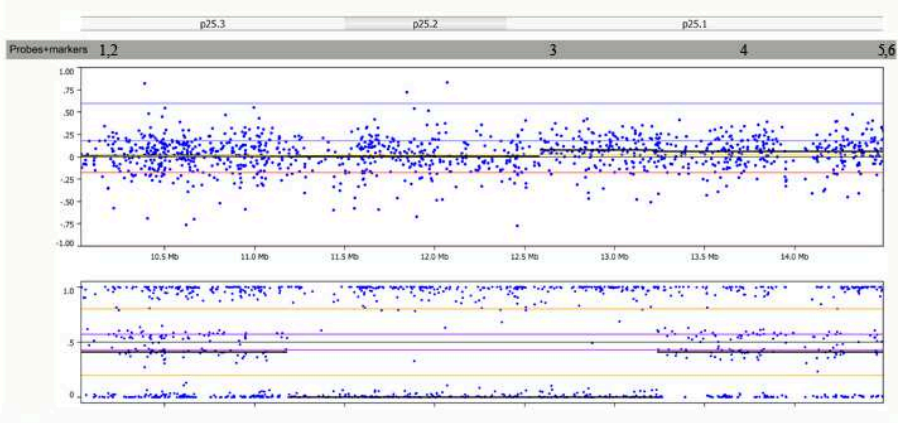


Figure 2: SNP-array overview of chromosome 3 of all cell lines with the earliest passages analyzed The Log-R ratio is displayed in the upper panel for each cell line and the B-allele frequency in the lower panel for each cell line. **(page 123)**

A



B

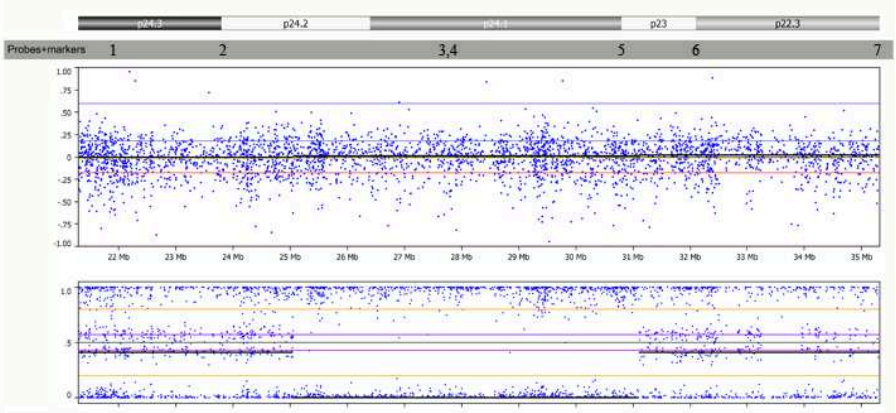
Location probes + markers	BAC	Position*	Copy number	Largest clone (%)	Marker	Position*	Number of alleles
1,2	481H17	3p25.3	2	85%	D3S1597	3p25.3	2
3	767C1	3p25.1	2	91%	D3S3693	3p25.1	1
4	-				D3S3608	3p25.1	2
5,6	255O19	3p24.3	2	83%	D3S1286	3p24.3	2

C

Position*	Candidate genes
3p25.3	*HRH1*
3p25.3-p25.2	*ATG7*
3p25.2	*VGLL4*, *SYN2*/ *TIMP4*, *C3orf31*
3p25.2-p25.1	*PPARγ*
3p25.1	*RAF1*, *TSEN2*, *MKRN2*, *IQSEC1*, *CAND2*, *RPL32*, *TMEM40*

Figure 3: Detail of the LOH region on 3p25.2-p25.1 with SNP-array results (A), FISH probe and microsatellite marker analysis (B), and candidate genes mapping to this region (C).
Figure A: Log-R ratio (upper panel) and B-allele frequency (lower panel) of SNP-array.
Figure B: Markers and probes mapping to the numbered locations displayed in figure A.
BAC = Bacterial Artificial Chromosome (used as FISH probe).
* = position according to the UCSC Humane Genome Browser (March 2006).
Figure C: Candidate genes mapping to the region identified by Endeavour prioritizing software.
(page 124)

