Molecular Prognostic Markers in Uveal Melanoma: Expression Profiling and Genomic Studies

Walter van Gils

Molecular Prognostic Markers in Uveal Melanoma: Expression Profiling and Genomic Studies

Walter van Gils

The research in this thesis was carried out at the Erasmus MC in Rotterdam and was financially supported by Grant EMCR 2002-2695 from the Dutch Cancer Society. The publication of this thesis was financially supported by the Dutch Cancer Society.

ISBN/EAN: 978-90-9022630-9

Cover, layout and graphical assistance: Tom de Vries Lentsch

Printed by: PrintPartners Ipskamp, Enschede

Molecular Prognostic Markers in Uveal Melanoma: Expression Profiling and Genomic Studies

Moleculaire prognostische markers in oogmelanomen:
gen expressie profilering en genomische studies

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof.dr. S.W.J. Lamberts

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
woensdag 30 januari 2008 om 15:45 uur

door

Walter van Gils

geboren te Roosendaal

Promotiecommissie

Promotoren:	Prof.dr. G. van Rij
	Prof.dr. G.P.M. Luyten
Overige leden:	Prof.dr. B.A. Oostra
	Prof.dr. J.C. van Meurs
	Dr. P.M.J.J. Berns
Copromotoren:	Dr. J.E.M.M. de Klein
	Dr. H.T. Brüggenwirth

Index

Part I

Introduction

Chapter 1

Introduction

Chapter 1
Introduction

Uveal Melanoma

Brief overview

Uveal Melanomas (UMs) arise from melanocytes. This cell type originates from neural crest cells and thereby uveal melanomas share their origin with pheochromocytomas, neuroblastomas, paragangliomas and cutaneous melanomas, other tumors that develop from neural crest originating cells. [1-3] Uveal melanoma is the most common primary tumor in the eye with an incidence of approximately 7 per million every year in the Western World. In adults, 80 percent of all intraocular tumors are uveal melanomas. The mean age at diagnosis is 60 years. Most uveal melanomas arise in the ciliary body (23%), or the choroid (72%) and a small fraction originates in the iris (5%).[4] Predispositions for UM are a light eye color, fair skin color and ability to tan, which are all related with a fair phenotype. Despite advances in treatment with enucleation, pre-enucleation radiotherapy, stereotactic radiotherapy, brachytherapy, charged particle irradiation, thermo therapy and local eye wall resection, the mortality rate has not changed significantly. As many as 50% of all newly diagnosed patients will die from distant metastases, which are mainly located in the liver.[5]

Prognostic parameters

The clinical outcome of patients with uveal melanoma depends on the development of metastases. A number of clinical and histopathological parameters to predict the outcome of the disease have been described. The most widely used prognostic parameters are tumor diameter, involvement of the ciliary body, cell type, nucleolar size and tumor vascular patterns. Tumor diameter and nucleolar size correlate negatively with prognosis and also ciliary body involvement, the presence of epitheloid cells and vascularization indicate a poorer prognosis. Other prognostic factors associated with a poor prognosis are extrascleral growth, presence of tumor-infiltrating lymphocytes, older age and male gender.[6, 7] However, the use of these parameters only is not conclusive; even combined, these parameters cannot provide a sound estimation of prognosis.

Molecular Tumor Cytogenetics

Different cytogenetic techniques have been applied to uveal melanoma in the search for candidate regions on chromosomes and, eventually, candidate genes related with poor prognosis or responsible for tumor initiation and development. With conventional karyotyping larger gains, deletions and translocations can be detected, but for analysis of more cryptic abnormalities and for higher resolution delineation, molecular cytogenetic techniques are indispensable. Molecular cytogenetic techniques that are nowadays widely used in studies on UM and a variety of other types of tumors are Fluorescence In Situ Hybridization (FISH), Spectral Karyotyping (SKY),

Comparative Genomic Hybridization (CGH) and Loss Of Heterozygosity (LOH) analysis with microsatellite markers.

FISH

With FISH specific translocations and gains and losses can be detected in both metaphase spreads and interphase nuclei. The technique is rapid, has a high specificity and makes it possible to detect gains and losses that are only present in smaller sub clones of the tumor.[8, 9] Thereby, this technique is also of value in estimating tumor heterogeneity. In spite of heterogeneity, known to occur in UM, it is demonstrated that FISH results in Fine Needle Aspiration Biopsies (FNABs) are in agreement with the results from the main tumor.[10] A drawback of FISH is that it only focuses on a very limited number of loci analyzed in a single experiment.

SKY

With SKY DNA of all chromosomes are differentially labeled with a combination of fluorochromes. Hybridization of the labeled DNA to metaphase spreads of the tumor and visualization using spectral imaging leads to a separate color for each chromosome. In this way the origin of derivative chromosomes can be discerned. This technique has been used to characterize balanced and unbalanced complex chromosomal abnormalities in UM and UM derived cell lines.[11, 12]

CGH

CGH allows a complete copy number analysis across the entire genome. It involves the competitive hybridization of DNA of the sample of interest with a reference DNA to a target sequence. In case of the classic CGH this target are normal human metaphase spreads. Since control and sample DNA are labeled with different fluorochrome tags, imbalances caused by gene deletion or amplification lead to a detectable and visible difference in fluorescence signals. In this way a higher resolution compared to conventional karyotyping can be archived (table 1). However, balanced rearrangements and abnormalities that are only present in smaller sub clones of a tumor cannot be detected with CGH.[13-15] A large CGH study on UM by Aalto *et al*. (2005) revealed concomitant loss of chromosome 3 and whole arm losses and gains of the respective chromosome 1, 6, and 8 in metastasizing primary uveal melanoma.[16] A CGH study by White *et al*. (2006) revealed gain of 18q22.1 as the most powerful predictor of a poor prognosis in UM. [17] However, this aberration occurs less frequent and these findings are not confirmed by other groups.

LOH

Microsatellites are tandem repeats of simple polymorphic sequences randomly distributed in non-coding regions of DNA. An extreme form of microsatellite instability (MSI), characterized by frequent length changes at simple tandem repeats, was first described in sporadic colon cancer and in HNPCC syndrome.[18-20] MSI is, however, not observed in UM and in most studies microsatellite markers are used to detect Loss Of Heterozygosity (LOH) as an indicator for chromosomal loss. A drawback of this technique is that only a limited number of markers can be analyzed in one single experiment.

Genomics

Micro array technology

A new era in biomedical science started with the completion of the human genome sequencing project and the introduction and development of DNA array technology. DNA arrays consist of a series of DNA segments regularly arranged on a support, to which a labeled DNA sample can be hybridized for copy number or expression measurements. In this way information on thousands of loci or expression of thousands of genes in a sample can be obtained in one single experiment. Although low-resolution DNA arrays already existed in the seventies as dot blots[21], the development of gridding robots in the late 1980s made it possible to produce high-density filters with 10,000 spots on a filter the size of A4 page. [22, 23] To increase the number of genes assayed and to decrease the required amounts of sample, smaller cDNA arrays were developed, resulting in the first micro arrays. Competing with the cDNA arrays, oligonucleotide micro arrays were developed to discriminate between a perfect match and a mismatch of the oligonucleotide present. These types of arrays are based on sequence knowledge alone and do not require the laborious maintenance of cDNA libraries and PCR amplification.[24-26] For DNA copy number approaches, arrays have been constructed with bacterial artificial chromosomes (BACs) as a DNA target with a length of each clone ranging from 80 to 200 kb.[27] Micro-array technology can be used for many different genomic approaches, of which array-based CGH, SNP analysis and gene expression analysis are the most frequently applied techniques. Also exon arrays and micro-RNA arrays are applied more and more frequent. A drawback of these array-based approaches is that the analyzed signal represents the average value of all cells in the analyzed sample. As a result, balanced anomalies and genomic abnormalities appearing in frequencies below 50% of the analyzed nuclei will not be detected. For these cases, the FISH approach is a better option. In table 1 the different cytogenetic approaches are compared on their detection limits.

Genomic micro array approaches

Array-CGH

The recent advances in micro array technologies offer the potential of CGH analysis with, compared to the classic chromosome CGH, an even higher resolution using many different highly specific oligonucleotides or BACs as a fixed template on a micro array slide. With array-CGH, regions of gain and loss as small as 10 kb can be detected. There are CGH micro arrays available that cover specific chromosomal regions with a resolution of approximately 1 kb, whereas other arrays cover the entire genome with a lower resolution. There are also tiled arrays available covering almost the entire genome with overlapping sequences.[28]

Micro array SNP analysis

Another, even more recently developed type of micro array makes use of single-nucleotide polymorphisms (SNPs) in the human genome. The discovery of SNPs lead to the construction of increasingly dense SNP maps, allowing innovations in genotyping technologies. Today, there are micro arrays on the market that genotype more than 7 million SNPs in one experiment. This offers a mapping tool designed to identify regions of the genome linked to or associated with a

particular trait or phenotype. Additionally, it is useful for the determination of allele frequencies in various populations and for mapping regions with loss of heterozygosity. Furthermore, SNP arrays can be used to define copy number changes in tumors using the combined information of signal intensities and SNPs.[29]

Cytogenetics and genomics in uveal melanoma

In contrast to hematological malignancies, it is difficult to get good quality metaphase spreads from solid tumors. Furthermore, solid tumors are often more complex and heterogeneous. This makes it harder to study solid tumors cytogenetically and to determine frequently occurring aberrations in a tumor type. However, uveal melanoma was found to be relatively accessible to cytogenetical analysis. From the late eighties on, several reports on the cytogenetic analysis of uveal melanomas have been published. Most of the studies are based on cytogenetic and FISH analysis. Only a minor fraction was analyzed with CGH. The most frequently found aberrations are loss of 1p36, loss of chromosome 3, abnormalities of chromosome 6 and gain of chromosome 8 or 8q. Also changes in chromosome 11, 16, loss of chromosome 2, 21 and the sex chromosomes were described, but those occurred less frequently.[16, 33, 34] Results of these molecular (cyto) genetic studies result in a delineation of commonly affected regions on chromosomes 1p, 3, 6 and 8. These data are summarized in the following sections.

Chromosome 1

Loss of chromosomal region 1p36 is frequently observed in various tumors, including the neural crest derived neuroblastoma and pheochromocytoma.[35-37] In neuroblastoma, loss of chromosome-arm 1p is known to be a predictor of unfavorable clinical outcome.[38, 39] In UM loss of 1p has been detected predominantly in primary tumors with monosomy of chromosome 3 and metastasizing tumors.[16, 40] Loss of chromosome 1p by itself is not of prognostic significance, but concurrent loss of chromosomes 1p and 3 is strongly associated with decreased survival of UM patients (discussed further in chapter 4).[41] This suggests that a tumor suppressor gene involved in UM is located on 1p36.

The common deleted regions on chromosome 1 described in the literature are summarized in figure 1, in which also the overlap between these regions, referred to as shortest region of overlap (SRO) total, is indicated. The candidate tumor suppressor gene *APITD1*, located in this SRO addressed in chapter 5 of this thesis is also depicted in this figure.

Chromosome 3

In UM, monosomy of chromosome 3 is the most frequently found non-random chromosomal aberration. It is a constant event in UMs that are characterized by different sub-populations and therefore the involvement of chromosome 3 is considered to be a primary event.[43] In most UM cases an entire copy of this chromosome is lost, although in some cases, isodisomy of this chromosome is acquired.[16, 44, 45] Monosomy of chromosome 3 correlates highly with a poor prognosis.[46-49] This effect becomes even stronger when besides chromosome 3, chromosome 1p36 is also lost.[41] This high incidence of monosomy of chromosome 3 is specific for UM and not

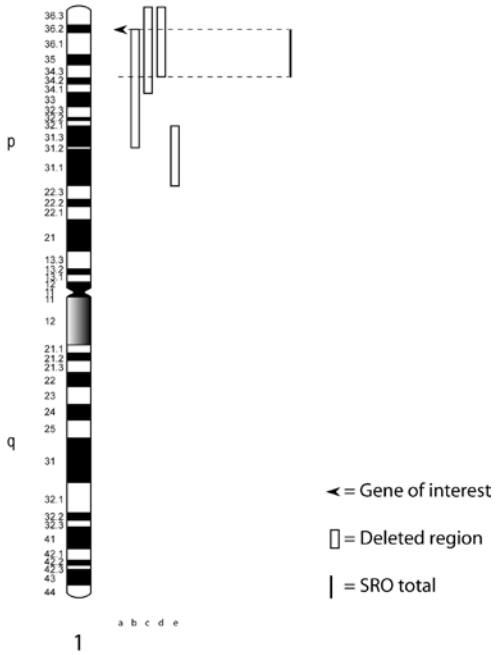


Figure 1. **Summary of previously described common regions of deletion on the short arm of chromosome one in UM.** Chromosome banding pattern is designated on the left according to ISCN (2005). On the right side of the ideogram, the vertical bars represent regions of deletion found in different studies. Letters under the respective bar indicate the different studies. **A)** Arrow: indicates the *APITD1* gene (see chapter 5); Smallest region of deletion overlap in UMs with **B)** monosomy of chromosome 3[42] or **C)** disomy of chromosome 3[42] in micro satellite analysis; Common region of deletion identified with **D)** array CGH [40] or **E)** conventional CGH. [16]

commonly observed in other tumor types. It is widely believed that chromosome 3 harbors tumor suppressor genes that play an important role typically in uveal melanoma development. Since in most cases of chromosome 3 losses there is a complete monosomy, only a few melanomas with partial deletions on chromosome 3 or translocations involving chromosome 3 have been described. This has hampered the mapping of putative tumor suppressor genes. Different candidate regions were reported on both the short arm and the long arm of this chromosome. These data are summarized in figure 2.
Common areas of 3p and 3q loss are observed by us (see chapter 6 and 7) and others. [44, 51-54] Remarkably some of these chromosomal loci overlap with the regions with low gene expression in tumors with a short survival, a study described in chapter 2. The analysis of genes in these regions and their role in tumor development and progression is in progress.

Chromosome 6

In UM abnormalities on chromosome 6 consist mostly of 6p gain and 6p loss. Aberrations, resulting in a relative increase of 6p material, either through an isochromosome of 6p or a deletion of 6q, have been found to be related with both a longer survival[46] or decreased survival[16] in uveal melanoma. Deletion of the long arm of chromosome 6 is a frequent event in many neoplasms, including carcinomas of the prostate and breast and melanomas. This suggests the presence of a tumor-suppressor gene or genes on 6q. In UM deletion of 6q appears to be a late event resulting from tumor progression.[55] Although in certain tumor types a smaller candidate region or regions for oncogenes on 6p and tumor suppressor genes on 6q have been identified, in UM only relatively large common regions of gain and loss have previously been described. Figure 3 represents a summary of the different regions on chromosome 6 that are described in these papers, together with the regions demarcated in our own study as described in chapter 8.

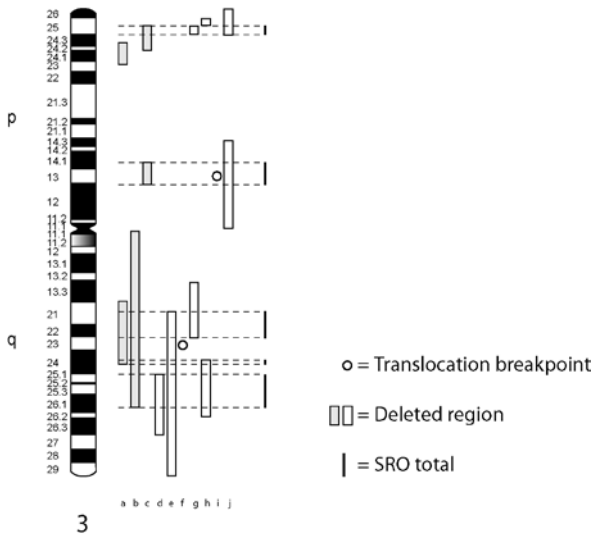


Figure 2. **Summary of previously described common regions of deletion on chromosome three in UM.** Chromosome banding pattern is designated on the left according to ISCN (2005). On the right side of the ideogram, the deleted regions observed in the different studies are represented by vertical bars, the breakpoints of a translocation by an open circle. The different studies are indicated by letters under the respective bar. **A)** Regions of loss in UM derived cell line Mel270 demarcated with FISH and micro satellite analysis (Chapter 6); **B)** SRO-region demarcated with FISH analysis (chapter 7); **C)** Regions with significant lower expression of genes in tumors with short survival (chapter 2); **D)** Overlap found with CGH[16]; **E, G, H and J)** Common deleted region identified with micro satellite markers in studies from the UK[44], USA[51]; Germany[50] and UK[52], respectively; **F)** Translocation t(3;14)(q23;q32)[53]; **I)** Translocation t(3;22)(p13;p11). [54]

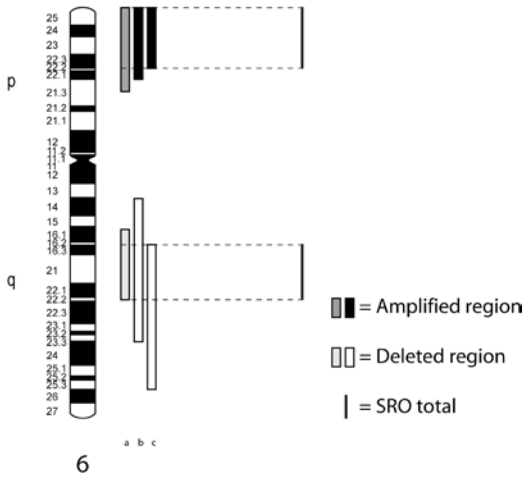


Figure 3. **Summary of previously described common regions of gain and deletion on chromosome six in UM.** Chromosome banding pattern is designated on the left according to ISCN (2005). On the right side of the ideogram, the vertical bars represent the regions of deletion found in different studies. The different studies are indicated by letters under the respective bar. **A)** Common regions of gain and loss identified with karyotyping, CGH and FISH described in chapter 8 of this thesis; **B)** Common regions of gain and loss identified with CGH[16] or **C)** with array CGH. [40]

Chromosome 8q

Gain of chromosome 8q is often seen as a non-random aberration. Several studies indicated gain of chromosome 8 as an independent prognostic marker for decreased patient survival.[46, 49] Acquisition of an isochromosome 8q seems to be a secondary event, indicated by the variable number of copies of chromosome 8q that is often observed in a tumor with gain of chromosome 8. Where involvement of chromosome 3 is considered to be a primary event, variable number of copies of chromosome 8q can be observed in different UM sub-populations.[43, 56] Chromosome 8 abnormalities occur more often in larger tumors, which supports the hypothesis that a certain

gene (or a combination of genes) present on 8q, when overexpressed in these tumors, plays a role in the development of the metastatic phenotype.[43] However, in our study described in chapter 3 of this thesis, the presence of chromosome 8q abnormalities correlated with largest tumor diameter and in univariate analysis there was no significant relation found between gain of 8q and the metastatic phenotype. We suggest that the acquisition of additional copies of 8q is

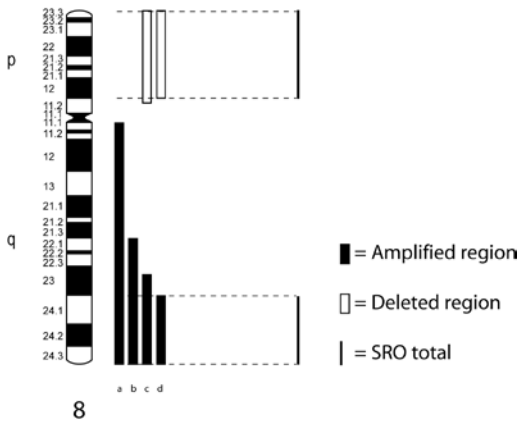


Figure 4. **Summary of previously described common regions of gain and deletion on chromosome 8 in UM.** Chromosome banding pattern is designated on the left according to ISCN (2005). On the right side of the ideogram, the regions deleted in the different studies are represented by vertical bars. The different studies are indicated by letters under the respective bar. **A)** Gain identified with array CGH[40] **B)** Multiplex FISH analysis of common region of gain[59]; **C)** Gain and loss identified with CGH analysis; **D)** Gain and loss identified with array CGH. [40]

related more with tumor growth than tumor aggressiveness. [57] In most cases, amplification on chromosome 8q involves the entire chromosome or isochromosome formation of the long arm of chromosome 8. Amplification of the long arm of chromosome 8 by isochromosome formation occurs very frequently in tumors with loss of chromosome 3 and concurrent abnormalities of chromosome 3 and 8 are associated with a poor outcome.[16, 46, 58] Most reported partial gains of chromosome 8 in UM are the result of unbalanced translocations. An overview of the observed loss and gain of chromosome 8 in the different reports is given in figure 4. Altogether, multiple breakpoints are found on chromosome 8q, with a common amplified region ranging from 8q24.1 to 8qter.

Gene expression profiling in uveal melanoma

With the emerging higher resolution views of gene expression, it became possible to classify human cancers based on their gene expression signature. The technique is now being used to predict the clinical outcome and to propose molecular targets for therapy. In melanoma research, gene expression profiling has already been applied in a number of studies, but in most cases the subject was cutaneous melanoma. In the year 2000, 31 skin melanomas were classified into 2 subgroups, which lead to the identification of WNT5A as a new disease gene for melanoma progression.[60] Other studies on cutaneous melanoma focused on primary tumors vs. their metastases and on treatment response.[61, 62]

Using cDNA micro array technology, highly invasive uveal melanoma tumor cells were compared with poorly invasive cells and a genetic reversion to a pluripotent embryonic-like genotype was found in the aggressive tumor cells. This suggests that aggressive uveal melanoma cells may

generate vascular channels that facilitate tumor perfusion independent of tumor angiogenesis in a process called vascular mimicry.[63] Tschentscher *et al*. (2003) reported that UMs could be classified in groups with or without monosomy 3 with use of oligonucleotide gene expression arrays.[64] In another study by Onken *et al*. (2005), uveal melanomas were found to cluster in two distinct entities on basis of their gene expression profile, on basis of which metastatic death could be predicted.[65] The existence of two distinct entities was also reported by Petrausch *et al.* (2007).[66] Recently a strong association was found between vascular looping patterns in the extra cellular matrix and the unfavorable molecular prognostic signature of the high grade uveal melanoma subgroup.[67] This is confirmed in chapter 2 of this thesis, which describes the analysis of 46 uveal melanomas with gene expression profiling. Additionally, our data revealed a strong association between an unfavorable prognostic signature and presence of closed vascular patterns. This proves that gene expression profiling is a very powerful technique that can be used to study a broad range of variables in uveal melanoma. Data generation in expression profiling is enormous and can be used for clustering analysis and prognostic subtyping the data. In addition, the data can also be used to answer more specific questions on uveal melanoma behavior from a mechanistical point of view.

Aim of this thesis

The work presented in this thesis is aimed to get a better understanding of uveal melanoma tumor biology by delineation of chromosomal regions and identification of genes responsible for a poor prognosis in uveal melanoma patients. This will also help to make advances in prognostic screening and might provide targets for treatment. To do so, two different approaches were combined. The first approach was to perform gene expression profiling on a large set of uveal melanomas. The second approach was to further investigate the known chromosomal regions with prognostic significance in uveal melanomas. A combination of these approaches will lead to the identification of differential expressed genes located on candidate regions that function in tumor-related functional pathways. The use of gene expression profiling in uveal melanoma is described in Chapter 2. In this chapter, the creation of gene expression signatures and a comparison with known prognostic parameters are outlined. In chapter 3 the relations of clinical, histopathological and cytogenetic parameters with prognosis are outlined. A study focusing on the concurrent loss of chromosomes 1p and 3 is described in Chapter 4. The next section reflects studies on different chromosomal regions of prognostic significance in uveal melanoma. Chapter 5 outlines a study on a possible candidate tumor suppressor gene located on 1p36, called *APITD1*, an apoptosis-inducing gene that is affected in many cases of neuroblastoma, another neural-crest cell-derived tumor. Delineation studies of regions of loss on chromosome 3 are described in Chapters 6 and 7, followed by a delineation study on chromosome 6 in Chapter 8. In Chapter 9, the results of the different studies are combined and discussed. In addition, different possibilities to use gene expression data to identify critical regions, genes and pathways in different uveal melanoma subgroups are discussed.

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

Method	Resolution	Detection of balanced anomalies?	Detection of unbalanced anomalies?
G-banding	~ 5 – 10 Mb	Yes	Yes
FISH	~ 0.1 Mb	Yes	Yes
SKY	~ 1 - 2 Mb	Yes	Yes
LOH	<1 kb	No	Yes
CGH	~ 5 – 20 Mb	No	Yes
MLPA	~ 1 – 40 kb	No	Yes
CGH array	~ 1 kb – 250 kb **	No	Yes
SNP array	~ 1 kb – 250 kb **	No	Yes

*Adapted from Feenstra *et al.*, Fan *et al.* and Peiffer *et al.*[30-32] ** Depending on type and number of probes (BAC, oligonucleotides or SNP) and/or their distribution.

References

1. Krona C, Ejeskar K, Caren H *et al.* A novel 1p36.2 located gene, APITD1, with tumour-suppressive properties and a putative p53-binding domain, shows low expression in neuroblastoma tumours. *Br J Cancer*. 2004;91:1119-1130
2. Sesterhenn AM, Folz BJ, Lippert BM, Janig U, Werner JA. Laser surgical treatment of laryngeal paraganglioma. *J Laryngol Otol*. 2003;117:641-646
3. Namiki T, Yanagawa S, Izumo T *et al.* Genomic alterations in primary cutaneous melanomas detected by metaphase comparative genomic hybridization with laser capture or manual microdissection: 6p gains may predict poor outcome. *Cancer Genet Cytogenet*. 2005;157:1-11
4. Zimmerman LE, McLean IW. Do growth and onset of symptoms of uveal melanomas indicate subclinical metastasis? *Ophthalmology*. 1984;91:685-691
5. Kujala E, Makitie T, Kivela T. Very long-term prognosis of patients with malignant uveal melanoma. *Invest Ophthalmol Vis Sci*. 2003;44:4651-4659
6. Mooy CM, De Jong PT. Prognostic parameters in uveal melanoma: a review. *Surv Ophthalmol*. 1996;41:215-228
7. Singh AD, Shields CL, Shields JA. Prognostic factors in uveal melanoma. *Melanoma Res*. 2001;11:255-263
8. van Dekken H, Pizzolo JG, Reuter VE, Melamed MR. Cytogenetic analysis of human solid tumors by in situ hybridization with a set of 12 chromosome-specific DNA probes. *Cytogenet Cell Genet*. 1990;54:103-107
9. Trask BJ. Fluorescence in situ hybridization: applications in cytogenetics and gene mapping. *Trends Genet*. 1991;7:149-154
10. Naus NC, Verhoeven AC, van Drunen E *et al.* Detection of genetic prognostic markers in uveal melanoma biopsies using fluorescence in situ hybridization. *Clin Cancer Res*. 2002;8:534-539
11. Naus NC, van Drunen E, de Klein A *et al.* Characterization of complex chromosomal abnormalities in uveal melanoma by fluorescence in situ hybridization, spectral karyotyping, and comparative genomic hybridization. *Genes Chromosomes Cancer*. 2001;30:267-273.
12. White JS, Becker RL, McLean IW, Director-Myska AE, Nath J. Molecular cytogenetic evaluation of 10 uveal melanoma cell lines. *Cancer Genet Cytogenet*. 2006;168:11-21