A



B

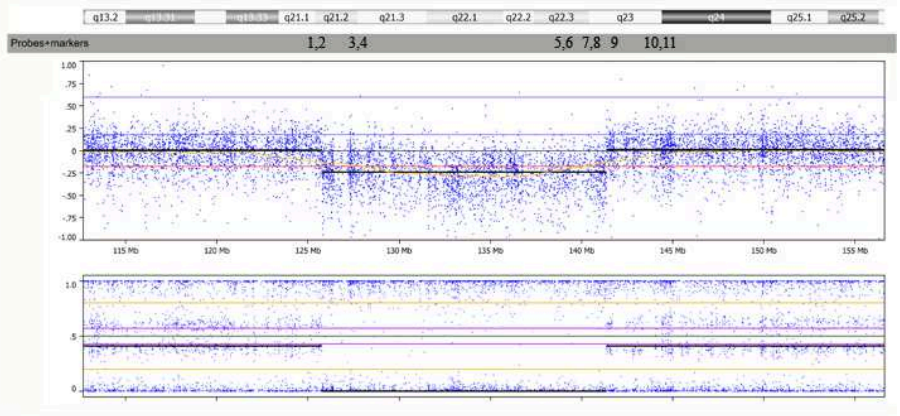
Location probes + markers	BAC	Position*	Copy number	Largest clone (%)	Marker	Position*	Number of alleles
1	-				D3S1293	3p24.3	2
2	41F5	3p24.3-p24.2	2	90%	-		
3,4	11L6	3p24.1	2	92%	D3S1266	3p24.1	1
5	-				D3S1211	3p23	1
6	-				D3S2432	3p23-p22.3	2
7	209O16	3p22.2	2	93%	-		

C

Position*	Candidate genes
3p24.2	*TOP2B*, *RARB*, *NGLY1*, *OXSM*
3p24.1	*TGFBR2*, *AZI2*, *SLC4A7*, *RBMS3*, *EOMES*, *LRRC3B*, *NEK10*, *ZCWPW2*
3p23	*GADL1*

Figure 4: Detail of the LOH region on 3p24.2-p23 with SNP-array results (A), FISH probe and microsatellite marker analysis (B), and candidate genes mapping to this region (C).
Figure A: Log-R ratio (upper panel) and B-allele frequency (lower panel) of SNP-array.
Figure B: Markers and probes mapping to the numbered locations displayed in figure A.
BAC = Bacterial Artificial Chromosome (used as FISH probe).
*** =** position according to the UCSC Humane Genome Browser (March 2006).
Figure C: Candidate genes mapping to the region identified by Endeavour prioritizing software.
(page 125)

A



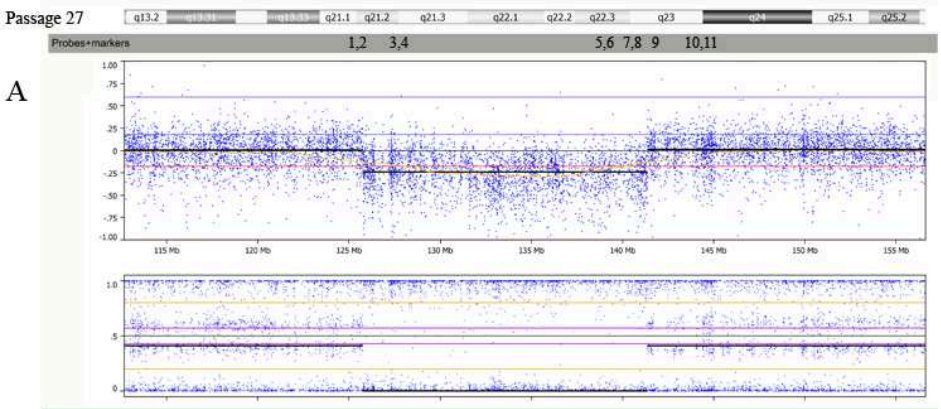
B

Location probes + markers	BAC	Position*	Copy number	Largest clone (%)	Marker	Position*	Number of alleles
1,2	95H16	3q21.2	2	79%	D3S1269	3q21.2	2
3,4	205A6	3q21.2	1	45%	D3S1589	3q21.2	1
5,6	162J10	3q22.3	1	41%	D3S1576	3q22.3	1
7,8	166D18	3q23	1	42%	D3S3554	3q23	1
9	-				D3S1309	3q23	2
10,11	160A13	3q23	2	80%	D3S3546	3q23	ni