13. Kallioniemi A, Kallioniemi OP, Sudar D *et al.* Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. *Science*. 1992;258:818-821
14. Kallioniemi OP, Kallioniemi A, Sudar D *et al.* Comparative genomic hybridization: a rapid new method for detecting and mapping DNA amplification in tumors. *Semin Cancer Biol*. 1993;4:41-46
15. Ried T, Liyanage M, du Manoir S *et al.* Tumor cytogenetics revisited: comparative genomic hybridization and spectral karyotyping. *J Mol Med*. 1997;75:801-814
16. Aalto Y, Eriksson L, Seregard S, Larsson O, Knuutila S. Concomitant loss of chromosome 3 and whole arm losses and gains of chromosome 1, 6, or 8 in metastasizing primary uveal melanoma. *Invest Ophthalmol Vis Sci*. 2001;42:313-317
17. White JS, McLean IW, Becker RL, Director-Myska AE, Nath J. Correlation of comparative genomic hybridization results of 100 archival uveal melanomas with patient survival. *Cancer Genet Cytogenet*. 2006;170:29-39
18. Ionov Y, Peinado MA, Malkhosyan S, Shibata D, Perucho M. Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. *Nature*. 1993;363:558-561
19. Thibodeau SN, Bren G, Schaid D. Microsatellite instability in cancer of the proximal colon. *Science*. 1993;260:816-819
20. Aaltonen LA, Peltomaki P, Leach FS *et al.* Clues to the pathogenesis of familial colorectal cancer. *Science*. 1993;260:812-816
21. Kafatos FC, Jones CW, Efstratiadis A. Determination of nucleic acid sequence homologies and relative concentrations by a dot hybridization procedure. *Nucleic Acids Res*. 1979;7:1541-1552
22. Hoheisel JD, Lennon GG, Zehetner G, Lehrach H. Use of high coverage reference libraries of Drosophila melanogaster for relational data analysis. A step towards mapping and sequencing of the genome. *J Mol Biol*. 1991;220:903-914
23. Lennon GG, Lehrach H. Hybridization analyses of arrayed cDNA libraries. *Trends Genet*. 1991;7:314-317
24. Wodicka L, Dong H, Mittmann M, Ho MH, Lockhart DJ. Genome-wide expression monitoring in Saccharomyces cerevisiae. *Nat Biotechnol*. 1997;15:1359-1367
25. Fodor SP, Read JL, Pirrung MC *et al.* Light-directed, spatially addressable parallel chemical synthesis. *Science*. 1991;251:767-773
26. Jordan B. Historical background and anticipated developments. *Ann N Y Acad Sci*. 2002;975:24-32
27. Bejjani BA, Saleki R, Ballif BC *et al.* Use of targeted array-based CGH for the clinical diagnosis of chromosomal imbalance: is less more? *Am J Med Genet A*. 2005;134:259-267
28. Shaffer LG, Bejjani BA. Medical applications of array CGH and the transformation of clinical cytogenetics. *Cytogenet Genome Res*. 2006;115:303-309
29. Bignell GR, Huang J, Greshock J *et al.* High-resolution analysis of DNA copy number using oligonucleotide microarrays. *Genome Res*. 2004;14:287-295
30. Feenstra I, Brunner HG, van Ravenswaaij CM. Cytogenetic genotype-phenotype studies: improving genotyping, phenotyping and data storage. *Cytogenet Genome Res*. 2006;115:231-239
31. Fan YS, Siu VM, Jung JH, Xu J. Sensitivity of multiple color spectral karyotyping in detecting small interchromosomal rearrangements. *Genet Test*. 2000;4:9-14
32. Peiffer DA, Le JM, Steemers FJ *et al.* High-resolution genomic profiling of chromosomal aberrations using Infinium whole-genome genotyping. *Genome Res*. 2006;16:1136-1148
33. Sisley K, Rennie IG, Cottam DW *et al.* Cytogenetic findings in six posterior uveal melanomas: involvement of chromosomes 3, 6, and 8. *Genes Chromosomes Cancer*. 1990;2:205-209
34. Sisley K, Cottam DW, Rennie IG *et al.* Non-random abnormalities of chromosomes 3, 6, and 8 associated with posterior uveal melanoma. *Genes Chromosomes Cancer*. 1992;5:197-200
35. Brodeur GM, Green AA, Hayes FA *et al.* Cytogenetic features of human neuroblastomas and cell lines. *Cancer Res*. 1981;41:4678-4686
36. Benn DE, Dwight T, Richardson AL *et al.* Sporadic and familial pheochromocytomas are associated with loss of at least two discrete intervals on chromosome 1p. *Cancer Res*. 2000;60:7048-7051
37. Brodeur GM, Sekhon G, Goldstein MN. Chromosomal aberrations in human neuroblastomas. *Cancer*. 1977;40:2256-2263
38. Caron H, van Sluis P, de Kraker J *et al.* Allelic loss of chromosome 1p as a predictor of unfavorable outcome in patients with neuroblastoma. *N Engl J Med*. 1996;334:225-230
39. Casciano I, Mazzocco K, Boni L *et al.* Expression of DeltaNp73 is a molecular marker for adverse outcome in neuroblastoma patients. *Cell Death Differ*. 2002;9:246-251
40. Hughes S, Damato BE, Giddings I *et al.* Microarray comparative genomic hybridisation analysis of intraocular uveal melanomas identifies distinctive imbalances associated with loss of chromosome 3. *Br J Cancer*. 2005;93:1191-1196
41. Kilic E, Naus NC, van Gils W *et al.* Concurrent loss of chromosome arm 1p and chromosome 3 predicts a decreased disease-free survival in uveal melanoma patients. *Invest Ophthalmol Vis Sci*. 2005;46:2253-2257

42. Hausler T, Stang A, Anastassiou G *et al.* Loss of heterozygosity of 1p in uveal melanomas with monosomy 3. *Int J Cancer*. 2005;116:909-913
43. Prescher G, Bornfeld N, Becher R. Two subclones in a case of uveal melanoma. Relevance of monosomy 3 and multiplication of chromosome 8q. *Cancer Genet Cytogenet*. 1994;77:144-146
44. Scholes AG, Liloglou T, Maloney P *et al.* Loss of heterozygosity on chromosomes 3, 9, 13, and 17, including the retinoblastoma locus, in uveal melanoma. *Invest Ophthalmol Vis Sci*. 2001;42:2472-2477
45. White VA, McNeil BK, Horsman DE. Acquired homozygosity (isodisomy) of chromosome 3 in uveal melanoma. *Cancer Genet Cytogenet*. 1998;102:40-45
46. White VA, Chambers JD, Courtright PD, Chang WY, Horsman DE. Correlation of cytogenetic abnormalities with the outcome of patients with uveal melanoma. *Cancer*. 1998;83:354-359
47. Prescher G, Bornfeld N, Hirche H *et al.* Prognostic implications of monosomy 3 in uveal melanoma. *Lancet*. 1996;347:1222-1225
48. Sisley K, Parsons MA, Garnham J *et al.* Association of specific chromosome alterations with tumour phenotype in posterior uveal melanoma. *Br J Cancer*. 2000;82:330-338
49. Sisley K, Rennie IG, Parsons MA *et al.* Abnormalities of chromosomes 3 and 8 in posterior uveal melanoma correlate with prognosis. *Genes Chromosomes Cancer*. 1997;19:22-28.
50. Tschentscher F, Prescher G, Horsman DE *et al.* Partial deletions of the long and short arm of chromosome 3 point to two tumor suppressor genes in uveal melanoma. *Cancer Res*. 2001;61:3439-3442
51. Parrella P, Fazio VM, Gallo AP, Sidransky D, Merbs SL. Fine mapping of chromosome 3 in uveal melanoma: identification of a minimal region of deletion on chromosomal arm 3p25.1-p25.2. *Cancer Res*. 2003;63:8507-8510
52. Cross NA, Ganesh A, Parpia M *et al.* Multiple locations on chromosome 3 are the targets of specific deletions in uveal melanoma. *Eye*. 2006;20:476-481
53. Dahlenfors R, Tornqvist G, Wettrell K, Mark J. Cytogenetical observations in nine ocular malignant melanomas. *Anticancer Res*. 1993;13:1415-1420
54. Blasi MA, Roccella F, Balestrazzi E *et al.* 3p13 region: a possible location of a tumor suppressor gene involved in uveal melanoma. *Cancer Genet Cytogenet*. 1999;108:81-83
55. Prescher G, Bornfeld N, Becher R. Nonrandom chromosomal abnormalities in primary uveal melanoma. *J Natl Cancer Inst*. 1990;82:1765-1769
56. Horsman DE, White VA. Cytogenetic analysis of uveal melanoma. Consistent occurrence of monosomy 3 and trisomy 8q. *Cancer*. 1993;71:811-819
57. Kilic E, van Gils W, Lodder E *et al.* Clinical and cytogenetic analyses in uveal melanoma. *Invest Ophthalmol Vis Sci*. 2006;47:3703-3707
58. Prescher G, Bornfeld N, Friedrichs W, Seeber S, Becher R. Cytogenetics of twelve cases of uveal melanoma and patterns of nonrandom anomalies and isochromosome formation. *Cancer Genet Cytogenet*. 1995;80:40-46
59. Sisley K, Tattersall N, Dyson M *et al.* Multiplex fluorescence in situ hybridization identifies novel rearrangements of chromosomes 6, 15, and 18 in primary uveal melanoma. *Exp Eye Res*. 2006;83:554-559
60. Bittner M, Meltzer P, Chen Y *et al.* Molecular classification of cutaneous malignant melanoma by gene expression profiling. *Nature*. 2000;406:536-540
61. Clark EA, Golub TR, Lander ES, Hynes RO. Genomic analysis of metastasis reveals an essential role for RhoC. *Nature*. 2000;406:532-535
62. Wang E, Miller LD, Ohnmacht GA *et al.* Prospective molecular profiling of melanoma metastases suggests classifiers of immune responsiveness. *Cancer Res*. 2002;62:3581-3586
63. Seftor EA, Meltzer PS, Kirschmann DA *et al.* Molecular determinants of human uveal melanoma invasion and metastasis. *Clin Exp Metastasis*. 2002;19:233-246
64. Tschentscher F, Husing J, Holter T *et al.* Tumor classification based on gene expression profiling shows that uveal melanomas with and without monosomy 3 represent two distinct entities. *Cancer Res*. 2003;63:2578-2584
65. Onken MD, Worley LA, Ehlers JP, Harbour JW. Gene expression profiling in uveal melanoma reveals two molecular classes and predicts metastatic death. *Cancer Res*. 2004;64:7205-7209
66. Petrausch U, Martus P, Tonnies H *et al.* Significance of gene expression analysis in uveal melanoma in comparison to standard risk factors for risk assessment of subsequent metastases. *Eye*. 2007
67. Onken MD, Lin AY, Worley LA, Folberg R, Harbour JW. Association Between Microarray Gene Expression Signature and Extravascular Matrix Patterns in Primary Uveal Melanomas. *Am J Ophthalmol*. 2005;140:748-749

Part II

Gene expression profiling

Chapter 2

Expression profiling in uveal melanoma provides a strong marker for survival and reveals two regions on chromosome 3p related to prognosis

Chapter 2

Expression profiling in uveal melanoma provides a strong marker for survival and reveals two regions on chromosome 3p related to prognosis

Expression profiling in uveal melanoma provides a strong marker for survival and reveals two regions on chromosome 3p related to prognosis

Walter van Gils, Elisabeth M. Lodder, Hanneke W. Mensink, Emine Kiliç, Nicole C. Naus, Hennie T. Brüggenwirth, Wilfred van IJcken, Dion Paridaens, Gregorius P. Luyten and Annelies de Klein

Submitted for publication

Abstract

Introduction: Although studies on uveal melanoma (UM) revealed prognostic significance of chromosomal aberrations, they resulted in many classification errors in survival prediction. To obtain a robust prognostic classifier with strong predictive value and to gain further insight in genes responsible for poor prognosis, we have applied gene-expression profiling on tumors of UM patients of which extensive clinical, histopathological, cytogenetic and follow-up data is available.

Methods: Gene-expression profiles of 46 UMs were obtained using Affymetrix Hu133 2.0 Plus GeneChips. Data was analyzed with Omniviz and PAM software and validated with Real-Time PCR. The prognostic significance of UMs with specific molecular signatures was determined. Furthermore, LAP analysis resulted in the identification of differentially expressed chromosomal regions.

Results: The primary UMs could be classified in two distinct molecular classes with a strong prognostic value ($p<0.001$; hazard ratio 7.7). Classifier gene sets for micro-array class and disease-free survival were validated with Real-Time PCR and the predictive value of the UM class marker set was validated with gene expression profiles of tumors provided by other institutions, showing a sensitivity of 0.93 and specificity of 1.00 for class two tumors. Using a locally adaptive statistical procedure two regions on chromosome-arm 3p with decreased gene-expression in tumors with shorter disease-free survival were identified.

Discussion: Micro-array classification outperforms known prognostic indicators for UM, such as clinical, histopathological and cytogenetic parameters. In addition, the identified regions with lower expressed genes on chromosome-arm 3p could harbor genes, responsible for the poor prognosis of UM patients.

Introduction

Uveal melanoma (UM) is the most frequently occurring primary tumor in the eye, with an incidence of 7 per million every year in the Western world. Approximately 50% of all patients will die of metastatic disease. Several prognostic factors have been reported in UM of which the most promising are cytogenetic anomalies. Structural abnormalities on chromosomes 3, 6, and 8q have been linked to metastatic death [1, 2] and concurrent loss of chromosomes 1 and 3 strongly correlates with poor survival. [3] Although these associations are rather strong, they are not specific or sensitive enough for the prediction of clinical outcome. Gene expression profiling might therefore be very helpful to improve predictive clinical testing. Tschentscher *et al.* already identified a correlation between gene expression profile and monosomy of chromosome 3 in UMs. However, they did not investigate a direct relation between gene expression and patient survival. [4] Onken *et al.* identified two different classes in UM expression profiles that correlate with metastatic disease [5], but until now no gene expression classifier set has been identified specific for UM survival. Here, we describe gene expression analysis of a cohort of 46 primary UMs and compared these data with extensive follow-up data, clinical, pathological and cytogenetic parameters. We identified gene expression signatures for array class as well as survival. Additionally, using locally adaptive statistical procedure (LAP analysis), we identified several chromosomal regions with differential gene expression.

Materials and methods

Patients and tumor samples

Ciliary body or choroidal melanomas were collected from patients who underwent enucleation of the tumor-containing eye at the Erasmus MC Rotterdam or Rotterdam Eye Hospital. Informed consent was given prior to enucleation and the study was performed according to the tenets of the Declaration of Helsinki. Fresh tumor material was obtained within 1 hour after enucleation and processed for FISH and cytogenetic analysis as described. [6] A fraction of each tumor was snap-frozen and stored in liquid nitrogen. The remainder of the eye was embedded in paraffin and sections were stained with Hematoxylin and Eosin staining (H&E) for evaluation. Patients (n=49) were selected from our extensive database with information on follow-up and clinical, cytogenetical and histopathological parameters. The selection was made such that numerical abnormalities of chromosomes 1, 3, 6 and 8 were all represented in at least ten patients, as well as UMs with no numerical chromosome anomalies.

Preparation of RNA and hybridization

From fresh frozen tumor material, a 5μm section was made for H&E staining and depending on the size of the tumor 5 to 8 sections of 50μm were used for RNA isolation with RNA-Bee (Teltest, Friendswood, TX, USA) according to the manufacturers' instructions. RNA quantity (20 to 80μg in 20μl) was measured using the NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware USA) and quality was assayed on the Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA). The Affymetrix GeneChip One-Cycle Target Labeling and Control Reagents package (Affymetrix, Santa Clara, CA, USA) was used to create

biotinylated cRNA out of 5μg of total RNA. The cRNA was used for hybridization on Affymetrix Hu133 2.0 Plus GeneChips with the Affymetrix Fluidics 450 station and the arrays were scanned with the Affymetrix GeneChip Scanner 3000 (Affymetrix, Santa Clara, CA, USA), following the manufacturer's instructions.

Normalization

Only gene chips with at least 30% present calls and no signs of degradation, were analyzed. For normalization Variance-Stabilizing Normalization (VSN) [7] was used with the Affy, VSN and BioConductor package in the open-source statistical language R, version 2.2.1. The MisMatch (MM) intensities were ignored and by doing so only the Perfect Match (PM) intensities from the created CEL file were taken for normalization. This strategy circumvents systematical over-estimation of intensities by implementation of a heuristic, when PM intensities are smaller than MM probe intensities. For each probe set, the geometric mean of the hybridization intensities of all tumor samples was calculated. To reflect differential expression, the level of expression of each probe set in every sample was determined relative to its geometric mean and logarithmically transformed (on a base 2 scale) to ascribe equal weight to gene-expression levels with similar relative distances to the geometric mean.

Unsupervised clustering and visualization

The 528 annotations that had a standard deviation of at least 1.25 were selected for unsupervised hierarchical clustering with a K-means algorithm. Pearson's correlation was used for unsupervised heat mapping cluster analysis. Probe sets that were differentially expressed in at least one patient were selected for further analysis. Hierarchical cluster analysis, heatmap cluster analysis and visualization were all performed with Omniviz software (OmniViz, Inc., Maynard, MA, USA).

PAM analysis

Supervised class-prediction analyses were performed by applying PAM (Predictive Analysis of Micro-arrays) software in Microsoft Excel (PAM version 2.1 Excel plug-in). This program uses the method of the nearest shrunken centroids to identify a subgroup of genes that best characterizes a predefined class. [8] The prediction error was calculated by 10-fold cross validation within a training set (two thirds of the patients) followed by analysis of a test set (one third of the patients).

Validation by Real-Time PCR

Ten RNA samples were selected from the gene expression analysis cohort. From 1.5 μg of total RNA cDNA was synthesized using the I-Script cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). For Real-Time assays iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) was used. Using geNorm software and following the instructions suggested by Vandesompele *et al.* [9], we selected the three most stable reference genes from a panel of eight genes. Primers for the genes of interest (GOI) were selected with Primer Express Software v1.7 (Applied Biosystems, Foster City, CA, USA) and checked for the absence of SNPs in the primer

sequences with the Ensembl Genome Browser, release 41. Genes and primers are listed in table 1. The reactions were run on the ABI PRISM™ 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with a protocol of subsequently 95 °C for 10 min. and 40 cycles of 95 °C for 15 s, followed by 60 °C for 1 min.

Table 1. **PCR primers used in this study**

Gene	Forward primer sequence (5'-> 3')	Reverse primer sequence (5'-> 3')	Ratio class2/ class1	P-value
4-year DFS				
DKFZp434A202	CAACTTGTGATGGCCTGCCT	AATTGTTCTCATCCCAACTGCC	5,7	*0,079
FDFT1	AAGGTGATGCCCAAGATGGA	AGTTTTCAGGCTGCTGCTGAG	0,2	0,003
GCNT2	TGCCACGGCCACTATGTACA	TTTAAGTCTCCGTTTCCATAGATACAAA	0,3	0,000
ULK4	GTTCACCACAGAAGACTTCTCCTCT	GCTGACTCAGGTGTCCACTTGTAAT	**0,3	0,015
SRD5A2L2	CTGATCCGCTATGGGAAAACC	GAGTAAGGCACCAAAGCAGGAA	1,5	*0,316
FZD7	CTGCTTTGTTGGAAAGAGGGA	ACTGCTTGACAAGCACACCG	1,9	*0,076
RIMS2	TCGACAGATGTGCATTGGTTG	TGGACACAGATGGGCTTCTTTT	0,0	*0,353
FER1L3	GCTCCAGCCCCAGGACA	CCAGTGTTATTTTTATGTAAGGGTCACA	2,0	*0,235
SLC1A4	TCCTGGCTGTGGACTGGATT	CTTCCACATTCACCACCGTG	2,9	0,021
ZNF447	TCCCGGCACCTTCTGAGAC	CTCCTCTGTTCCAGAAACAGGG	0,2	0,001
UM Class				
MEGF10	GTGTGTAGCCACTGGGAAAGC	TGGGTATGACTCTTGCACAGTCA	0,0	0,002
LMCD1	AAAAGATGTCCCTGGGCCAG	CCAAACATGCCACACCTCTTG	0,1	0,002
ICRFp507I1077	TGCACACCGTCACACTGTTAAC	GCGGAAGATACAGCACTGCC	0,2	0,024
MGC9913	TCACCTACTTCAGTGTGGTTTCAAC	CCTGCACAGTGTCCAACGTT	0,0	*0,322
CUGBP2	ACCTGATGGGCTGAGTCGAG	TTGCCCTTGTAGAAAATGTGACA	5,3	0,007
RaLP	GTGAAGCTGTCCCCGGG	TGCCAAGGACTGTTGATAGGAA	0,0	0,000
HTR2B	CAAAATAACAACGAAACCAGAGGG	GTTTGAACTTGCATGCCAGAGAG	55,6	0,027
PDE3A	CAGTGAAAACTATACCTGTTCTGACTCTG	GAGGCAAACTCCTTCTCAGGC	0,1	0,002
SORBS2	CAAATGTGGAGCTGTCACTGAGA	TTTTACCTTCATACCAGTTTTGATCAA	0,1	0,005
OVOS2	ACAAGGGCCAAGTGATGAAGAC	ACAAGGTTGCTCTGCTCAACAG	0,0	0,003
UM Class add.				
PTGER4	TGCCGCTACAGACCCAGC	AATTGACCCCGGGAGTGG	16,5	0,004
HTRA1	AATTGTTTCGCAAGCTTCCG	CACTAGCCACCGGCACCT	5,0	0,002
HTATIP2	CAGCATGGCCGAAACAGAA	GCATCCTGAAGTCTTCCCGA	2,7	0,028
MTUS1	CAATTGCAAGAGCAGTTTGACAA	CTTAGAGGTTTCATGCGCAGC	0,1	0,002
TIMP3	CACCTGGGTTGTAACTGCAAGTC	CTGCAATTAGATAACAGGCAGCA	0,1	0,023
LIMS2	CAACAGCAAGCTCACCCTGA	GGCTTCATGTCGAACTCCACA	0,1	0,001
TBC1D8	GCCAAAGAAAAAGATAAAACTGAGAAA	TGGATCTTCATGGAACATACTGTACAG	0,8	*0,516
RAB31	TGAGACAAGTGCAAAAAATGCTATT	GGTGGGATCTGGCGGC	3,0	0,005
TNFRSF19	TGCATTCTGCAGCCAGTCTT	CATCTCCCCGGCTGGG	6,9	0,006
Other genes				
DDEF1	GTACCACTGCCCAGAAAAATCAA	CACTCGCCTCACTTTATTTTTCC	1,9	0,011
ENPP2	ACGAGGAGAGCTGCAATAGCTC	GTTCTTCTACCCATTTTGATTCGTC	0,1	0,029
CUL2	CAATGCAGAAAGACACACCACA	CAACTGCACTTCTAGTCTGCTCCAT	0,6	*0,194
LAMR1	AGGTCATGCCTGATCTGTACTTCTAC	GCAGCCTGCTCTTCTTTTTCA	0,2	0,001
FZD6	CCAAGAGCTTCAAAAAATCCTTCT	TGAAAATGAGTCCTGGGTCAATTA	0,3	*0,128
ID2	CCTAGAGGCGGAGTGATGAACT	AATCACAGCTACACGGGCG	3,2	*0,131
PHLDA1	GGGTTGTTGCAGCTCTGGA	CCCTTCCTCGGTGAGGATG	0,1	0,050
VBP1	GAGGCCGTGTTTGTGGAAGA	ATTCCCAGGCTGTTTCATGAA	0,7	*0,081
MITF	TTGATGGATCCTGCTTTGCA	ACAGGAGTTGCTGATGGTGAGG	0,4	0,043
ET2	TGAAGGGAAGGGCCAGG	TGATGTCCAAGTGGCAGAAGTAGA	0,0	0,000

*=not significant; **=result contradictory to gene expression profile

The categories 4-year DFS and UM Class contain the most differential expressed genes of the respective classifier sets. The category of UM Class add. consists of candidate tumor genes that belong to the UM Class predictor set but with a less strong differential expression. The category of Other genes harbors those genes that are described in other gene expression profiling studies on uveal melanoma. Ratio and p-value data are derived from Real-Time PCR analysis on 10 of the UMs in the micro-array cohort.

The efficiency of all assays was determined with a pool of cDNA of all ten selected samples in dilutions of 1, 10, and 100 times. A standard curve of mean Ct for two replicates at each dilution versus log10 amount of cDNA was determined. The efficiency of the reaction was calculated from the slope of this standard curve using the formula $E^{target} = 10^{-1/slope}$. This efficiency value was included in the calculation of the relative quantity in each sample for the respective assay. After selection of three reference genes from a panel of eight possible reference genes and determination of a normalization factor for all samples in geNorm, the normalized GOI expression levels were calculated by dividing the raw GOI quantities for each sample by their normalization factor. All reference genes, assayed genes and primers are presented in table 1, together with the results.

Identification of differentially expressed chromosomal regions

Differentially expressed chromosomal regions were identified with the computational tool called Locally Adaptive statistical Procedure (LAP) [10], which combines transcriptional data with structural information and estimates the differential expression of chromosomal regions accounting for variations in the distance between genes and gene density. LAP analysis was performed in R, version 2.2.1. Functions in R for implementing the LAP method were obtained from the website http://www.dpci.unipd.it/Bioeng/Publications/LAP.htm of the University of Padua.

Statistical analysis

Statistical analyses were performed with SPSS software, release 11.0. Odds-ratios with corresponding p-values were calculated to identify associations between the different parameters. Actuarial probabilities of disease-free survival (DFS; with an event defined as development of metastatic disease or death by disease) were estimated according to the Kaplan–Meier method. To examine the possibility that other clinical, histopathological or chromosomal variations affected the prognosis, we performed Cox proportional hazard analysis for each confounding variable. An effect was considered significant if the p-value was 0.05 or less.

Results

Selection

Patients (n=49) were selected from the tumor database such that numerical abnormalities of chromosomes 1, 3, 6 and 8 were all represented in at least ten patients, as well as UMs with no numerical chromosome anomalies. From frozen sections RNA was isolated, labeled and hybridized on gene chip arrays. After scanning the expression profiles were normalized and analyzed using different software packages.