C

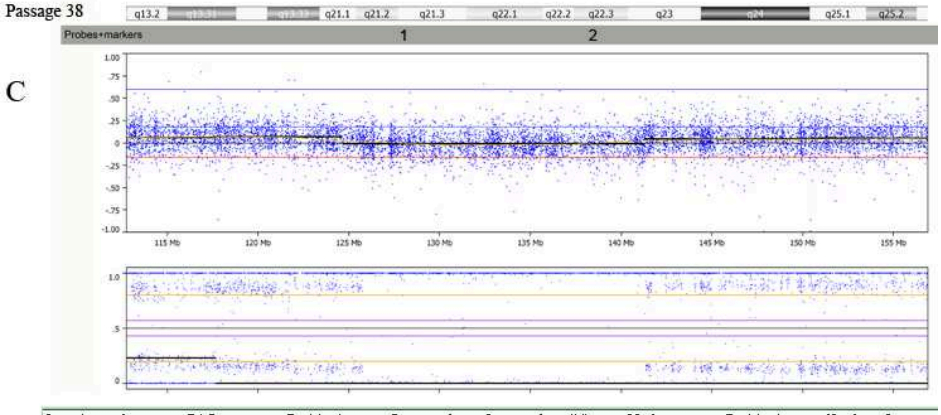
Position*	Candidate genes
3q21.2	*SLC12A8*, *ZNF148*
3q21.3	*MCM2*, *RUVBL1*, *MBD4*, *RHO*
3q22.1	*TOPBP1*, *RYK*, *NEK11*, *ATP2C1*
3q22.2	*PPP2R3A*
3q22.3	*PIK3CB*, *MCK1*, *STAG1*, *MRAS*

Figure 5: Detail of the deleted region 3q21.2-q23 with SNP-array results (A), FISH probe and microsatellite marker analysis (B), and candidate genes mapping to this region (C).
Figure A: Log-R ratio (upper panel) and B-allele frequency (lower panel) of SNP-array.
Figure B: Markers and probes mapping to the numbered locations displayed in figure A.
BAC = Bacterial Artificial Chromosome (used as FISH probe).
* = position according to the UCSC Humane Genome Browser (March 2006).
ni = noninformative marker
Figure C: Candidate genes mapping to the region identified by Endeavour prioritizing software.
(page 126)