Unsupervised clustering

After applying a hierarchical clustering algorithm on the 528 probe sets with the highest differential expression, the UMs clustered into two discrete molecular classes (UM class 1: n =23; UM class 2: n=23). This unsupervised clustering is shown in figure 1A. With Pearson's correlation analysis the same two blocks of related UMs were found (fig. 1B). Next to this correlation plot cytogenetic

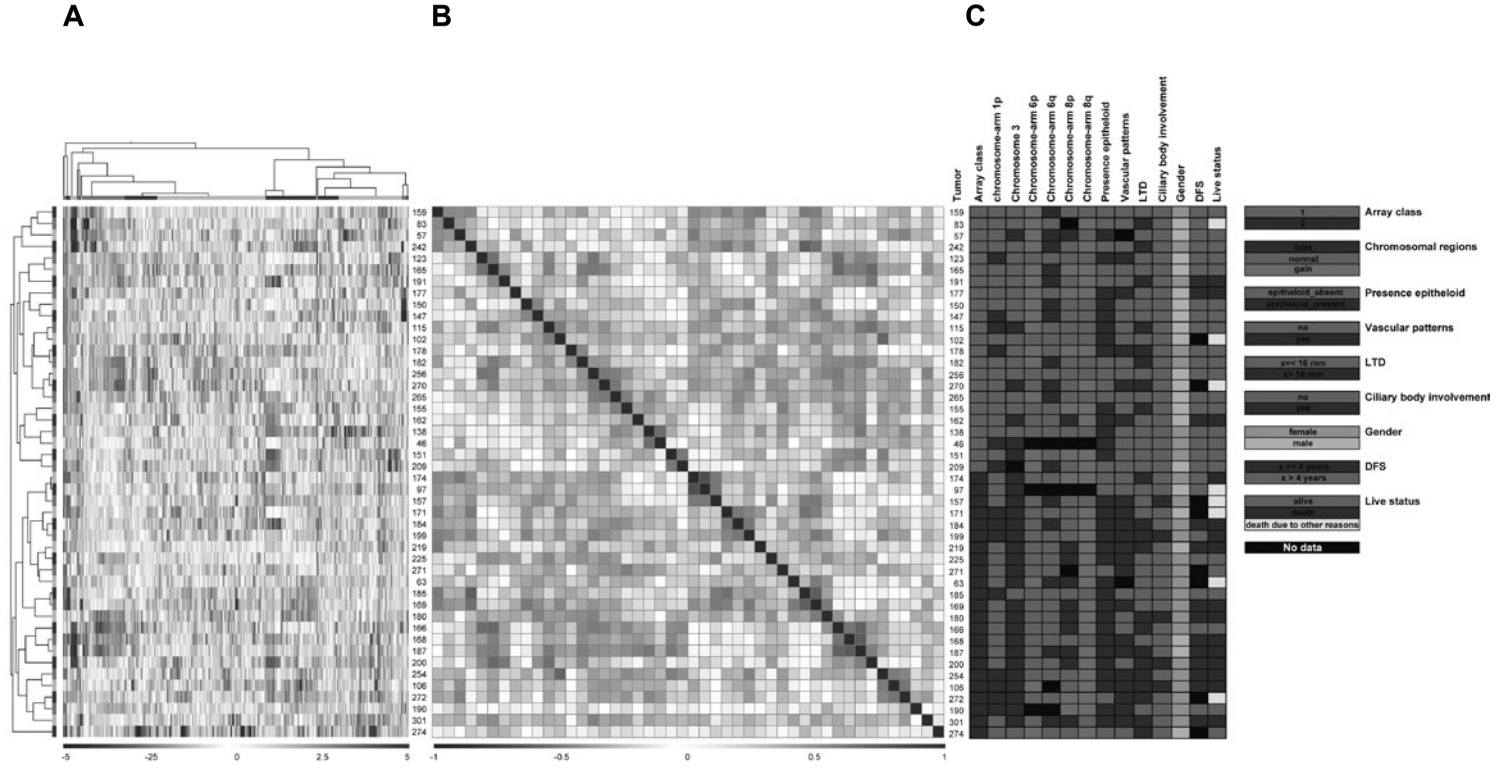


Figure 1. **Correlation View of Specimens from 46 UM patients involving 528 probes combined with data of follow-up, clinical, pathological and cytogenetic parameters. A)** Results of unsupervised hierarchical clustering of probes and tumors with a K-means algorithm is shown. **B)** The results of heat mapping cluster analysis with Pearson's correlation algorithm. The figure shows 2 clusters identified on the basis of the Correlation View, separated by a black line. **C)** Data of follow-up, clinical, pathological and cytogenetic parameters are shown schematically.
Color figure can be found on page 140.

and clinical prognostic markers as status of chromosomes 1, 3, 6p, 6q, 8p and 8q, presence of epitheloid cells, vascular patterns, largest tumor diameter (LTD), ciliary body involvement and gender are shown (fig.1C). Odds ratios of UM class with the different parameters were calculated and the corresponding p-values are displayed in table 2. The parameters 4-year DFS, monosomy of chromosome 3, gain of 6p and vascular patterns correlated with UM class with the highest significance. To assess the prognostic significance of UM class and the other parameters, we performed a multivariate analysis. The parameter UM class remained significant after correction for possible confounders and near significant when corrected for 8q-gain (p=0.053). Also chromosome 3 loss, gain of 6p, 8p loss, 8q gain, ciliary body involvement and vascular patterns were significant when corrected for almost all confounders (table 3).

Table 2. **Correlation between prognostic parameters for uveal melanoma in the analyzed cohort†‡**

	array class	4 year-survival	1p loss	3 loss	6p gain	6q loss	8q gain	age	epitheloid present	vascular patterns	LTD	involv. ciliary body	m/f
array class		0,003	NS	0,000	0,000	NS	0,026	0,018	NS	0,003	NS	0,018	0,008
4 year-survival	0,003		NS	0,016	0,036	NS	NS	NS	NS	0,001	NS	NS	NS
1p loss	NS	NS		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
3 loss	0,000	0,016	NS		0,003	NS	0,016	NS	0,025	0,002	0,001	NS	NS
6p gain	0,000	0,036	NS	0,003		0,003	NS	NS	0,038	0,002	NS	NS	NS
6q loss	NS	NS	NS	NS	0,003		NS	NS	NS	NS	NS	NS	NS
8q gain	0,026	NS	NS	0,016	NS	NS		NS	NS	NS	0,008	NS	NS
age	0,018	NS	NS	NS	NS	NS	NS		0,046	0,008	NS	NS	NS
epitheloid present	NS	NS	NS	0,025	0,038	NS	NS	0,046		0,025	NS	NS	NS
vascular patterns	0,003	0,001	NS	0,002	0,002	NS	NS	0,008	0,025		NS	NS	NS
LTD	NS	NS	NS	0,001	NS	NS	0,008	NS	NS	NS		NS	NS
involv. ciliary body	0,018	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS		0,030
m/f	0,008	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	0,030	

† The odds ratio's of the parameters were calculated with Chi-square analysis based on the available data of the 46 included uveal melanomas; correlation expressed in p-values.
‡ Dark gray boxes/NS: Not-significant ($p>0.05$); light gray boxes: $0.01 < p \leq 0.05$; white boxes: $p \leq 0.01$.

Gene expression signatures

To create classifier sets we applied PAM analysis for the categorical variables 4-year disease free survival (DFS) and UM class (table 4).The 61-gene annotation classifier set for 4-year DFS has a sensitivity of 0.89 and a specificity of 0.6. For UM class we selected 69 gene annotations that classified all samples correctly in training and cross validation. This 69 classifier set predicts with the maximum score of 1 for both sensitivity and specificity.

Real-Time PCR

The 8 most differentially expressed known classifying genes for UM class and the 4-year DFS classifier were tested in real-time PCR together with a candidate tumor suppressor and oncogenes that were present in the micro-array class and other genes that were described to be associated with prognosis in other gene expression profiling studies on UM. Ten patient samples were selected from the gene expression analysis cohort, five from UM class 1 with

predicted DFS >4 years and five from class 2 with predicted DFS ≤4 years. The results of real-time PCR are shown in table 1. For UM class the differential expression of the top 8 genes was confirmed in real-time PCR (HTATIP2 borderline-significant). For the 4-year DFS classifier, five out of nine assays indicated differential expression between the two different tumor groups, of which four were differentially expressed according to the gene expression signatures of both classes. One gene, ULK4, where a higher expression was expected according to the 4-year DFS gene expression signature, showed lower expression in the poor survival group, where a higher expression was expected according to the 4-year DFS gene expression signature. The 5 other assays did not show significant differences. We further tested expression of the tumor suppressor genes TIMP3 and MTUS1 that were present in both classifier sets, the candidate tumor suppressor gene MITF that acts in the development of melanocytes and optic cup-derived

Table 3. **Prognostic significance of different histopathological, clinical and chromosomal aberrations in the uveal melanoma cohort analyzed in this study on gene expression profile[†‡]**

	array class	4-year DFS class	1p loss	3 loss	6p gain	6q loss	8p loss	8p gain	8q gain	age	epitheloid present	vascular patterns	LTD	involv. ciliary body	m/f
p-value*	0,000	0,014	0,513	0,006	0,008	0,363	0,006	0,397	0,003	0,067	0,057	0,000	0,121	0,003	0,241
array class		0,952	0,593	0,287	0,577	0,369	0,255	0,461	0,102	0,535	0,160	0,027	0,872	0,142	0,829
4-year DFS class	0,942		0,139	0,247	0,465	0,773	0,392	0,983	0,424	0,472	0,809	0,207	0,061	0,291	0,491
1p loss	0,020	0,028		0,013	0,016	0,297	0,017	0,402	0,013	0,059	0,043	0,000	0,149	0,005	0,251
3 loss	0,037	0,477	0,339		0,073	0,871	0,280	0,378	0,040	0,726	0,233	0,008	0,332	0,066	0,570
6p gain	0,021	0,253	0,701	0,023		0,056	0,055	0,648	0,020	0,035	0,214	0,026	0,531	0,077	0,525
6q loss	0,002	0,156	0,351	0,015	0,003		0,008	0,461	0,012	0,023	0,152	0,006	0,158	0,072	0,395
8p loss	0,010	0,037	0,597	0,042	0,044	0,373		0,881	0,021	0,354	0,391	0,003	0,246	0,041	0,456
8p gain	0,002	0,073	0,536	0,010	0,018	0,275	0,019		0,003	0,031	0,130	0,002	0,106	0,020	0,214
8q gain	0,053	0,111	0,854	0,053	0,051	0,939	0,111	0,048		0,258	0,074	0,016	0,895	0,048	0,947
age	0,005	0,030	0,361	0,036	0,220	0,341	0,168	0,491	0,025		0,137	0,004	0,328	0,025	0,407
epitheloid present	0,004	0,123	0,204	0,036	0,046	0,781	0,046	0,430	0,012	0,180		0,008	0,076	0,032	0,340
vascular patterns	0,017	0,096	0,061	0,048	0,203	0,594	0,013	0,295	0,023	0,292	0,389		0,075	0,047	0,383
LTD	0,030	0,911	0,598	0,023	0,025	0,730	0,019	0,398	0,014	0,147	0,058	0,001		0,029	0,351
involv. ciliary body	0,006	0,062	0,342	0,037	0,041	0,542	0,025	0,629	0,018	0,192	0,165	0,004	0,509		0,613
m/f	0,003	0,079	0,523	0,022	0,028	0,477	0,017	0,355	0,015	0,116	0,093	0,002	0,197	0,021	

*Log-rank test

†Multivariate analysis using Cox proportional hazard analysis

‡Likelihood ratio test, p-value represented by color: Dark gray boxes $p>0.05$; light gray boxes: $0.01 < p \leq 0.05$; white boxes: $p \leq 0.01$

retinal pigment epithelial cells [11], PTGER4 that was described in the context of tumor formation in cervical cancer [12], the potential tumor suppressor HTRA1 [13], HTATIP2 that is associated with metastasis suppression [14], LIMS2 that inhibits cell spreading and migration when overexpressed [15], TBC1D8 that contains a TBC domain presumed to be involved in regulation of cell growth and differentiation [16], the ras binding protein family member RAB31 and tumor necrosis factor receptor superfamily member 19, TNFRSF19. The results of these genes are shown in table 1, referred to as UM class add.

Table 4**. Gene expression classifier sets created in this study.** The list contains all different genes in the classifiers, of which some are represented more than one time in the respective classifier.

4-year DFS	UM class	UM class adjusted
236081_at	237651_x_at	ARID5B
cDNA: FLJ21271 fis, clone COL01751	ARID5B	Clone IMAGE:5538654, mRNA
CITED1	C10orf86	COL9A3
Clone IMAGE:5301129, mRNA	C6orf149	CUGBP2
CNTN3	cDNA FLJ39164 fis, clone OCBBF2002656	ENPP2
DLC1	Clone IMAGE:5538654, mRNA	HSD17B8
ERBB3	COL9A3	HTATIP2
FBXL7	CUGBP2	HTR2B
FDFT1	ENPP2	HTRA1
FER1L3	ENTPD1	IL12RB2
FZD7	FBXO17	ITPR2
GCNT2	FLJ25477	MID1
Hypothetical LOC389634	GALNTL4	MTUS1
JAG1	GPR27	NEDD9
MBNL2	HSD17B8	PAM
MRNA; cDNA DKFZp434A202	HTATIP2	PHLDA1
MTUS1	HTR2B	PLN
RIMS2	HTRA1	PTGER4
RNF19	IL12RB2	RAB31
SEMA3C	ITPR2	SDC2
SLC1A4	LIMS2	SORBS2
SLC6A6	LMCD1	TBC1D8
SNCA	MEGF10	TIMP3
SRD5A2L	MGC40222	ZNF11B
SULF2	MGC9913	
TIMP3	MID1	
ULK4	Clone ICRFp50711077, mRNA	
UQCRB	MTUS1	
ZNF395	NEDD9	
ZNF447	OVOS2	
	PAM	
	PARP8	
	PCDH20	
	PDE3A	
	PHLDA1	
	PLN	
	PPM1K	
	PTGER4	
	RAB31	
	RaLP	
	ROPN1	
	ROPN1	
	ROPN1B	
	SDC2	
	SIPA1L2	
	SLC44A3	
	SORBS2	
	SYNPR	
	TBC1D8	
	TIMP3	
	TNFRSF19	
	ZNF11B	
	ZNF667	

Finally, we have analyzed a set of genes that were described to be associated with prognosis in other gene expression profiling studies on UM, namely DDEFF1, PHLDA1, FZD6, ENPP2, ID2, LAMR1, ET2, CUL2 and VBP1. [5, 17-20] Real-time PCR showed significant higher expression of DDEFF1 in class 2 tumors; PHLDA1, ENPP2 and LAMR1 were significantly lower expressed in class 2 tumors. The lower expression of CUL2 and VBP1 in class 2 tumors was not significant. FZD6, ID2 and ET2 were not conclusive because no expression was detected in 5 or more tumors. MITF showed significant lower expression in class 2 tumors.

Survival analysis

We evaluated the predictive value of the classifiers for patient survival using Kaplan-Meier life table analysis (data not shown). Survival analysis of all 46 patients showed that all patients in UM class 2 developed metastatic disease within 7 years of follow-up whereas 86% of the patients in class 1 did not suffer from metastatic disease in this time period (p-value 0.0004). Survival analysis of the groups predicted with the 4-year DFS classifier also showed no 7-year DFS survival in the shorter survival class versus 80% DFS of the patients in the longer survival class (p-value 0.04). Kaplan-Meier survival analysis was also performed for the parameters chromosome 3 loss, 6p gain, 8p loss, 8q gain, vascular patterns and ciliary body involvement, all significant after correcting for possible confounders. UM class outperformed all other cytogenetic, clinical and pathological prognostic factors (data not shown).

Analysis of other UM expression data sets

To evaluate the predictive value of the UM classifier we were able to use the expression profiles, kindly provided by Tschentscher *et al.* [4], and Onken *et al.* [5] All annotations in our classifier were present on the Affymetrix U133A and B chips that were used by the group of Onken *et al.*, but on the Affymetrix U95Av2 chips that were used by Tschentscher *et al*., only 32 gene annotations of our classifier could be identified. For this reason, we have analyzed the complete data set of the three different research groups with an adjusted UM classifier containing the 32 annotated genes, present in all datasets. As a training set, we have chosen the same set of tumor samples that was used earlier in the UM array class prediction, the remaining samples of our dataset were analyzed together with the other datasets. Results are visualized in figure 2. All 62 tumors in the test set were classified according to the results presented by Tschentscher *et al.* [4] and Onken *et al.* [5] with the exception of two samples (MM27 and M18672).

LAP analysis

The Locally Adaptive statistic Procedure (LAP analysis) was used to identify differentially expressed chromosomal regions for chromosome 3 status, UM class and 4-year DFS. LAP analysis using monosomy 3 as discriminator confirmed a lower expression of genes over the entire chromosome 3 in tumors with monosomy 3. These monosomy 3 tumors appear to have a decreased expression of genes in the telomeric 2 Mb of chromosome 1p36 and on chromosome 8q large regions of higher expression. Other regions of higher expression in tumors with chromosome 3 monosomy were detected on chromosomes 1q, 7, 9, 14, 15, 17, 20 and 21 (fig. 3A). In tumors with two copies of chromosome 3 a chromosome 6p region (15 Mb from 6p24.3 to 6p22.2) showed a significant higher expression.

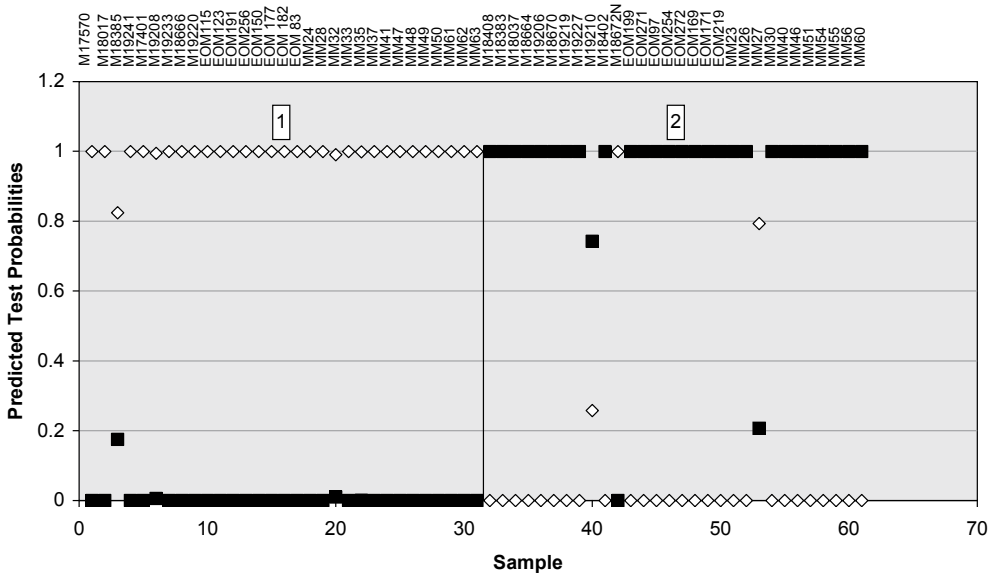


Figure 2. **Test Probabilities (threshold=2) in multiplatform PAM analysis of UMs with the 32 UM class gene classifier.** Tumors belonging to class 1, according to the literature, are placed on the left side, the tumors belonging to class 2 on the right side. The class 1 prediction probability of a sample with our 32-gene classifier is indicated with open diamonds, the class 2 probability with filled boxes. The samples were classified according to the highest prediction probability. Tumors: M1 numbers from data set Onken *et al.* [5]; MM numbers from Tschentscher *et al.* [4]; EOM numbers from our own data set. Class 1: sensitivity 1 and specificity 0,94; Class 2: sensitivity 0,93 and specificity 1.

LAP analysis on UM class revealed lower expression of almost the entire p-arm of chromosome 3 in class 1 tumors, whereas no significant differential expression of chromosome 3q was found between the two tumor classes. Additionally, a part of chromosome 6p (6 Mb from 6p24.3 to 6p23) showed significantly higher expression in class 1 and chromosome 8q had large regions of higher expression in class 2. Also higher expression of 1q in the poor prognosis class and other smaller regions of differential expression were found (fig. 3B).

Less differentially expressed chromosomal regions were found when comparing tumors from patients ≥4-year DFS with tumors from patients <4-year DFS. Remarkably, on the p-arm of chromosome 3, two smaller regions with lower expression in the short survival group were found; one region of 8 Mb ranging from the end of 3p23 to 3p25.3 and one region of 9 Mb ranging from 3p12 to 3p14.1. A small region of 0.5 Mb on chromosome 6p (6p23-6p24.1) showed significantly higher expression in tumors with a longer DFS. On chromosome 8, a large fraction of the p-arm showed significantly lower expression in the short DFS group whereas large regions on the q-arm were up regulated in this group. Additionally, regions of significantly higher expression in the short survival group are located on chromosome 10, 12, 13 and regions on chromosome 15 (fig. 3C).

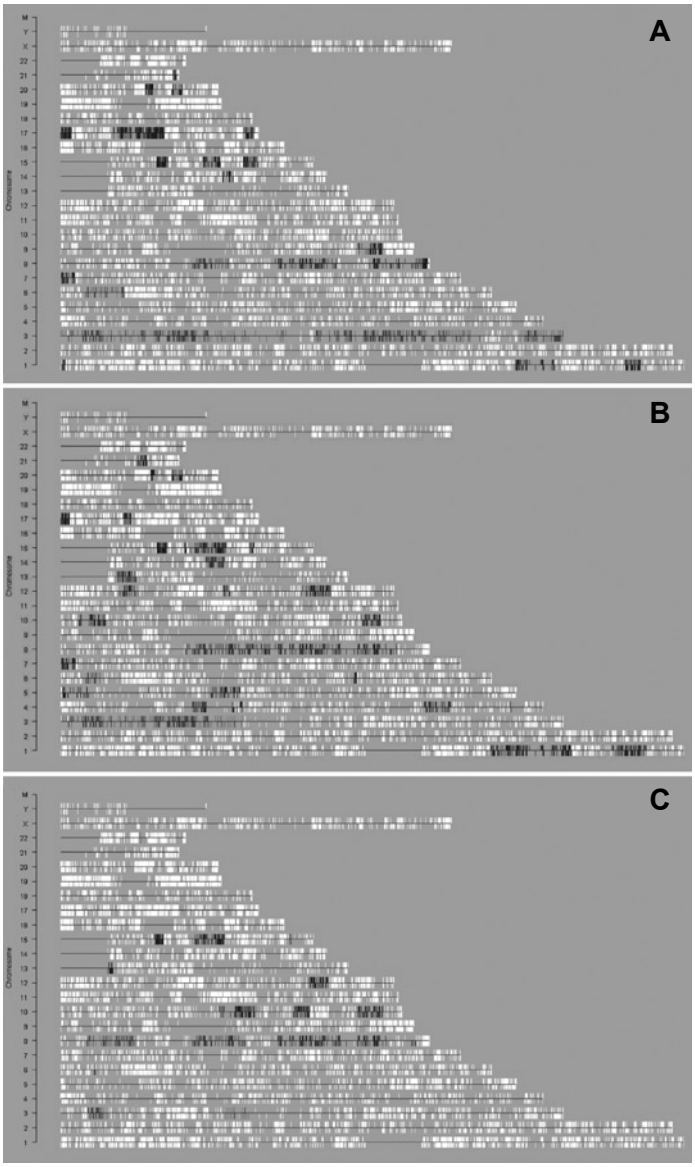


Figure 3. **Whole genome plot of the differentially expressed genes in LAP analysis at a q-value of <0.05.** The colored perpendicular lines represent the exact chromosomal locations, orientations, and up- (red) or down-regulation (green) states of the differentially expressed genes, while the white bars indicate locations and orientations of all probe sets in the microarray. Positions for both the sense and antisense strands are expressed in numbers of base pairs measured from the p (5' end of the sense strand) to q (3' end of the sense strand) arms; upper and lower bars stand for genes on sense and antisense strands, respectively. **A)** Differential expression of tumors with monosomy of chromosome 3 compared to tumors with normal copy numbers of chromosome 3. **B)** Differential expression of tumors in class 2 compared to tumors in class 1. **C)** Differential expression of tumors from patients with DFS ≤ 4 years compared to tumors from patients with DFS >4 years.
Color figure can be found on page 141.

Discussion

We provide results of gene expression profiling analysis of primary tumors from 46 selected patients with UM of all age groups and tumor sizes. Unsupervised clustering of the gene expression profiles grouped the tumors into two distinct classes that are strongly related with prognosis and 4-year DFS. Other parameters that have a strong correlation with this micro-array based classification are chromosome 3 loss, chromosome 6 abnormalities, vascular patterns and, surprisingly, gender. Chromosome 3 loss and the presence of closed vascular patterns, both more occurring in class 2, are known predictors of poor survival. [2, 21-23] The higher frequency of gains of chromosome 6p in class 1 corresponds with the earlier observed positive relation of prognosis with abnormalities of chromosome 6, resulting in a relative increase of 6p material compared to the q-arm [22]. We were able to confirm these findings in our analyzed cohort using Kaplan-Meier survival analysis. Array class, chromosome 3 loss, 6p gain, 8p loss, 8q gain, vascular patterns and ciliary body involvement were strongly related with prognosis in multivariate analysis using Cox proportional hazard analysis. Due to the tumor selection the results differ from previous results by our group [24] on a larger cohort of UMs. In this latter study, chromosome 3 loss and 8q gain were also significant after correcting for most confounding parameters, but LTD was also significant. In contrast, 6p gain, 8p loss, presence of vascular patterns and ciliary body involvement were not significant after correcting for confounders in that study. The relatively small size of our currently analyzed cohort and an apparent overrepresentation of tumors with structural abnormalities on chromosomes 1, 3, 6 and 8 could explain this discrepancy.

Because of the very strong relation of micro-array based UM class with survival, that outperforms all known prognostic clinical, histopathological and cytogenetic parameters in the studied patients, we used PAM analysis to obtain a gene signature that accurately predicts for UM micro-array class. The created 69-gene classifier flawlessly predicted for UM class. We also developed a gene-expression signature for 4-year DFS. This 61-gene classifier set had a sensitivity of 0.89 and a specificity value of 0.60. The low specificity value could be a result of the low number of patients in the poor survival group in the test set (only four patients), thereby increasing the influence of one misclassified patient. However, when the tumors are classified with this signature, this results in two groups of patients of which one has a survival rate of 80% after 120 months of follow-up, whereas all patients in the other group suffered from metastatic disease in this period ($p=0.014$). This classification is also outperformed by UM based tumor classification (86% vs. 0% of the patients with DFS after 120 months; $p<0.001$; hazard ratio 7.7). We confirmed differential expression in the 8 most differentially expressed genes of the array class classifier, indicating that the micro-array findings are truly representative for the UM samples. Additionally, we confirmed 7 potentially interesting tumor suppressor genes and oncogenes, based on literature, that were also represented by our classifier and we analyzed a set of genes that were previously described to be differentially expressed in micro-array analyses on UM. *PHLDA1*, *FZD6* and *ENPP2* form the optimal three-gene set that was used for additional screening of UMs with Real-time PCR by Onken *et al.* and loss of *ID2* expression, another highly differentially expressed gene, has been shown to increase anchorage-independent growth in UM. [5, 25] *DDEFF1* was described by Ehlers *et al.* [17] to be the gene that correlates most strongly with gain of chromosome 8q in their gene expression analysis. In our dataset *DDEFF1*, *PHLDA1*, *ENPP2* and *ID2* are also differentially expressed, corresponding to the data of Onken *et al.* and

Ehlers *et al.* The gene *PHLDA1* (pleckstrin homology-like domain, family A, member 1) is of particular interest, since it is also represented in the gene expression classifier set Tschentscher *et al.* [4] and there is strong evidence that reduced expression of this apoptosis related gene is important in breast cancer progression. [26]

To assess the robustness of micro array classification of UMs, we also applied our UM class gene-expression signature to UMs that had been analyzed by two other institutions [4, 5]. Our UM classification was in agreement with their earlier predicted class, with the exception of two tumors, M18672 described by Tschentscher *et al.* [4] and MM27 described by Onken *et al* [5]. In the original studies, both tumors differed the most from all other tumors in their respective tumor class and clustered to the class containing predominantly tumors with monosomy of chromosome 3, whereas both tumors showed retention of both alleles of this chromosome [4, 5]. This indicates that these two tumors are difficult to classify based on unsupervised clustering with gene-expression profiling and that with our classifier they were classified in concordance with their chromosome 3 status. These data underline the robustness of micro-array based UM classification, that was also reported by Onken *et al.*, Worley *et al.* and Petrausch *et al.* [5, 27, 28]