B

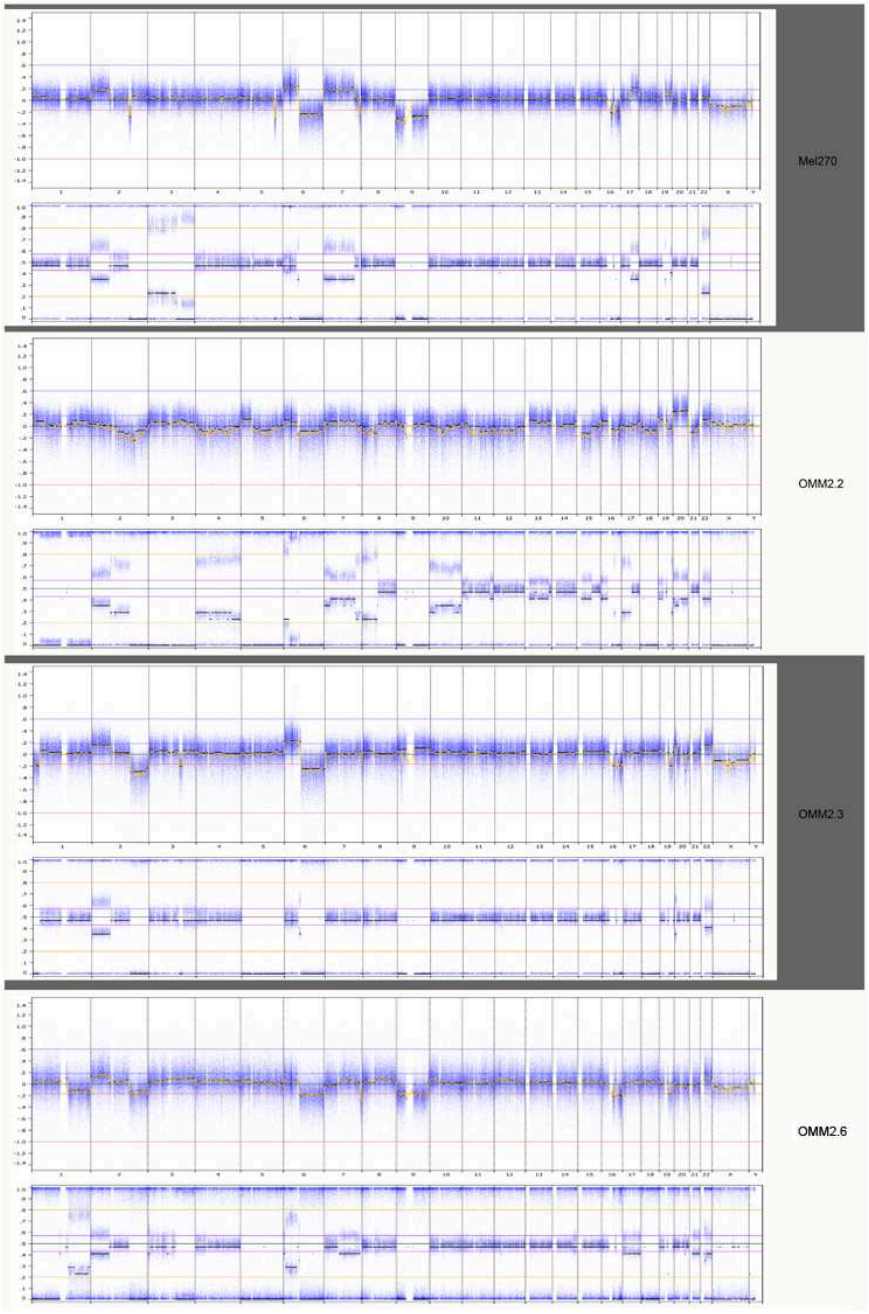
Location probes + markers	BAC	Position*	Copy number	Largest clone (%)	Marker	Position*	Number of alleles
1,2	95H16	3q21.2	2	79%	D3S1269	3q21.2	2
3,4	205A6	3q21.2	1	45%	D3S1589	3q21.2	1
5,6	162J10	3q22.3	1	41%	D3S1576	3q22.3	1
7,8	166D18	3q23	1	42%	D3S3554	3q23	1
9	-				D3S1309	3q23	2
10,11	160A13	3q23	2	80%	D3S3546	3q23	ni



D

Location probes + markers	BAC	Position*	Copy number	Largest clone (%)	Marker	Position*	Number of alleles
1	59J16	3q21.3	2	89%			
2	220J13	3q22.2	2	87%			

Figure 6: Detail of the deleted region 3q21.2-q23 for the earlier passage of Mel270 (passage 27) and the later passage of Mel270 (passage 38).
SNP-array results for passage 27 (A), passage 38 (C), and FISH probe and microsatellite marker analysis for passage 27 (B), and FISH analysis for passage 38 (D).
Figure A: Log-R ratio (upper panel) and B-allele frequency (lower panel) of SNP-array.
Figure B: Markers and probes mapping to the numbered locations displayed in figure A.
BAC = Bacterial Artificial Chromosome (used as FISH probe).
* = position according to the UCSC Humane Genome Browser (March 2006).
ni = noninformative marker
Figure C: Candidate genes mapping to the region identified by Endeavour prioritizing software.
Figure D: Markers and probes mapping to the numbered locations displayed in figure C. **(page 127)**