LAP analysis was used to determine whether the differential expression was associated with specific genomic regions. We used chromosome 3 status, UM class and 4-year DFS as discriminatory factors in the LAP analysis.
As could be expected, tumors with monosomy 3 showed lower expression of all the chromosome 3 genes. The results of LAP analysis on UM class showed that only chromosome-arm 3p and not 3q is significantly down regulated in the poor survival group.
In both these LAP analysis a large part of the genes on chromosome 8q are up-regulated and down-regulation of genes in a region on chromosome 6p was observed.
Interestingly, LAP analysis with 4-year DFS as discriminator revealed in the group with a shorter DFS two small regions of down-regulated genes on chromosome 3, one region of 8 Mb ranging from the end of 3p23 to 3p25.3 and one region of 9 Mb ranging from 3p12 to 3p14.1. These regions overlap with the two regions, 3p25-26 and 3p11-14, identified by Cross et al. [29] using micro satellite analysis. Surprisingly no regions on chromosome-arm 3q showed differential expression. The adverse effect on survival of monosomy of chromosome 3 is probably caused by decreased expression of tumor suppressor genes located on the identified regions on 3p.
An interesting candidate gene in the most proximal region is *MITF*. This gene is located at 3p13 translocation breakpoint of a t(3;14) reported in UM [30] and is possible disrupted by this translocation. Real-Time PCR analysis showed that *MITF* expression was significantly lower in class 2 tumors (table 1). *MITF* acts in the development of various cell types, including neural crest-derived melanocytes and optic cup-derived retinal pigment epithelial cells. [31] It transactivates the tyrosinase gene, a key enzyme for melanogenesis, known to be critically involved in melanocyte differentiation. [11] However, no correlation between *MITF*-positivity and the parameters cell type, largest tumor diameter, sclera invasion and mitotic figures was observed in UM as reported by Mouriaux *et al*. [32]. In melanoma cell lines in which the gene was repressed, induced expression of *MITF-M* showed growth-inhibitory effects and led to a change from epitheloid toward a spindle-cell type *in vivo*. [33] This suggests that decreased expression of *MITF* would lead to a more epitheloid phenotype, which is related with poor prognosis in UM. Interestingly, although

MITF was also significantly lower expressed in the shorter DFS group, its expression was not significantly lower in tumors with an epitheloid cell type (data not shown). Our data point to association of *MITF* expression and survival and it would be interesting to corroborate this in a larger UM cohort. Other candidate tumor suppressor genes on the proximal 3p-region are the Tata element Modulatory Factor 1 (*TMF1)* and EGF receptor antagonist leucine-rich repeats and immunoglobulin-like domains 1 (*LRIG1)*. *TMF* is a transcription factor that likely regulates the expression of genes via the TATA element. [35] Down-regulation of *LRIG1* increases cell-surface EGF receptor levels, enhances activation of downstream pathways, and stimulates epidermal cells proliferation. [36] The other region, ranging from 3p23 to 3p25.3, harbors multiple candidate TSGs, of which *XPC*, *WNT7A*, *PPARG* and *TIMP4* are the most promising genes. XPC is a well-described DNA repair gene that functions via nucleotide excision repair (NER) and is linked to type C xeroderma pigmentosum that concurs with a high incidence of malignant melanoma. [37] WNT7A belongs to the Wnt gene family, which genes are implicated in oncogenesis and several developmental processes. [38] Peroxisome proliferator-activated receptor g (PPARG) is a nuclear hormone receptor that acts in differentiation of adipocytes in particular, although this gene is also expressed in other tissue types. Activation of PPARG in different cancer cell types induces cell growth inhibition and differentiation. [39] This inhibitory effect would be impaired by decreased expression of this gene. *TIMP4* belongs to the tissue inhibitors of metalloproteinases (TIMPs) that inhibit matrix metalloproteinases (MMPs), which are involved in degradation of the extracellular matrix. Overexpression of recombinant *TIMP4* in breast cancer cells inhibited the invasion potential of the cells *in vitro*. [40] Decreased expression of *TIMP4* might therefore lead to an increased invasive potential.

The selective decreased expression of genes in the two regions on chromosome 3p in poor prognosis uveal melanomas could, besides loss of chromosomal DNA, also be caused by epigenetic mechanisms like methylation of gene promoter regions. Promoter methylation has been identified as the mechanism responsible for lower expression of the classifier gene *TIMP3* in uveal melanoma. [20] In addition, methylation of *RASSF1A* has been shown to be a sensitive prognostic marker in uveal melanoma. [41] Lower expression of blocks of genes in those regions on 3p in the absence of deletions could indicate epigenetics as an alternative mechanism of regulation of gene expression. It is therefore interesting to look at the methylation status of classifier genes in tumors of patients in the poor prognosis UM class without chromosome 3 aberrations. This could explain the presence of two UM with normal copy numbers of chromosome 3 in our UM class 2 group and the misclassification of the MM27 and M18672 from the Tschentscher [4] and Onken [5] cohorts, respectively.

In summary, we have created a very robust gene expression signature predicting for micro-array class that classifies correctly tumors analyzed on different micro-array platforms. Survival analysis with our extensive follow-up data revealed a very strong relation between this classification and DFS that is superior to all other known UM prognostic classifiers. Recently, the superiority of this classification was confirmed by Worley *et al.* [27] and Petrausch *et al.* [28] This offers great perspectives for predictive screening, and prospective studies using UM biopsies as source of RNA are being set up. [42]

Furthermore, the genes in the micro-array classifier set that are located at the plasma membrane

are especially potential markers for prognostic screening. There are 9 such genes in the classifier, of which 4 (*ENPP2*, *ENTPD1*, *IL12RB2* and *TBC1D8*) have lower and 5 (*HTR2B*, *PAM*, *PTGER4*, *SDC2* and *TNFRSF19*) have higher expression levels in class 2 tumors. Immunohistochemical studies might reveal their value for prognostic screening and their potential use in treatment strategies.

Using expression profiling, we have identified two small regions on chromosome 3 of which the lower expression correlates with poor survival. If indeed epigenetic mechanisms as methylation are the cause of this decreased expression, it offers an excellent starting point for a better predictive, non-invasive test for the early detection of metastatic disease.

References

1. Prescher G, Bornfeld N, Friedrichs W, Seeber S, Becher R. Cytogenetics of twelve cases of uveal melanoma and patterns of nonrandom anomalies and isochromosome formation. *Cancer Genet Cytogenet*. 1995;80:40-46
2. Sisley K, Rennie IG, Parsons MA *et al.* Abnormalities of chromosomes 3 and 8 in posterior uveal melanoma correlate with prognosis. *Genes Chromosomes Cancer*. 1997;19:22-28.
3. Kilic E, Naus NC, van Gils W *et al.* Concurrent loss of chromosome arm 1p and chromosome 3 predicts a decreased disease-free survival in uveal melanoma patients. *Invest Ophthalmol Vis Sci*. 2005;46:2253-2257
4. Tschentscher F, Husing J, Holter T *et al.* Tumor classification based on gene expression profiling shows that uveal melanomas with and without monosomy 3 represent two distinct entities. *Cancer Res*. 2003;63:2578-2584
5. Onken MD, Worley LA, Ehlers JP, Harbour JW. Gene expression profiling in uveal melanoma reveals two molecular classes and predicts metastatic death. *Cancer Res*. 2004;64:7205-7209
6. Naus NC, Zuidervaart W, Rayman N *et al.* Mutation analysis of the PTEN gene in uveal melanoma cell lines. *Int J Cancer*. 2000;87:151-153.
7. Huber W, von Heydebreck A, Sultmann H, Poustka A, Vingron M. Variance stabilization applied to microarray data calibration and to the quantification of differential expression. *Bioinformatics*. 2002;18 Suppl 1:S96-104
8. Tibshirani R, Hastie T, Narasimhan B, Chu G. Diagnosis of multiple cancer types by shrunken centroids of gene expression. *Proc Natl Acad Sci U S A*. 2002;99:6567-6572
9. Vandesompele J, De Preter K, Pattyn F *et al.* Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol*. 2002;3:RESEARCH0034
10. Callegaro A, Basso D, Bicciato S. A locally adaptive statistical procedure (LAP) to identify differentially expressed chromosomal regions. *Bioinformatics*. 2006;22:2658-2666
11. Tachibana M, Takeda K, Nobukuni Y *et al.* Ectopic expression of MITF, a gene for Waardenburg syndrome type 2, converts fibroblasts to cells with melanocyte characteristics. *Nat Genet*. 1996;14:50-54
12. Sales KJ, Katz AA, Davis M *et al.* Cyclooxygenase-2 expression and prostaglandin E(2) synthesis are up-regulated in carcinomas of the cervix: a possible autocrine/paracrine regulation of neoplastic cell function via EP2/EP4 receptors. *J Clin Endocrinol Metab*. 2001;86:2243-2249
13. Chien J, Staub J, Hu SI *et al.* A candidate tumor suppressor HtrA1 is downregulated in ovarian cancer. *Oncogene*. 2004;23:1636-1644
14. Shtivelman E. A link between metastasis and resistance to apoptosis of variant small cell lung carcinoma. *Oncogene*. 1997;14:2167-2173
15. Zhang Y, Chen K, Guo L, Wu C. Characterization of PINCH-2, a new focal adhesion protein that regulates the PINCH-1-ILK interaction, cell spreading, and migration. *J Biol Chem*. 2002;277:38328-38338
16. Richardson PM, Zon LI. Molecular cloning of a cDNA with a novel domain present in the tre-2 oncogene and the yeast cell cycle regulators BUB2 and cdc16. *Oncogene*. 1995;11:1139-1148
17. Ehlers JP, Worley L, Onken MD, Harbour JW. DDEF1 is located in an amplified region of chromosome 8q and is overexpressed in uveal melanoma. *Clin Cancer Res*. 2005;11:3609-3613
18. Onken MD, Lin AY, Worley LA, Folberg R, Harbour JW. Association Between Microarray Gene Expression Signature and Extravascular Matrix Patterns in Primary Uveal Melanomas. *Am J Ophthalmol*. 2005;140:748-749
19. Zuidervaart W, van der Velden PA, Hurks MH *et al.* Gene expression profiling identifies tumour markers potentially playing a role in uveal melanoma development. *Br J Cancer*. 2003;89:1914-1919
20. van der Velden PA, Zuidervaart W, Hurks MH *et al.* Expression profiling reveals that methylation of TIMP3 is involved in uveal melanoma development. *Int J Cancer*. 2003;106:472-479

21. Prescher G, Bornfeld N, Hirche H *et al.* Prognostic implications of monosomy 3 in uveal melanoma. *Lancet*. 1996;347:1222-1225
22. White VA, Chambers JD, Courtright PD, Chang WY, Horsman DE. Correlation of cytogenetic abnormalities with the outcome of patients with uveal melanoma. *Cancer*. 1998;83:354-359
23. Foss AJ, Alexander RA, Hungerford JL *et al.* Reassessment of the PAS patterns in uveal melanoma. *Br J Ophthalmol*. 1997;81:240-246; discussion 247-248
24. Kilic E, van Gils W, Lodder E *et al.* Clinical and cytogenetic analyses in uveal melanoma. *Invest Ophthalmol Vis Sci*. 2006;47:3703-3707
25. Onken MD, Ehlers JP, Worley LA *et al.* Functional gene expression analysis uncovers phenotypic switch in aggressive uveal melanomas. *Cancer Res*. 2006;66:4602-4609
26. Nagai MA, Fregnani JH, Netto MM, Brentani MM, Soares FA. Down-regulation of PHLDA1 gene expression is associated with breast cancer progression. *Breast Cancer Res Treat*. 2007
27. Worley LA, Onken MD, Person E *et al.* Transcriptomic versus chromosomal prognostic markers and clinical outcome in uveal melanoma. *Clin Cancer Res*. 2007;13:1466-1471
28. Petrausch U, Martus P, Tonnies H *et al.* Significance of gene expression analysis in uveal melanoma in comparison to standard risk factors for risk assessment of subsequent metastases. *Eye*. 2007
29. Cross NA, Ganesh A, Parpia M *et al.* Multiple locations on chromosome 3 are the targets of specific deletions in uveal melanoma. *Eye*. 2006;20:476-481
30. Blasi MA, Roccella F, Balestrazzi E *et al.* 3p13 region: a possible location of a tumor suppressor gene involved in uveal melanoma. *Cancer Genet Cytogenet*. 1999;108:81-83
31. Fuse N, Yasumoto K, Takeda K *et al.* Molecular cloning of cDNA encoding a novel microphthalmia-associated transcription factor isoform with a distinct amino-terminus. *J Biochem (Tokyo)*. 1999;126:1043-1051
32. Mouriaux F, Vincent S, Kherrouche Z *et al.* Microphthalmia transcription factor analysis in posterior uveal melanomas. *Exp Eye Res*. 2003;76:653-661
33. Selzer E, Wacheck V, Lucas T *et al.* The melanocyte-specific isoform of the microphthalmia transcription factor affects the phenotype of human melanoma. *Cancer Res*. 2002;62:2098-2103
34. Garcia JA, Ou SH, Wu F *et al.* Cloning and chromosomal mapping of a human immunodeficiency virus 1 "TATA" element modulatory factor. *Proc Natl Acad Sci U S A*. 1992;89:9372-9376
35. Jensen KB, Watt FM. Single-cell expression profiling of human epidermal stem and transit-amplifying cells: Lrig1 is a regulator of stem cell quiescence. *Proc Natl Acad Sci U S A*. 2006;103:11958-11963
36. Nusse R, Varmus HE. Wnt genes. *Cell*. 1992;69:1073-1087
37. Li L, Bales ES, Peterson CA, Legerski RJ. Characterization of molecular defects in xeroderma pigmentosum group C. *Nat Genet*. 1993;5:413-417
38. Ikegawa S, Kumano Y, Okui K *et al.* Isolation, characterization and chromosomal assignment of the human WNT7A gene. *Cytogenet Cell Genet*. 1996;74:149-152
39. Mueller E, Smith M, Sarraf P *et al.* Effects of ligand activation of peroxisome proliferator-activated receptor gamma in human prostate cancer. *Proc Natl Acad Sci U S A*. 2000;97:10990-10995
40. Wang M, Liu YE, Greene J *et al.* Inhibition of tumor growth and metastasis of human breast cancer cells transfected with tissue inhibitor of metalloproteinase 4. *Oncogene*. 1997;14:2767-2774
41. Maat W, van der Velden PA, Out-Luiting C *et al.* Epigenetic inactivation of RASSF1a in uveal melanoma. *Invest Ophthalmol Vis Sci*. 2007;48:486-490
42. Onken MD, Worley LA, Davila RM, Char DH, Harbour JW. Prognostic testing in uveal melanoma by transcriptomic profiling of fine needle biopsy specimens. *J Mol Diagn*. 2006;8:567-573

Part III

Clinical and cytogenetic analyses and relations with prognosis

Chapter 3

Clinical and cytogenetic analyses in uveal melanoma

Chapter 3

Clinical and cytogenetic analyses in uveal melanoma

E. Kilic, W. van Gils, E. Lodder, H.B. Beverloo, M.E. van Til, C.M. Mooy, D. Paridaens, A. de Klein, G.P.M. Luyten

Invest Ophthalmol Vis Sci. 2006 Sep;47(9):3703-7.