Supplementary figure 1: Whole genome SNP-array overview of cell lines with the highest passage numbers analyzed (OMM2.5 not available).
The Log-R ratio is displayed in the upper panel for each cell line (except OMM2.5), and the B-allele frequency in the lower panel for each cell line.
(page 135)

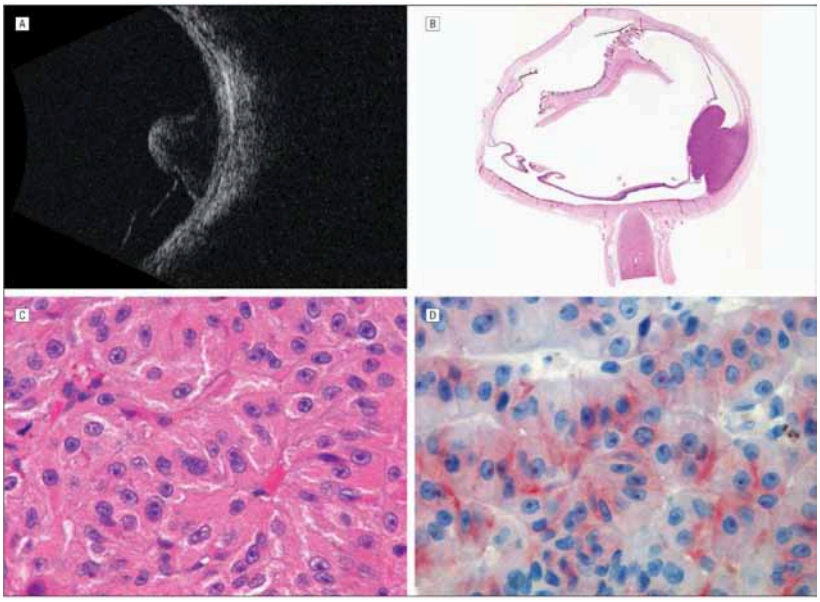


Figure ABCD: Ultrasonographic, whole-mount, histologic, and immunohistochemical appearance of the tumor. A B-scan of the tumor (A) and a whole-mount hematoxylin-eosin-stained section (B) of the left eye show a mushroom-shaped subretinal mass in the posterior pole. C, The tumor was exclusively composed of a nested and trabecular pattern of polygonal epithelioid cells with distinct borders and a granular eosinophilic cytoplasm that sometimes contained brown pigment. The nests and trabeculae were surrounded by a delicate capillary network. Nuclei were enlarged with coarse open chromatin and prominent irregular nucleoli (hematoxylin-eosin, original magnification x400). D, The cells stained positive for the melanocytic marker HMB-45 (original magnification x400). **(page 141)**

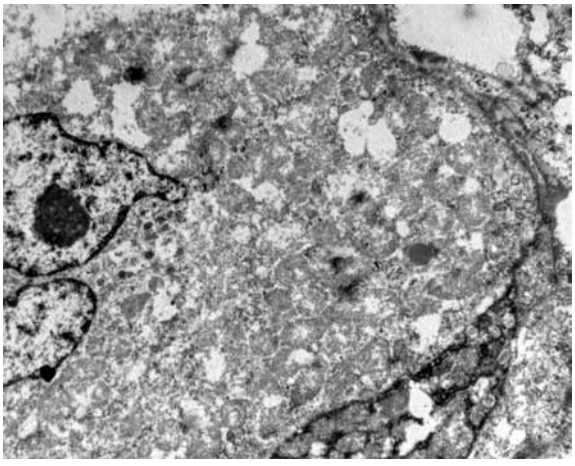


Figure E: Ultrastructural appearance of the tumor. Ultrastructural examination of formalin-fixed, deparaffinized, plastic re-embedded tumor tissue shows early and late melanosomes in cytoplasm, densely packed with structures that can be identified as mitochondria. Due to improper fixation many artefacts can be seen. **(page 141)**