Abstract

Purpose: Uveal melanoma is one of the most frequent primary intraocular malignancies in the western world. Cytogenetically these tumors are characterized by typical chromosomal losses and gains, such as loss of chromosome 1p, 3, 6q and gain of chromosome 6p, and 8q. Whereas most studies focus on known aberrations we characterized cytogenetic changes and correlated them with clinical and histopathological parameters.

Patients and methods: Karyotypes of 74 primary uveal melanomas were analyzed with respect to the presence or absence of chromosomal gains and losses. In the analysis classical clinical and histopathological parameters were analyzed together with the chromosomal aberrations.

Results: At a median follow up of 43 months 34 patients had died or were suffering from metastatic disease. Clonal chromosomal abnormalities were present in 59 tumors. The most frequent chromosomal abnormalities involved chromosome 8 (53%), loss of chromosome 3, p-arm (41%) and q-arm (42%), partial loss of chromosome 1p (24%) and abnormalities of chromosome 6, which resulted in gain of material of 6p (18%) and/or loss of 6q (28%). Less frequent aberrations were abnormalities of chromosome 16, in particular loss of chromosome 16q (16%). In the univariate analysis loss of chromosome 3, largest tumor diameter, gain of 8q and mixed/epithelioid cell type in the tumor compared to tumors without these chromosomal changes or with a spindle cell type was associated with a decreased disease free survival. When corrected for another confounding variable significance of gain of 8q and cell type was decreased, whereas the significance of loss of chromosome 3p or 3q and largest tumor diameter remained.

Conclusions: Monosomy 3 and largest tumor diameter are the most significant in determining survival for uveal melanoma patients. Abnormalities of chromosome 16q are relatively common in uveal melanoma, but are not associated with survival or other cytogenetic or histopathological parameters.

Introduction

Uveal melanoma (UM) is the most common primary intra-ocular tumor in the western world, affecting approximately 7 per million people each year. Tumorigenesis and progression of cancer is in general preceded by the occurrence of genetic changes in normal cells. [1] In this respect UMs are quite homogenous with a few tumor specific cytogenetic aberrations. Some of these aberrations are correlated with the metastatic potential of the tumor resulting in metastatic disease followed by death. Recurrent aberrations in UM concern loss of chromosome 1p, monosomy of chromosome 3, loss of chromosome 6q and 8p, gain of chromosome 6p and 8q.

Loss of chromosome 1p was observed in metastases [2] and concurrent loss of chromosome 1p and 3 is associated with decreased survival. [3,4] Furthermore, monosomy 3 is considered to be an early event in UM and several studies have shown that it is a strong predictor for survival. [5-7] Loss of chromosome 3 is frequently associated with amplification of chromosome 8q, often seen as an isochromosome 8q. [8,9]
Recently, Hoglund *et al*. elucidated a common genetic pathway for both uveal as cutaneous melanoma.[10] Monosomy 3 occurs probably as an early event and loss of chromosome 1p, 8p and gain of 8q as secondary events.

Regions of chromosomal loss are thought to harbor tumor suppressor genes and regions of gain oncogenes. Previous cytogenetic analyses focus in general on the known aberrations. In this study we performed cytogenetic analysis on short-term cell cultures of fresh tissue from 74 primary UMs to characterize all chromosomal changes and correlate these changes with clinical and histopathological parameters. Significant prognostic parameters for UM, at high-risk for metastases, were identified.

Material and methods

Patients and tumor samples

From March 1992 to April 2003, we collected tumor material of patients who underwent enucleation for ciliary body or choroidal melanoma. 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 obtained within 1 hour after enucleation and processed as described before. [3] Conventional histopathologic examination was performed on all tumors and confirmed the origin of the tumor. Cytogenetic studies were also carried out on stimulated peripheral blood samples of each patient to exclude the presence of congenital chromosome abnormalities. Follow-up data from time of diagnosis till the end of the study in December 2005 were obtained by reviewing patient's charts and contacting their general physician.

Cytogenetic analysis

Chromosome preparations were made according to standard procedures and stained with acridine orange or atebrine to obtain R or Q banding. Cytogenetic abnormalities were described in accordance with the ISCN (1995).[11]

Data classification

Based on the cytogenetic analysis tumors were classified for gain and or loss for all chromosomal regions, p-arm or q-arm. When different subclones were identified, only the cytogenetic findings of the largest clone were classified. Chromosomal regions with loss in more than 10% of all tumors and gain in more than 15% of all tumors were included for analysis. Tumors were identified as small (<12mm) and large (> 12mm).

Statistical analysis

The primary end point for disease free survival (DFS) was the time to development of metastatic disease, whereby death due to other causes was treated as censored. The influence of single prognostic factors on DFS was assessed using the log rank test (for categorical variables) or Cox proportional hazard analysis (for continuous variables) and Kaplan-Meier curves were made to illustrate the differences in survival. To examine the possibility that other clinical, histopathological or chromosomal variations may affect the prognosis we performed Cox proportional hazard analysis for each confounding variable. An effect was considered significant if the p-value was 0.05 or less. The odds-ratios with corresponding p-values were calculated to identify association between the different parameters. The statistical analyses were performed with the SPSS-11 software.

Results

Patients

From March 1992 to April 2003 152 patients were available for this study, but chromosome analysis was successfully performed in 74 cases. The clinical and histopathological features of the 74 primary UMs are listed in the supplementary information. The median age of the patients at time of enucleation was 60 years (range 21-87 years), 29 women and 45 men. One patient was lost to follow-up after 27 months. At the end of follow-up time 31 patients had died of melanoma-related disease, 3 patients were diagnosed with metastases, 9 patients had died due to other causes and 31 patients were still alive without metastases. The median follow-up time was 42.8 months (range 6.4 - 164.4 months).

Histopathology

All tumors were confirmed histopathologically as UM. Based on their cell type 16 tumors were classified as epithelioid cell type, 24 as mixed cell type and 34 tumors as spindle cell type. The mean tumor diameter and thickness were 13.2 mm (range 6-19 mm) and 8.4 mm (range 2-22 mm), respectively. Four tumors were located in the ciliary body and 70 were located in the choroid. From the tumors located in the choroid four showed involvement of the ciliary body.

Cytogenetic

Seventy-four UMs were analyzed for cytogenetic changes (see supplementary information) and classified for gain and loss for all chromosomal regions (Table 1). Clonal chromosomal abnormalities were present in 59 tumors. The most frequent chromosomal abnormality involved

chromosome 8, trisomy of chromosome 8 or gain of material from 8q, most often in the form of an i(8q) (53%). Other abnormalities involved loss of chromosome 3, p-arm (41%) and q-arm (42%). Partial loss of chromosome 1p (24%) and abnormalities of chromosome 6, resulting in gain of material of 6p (18%) and/or loss of 6q (28%). Other less frequent aberrations were abnormalities of chromosome 16, in particular loss of chromosome 16q (16%) (Figure 1).
Other chromosomal aberrations, such as loss of 6p, 9p, 15p, 15q, 21p, 22p and gain of 2p, 2q, 7q, 9p, 11q were present but did not reach the 10%.

Statistical analysis

Univariate analysis was performed for all clinical, histopathological and cytogenetic parameters (Table 2, Figure 2). Univariate analysis of the single prognostic factors showed significant lower disease free survival (DFS) for patients with loss of chromosome 3, largest tumor diameter, gain of 8q and with a mixed/epithelioid cell type in the tumor compared to tumors without these chromosomal changes or with a spindle cell type. Other potential prognostic factors such as gender, age at time of diagnosis and tumor location (i.e. involvement of ciliary body) did not reach significance. Also chromosomal changes such as loss of chromosome 1p, gain of chromosome 6p and loss of chromosome 6q were not significantly associated with disease free survival. To examine the possibility that other clinical, histopathological or chromosomal variations may affect the prognosis we performed Cox proportional hazard analysis for each confounding variable (Table 2). Parameters presented in the columns are the investigated prognostic parameters; in the rows the same parameters resemble the confounders with a possible modifying effect. Significance of loss of chromosome 3p/3q did not alter after correcting for the possible confounders. A similar pattern was observed for largest tumor diameter and cell type. Odds ratios were calculated to identify association between the different parameters (Table 3). Associations were shown for loss of chromosome 3 with gain of 8q, loss of chromosome 8p, vascular patterns and largest tumor diameter (>12 mm), and a weak association with mixed/epithelioid cell type. Presence of vascular patterns and largest tumor diameter (>12 mm) showed also association with gain of chromosome 8q. Associations were also present for loss of chromosome 1p with loss of 16q and loss of chromosome 3p, and weak association with cell type, vascular patterns largest tumor diameter, chromosome 3q loss and 8q gain. Loss of chromosome 6q was weakly associated with gain of chromosome 8q. Loss of chromosome 16q was weakly associated with gain of chromosome 8p.

Discussion

By means of karyotyping we have analyzed chromosomal aberrations in UM. Previous reports have revealed that abnormalities of chromosome 1, 3, 6 and 8 occur in a nonrandom fashion in UM. Some of these tumor specific aberrations have been associated with the metastatic potential of the tumor. In this study loss of chromosome 1p, chromosome 3, aberrations of chromosome 6, 8 and 16 are most often encountered. Furthermore, we have demonstrated that tumors with abnormalities of chromosome 3, gain of chromosome 8q, epithelioid/mixed cell type and a larger tumor diameter are strongly associated with a poor prognosis.
In UM numerous parameters have been used to predict survival, with the conventional parameters

Table 1. **Recurrent changes in karyotype of primary uveal melanoma**

Loss and gain >10% of all tumors	**N=74**
1p loss	18 (24%)
3p loss	30 (41%)
3q loss	31 (42%)
6p gain	13 (18%)
6q loss	21 (28%)
8p gain	13 (18%)
8p loss	18 (24%)
8q gain	39 (53%)
16q loss	12 (16%)
Loss and gain <10% of all tumors	
2p gain	4 (5%)
2q gain	4 (5%)
6p loss	7 (9%)
7q gain	4 (5%)
9p gain	4 (5%)
9p loss	7 (9%)
11q gain	7 (9%)
15p loss	7 (9%)
15q loss	7 (9%)
21p loss	7 (9%)
22p loss	7 (9%)

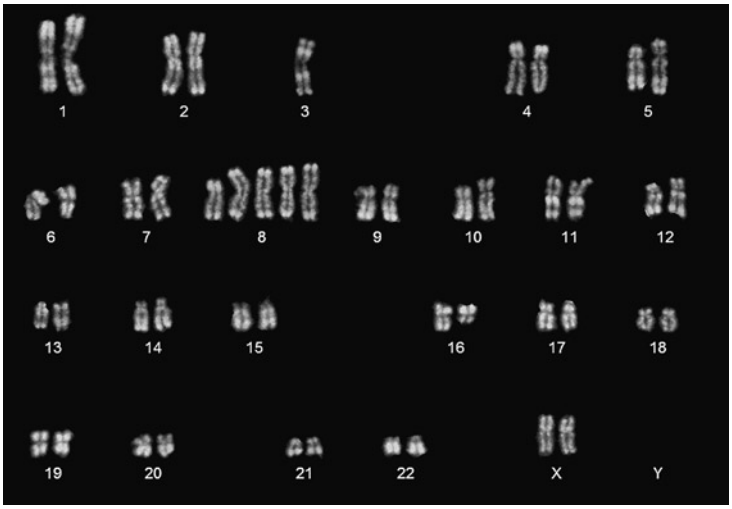


Figure 1. **Karyotype of tumor EOM 63.** This tumor showed chromosomal changes for UM: -3, i(6)(p), i(8)q (multiple copies) and del(16)(q21).

being tumor size, tumor location, cell type and vascular patterns. [12] None of these factors are entirely solid, and there has been considerable variation in interpretation among observers. In contrast to some previous reports [13] we did not find chromosome 11 and 21 to occur very

often (Table 1) and therefore these aberrations were not included in the analysis. In addition, we identified loss of chromosome 16q. Chromosome 16 loss, in particular 16q, also mentioned in earlier reports [10,13] occurred in more than 10% of the UMs. Even though it was not significantly associated with disease free survival it still might be involved in tumor progression. A remarkable association was shown for loss of chromosome 16q with loss of chromosome 1p. Delineation of a region on chromosome 16q may depict a region of interest with possible candidate genes.

Table 2. **Prognostic significance of clinical, histopathological and chromosomal aberrations in uveal melanoma[†‡]**

	m/v	age	epitheloid present	involv. ciliary body	vascular pattern	cell type	LTD	1p loss	3p loss	3q loss	6p gain	6q loss	8p gain	8p loss	8q gain	16q loss
p-value*	,897	,063	,298	,840	,116	,018	,007	,284	,0009	,0021	,424	,6997	,6192	,117	,023	,233
m/v		,063	,304	,849	,125	,020	,008	,272	,001	,003	,427	,698	,613	,118	,020	,195
age	,964		,443	,643	,289	,039	,026	,376	,004	,009	,405	,488	,557	,149	,014	,262
epithelioid present	,962	,086		,649	,257	,046	,009	,309	,003	,005	,490	,576	,594	,161	,026	,186
involv. ciliary body	,912	,057	,264		,091	,015	,010	,269	,001	,003	,421	,716	,616	,118	,025	,218
vascular pattern	,701	,034	,419	,502		,141	,070	,531	,012	,027	,283	,157	,816	,438	,172	,687
cell type	,671	,105	,771	,458	,248		,004	,112	,008	,013	,597	,688	,427	,512	,053	,145
LTD	,498	,205	,256	,797	,198	,005		,775	,022	,062	,272	,580	,561	,446	,128	,511
1p loss	,776	,075	,322	,727	,167	,009	,016		,003	,004	,521	,647	,935	,093	,042	,420
3p loss	,543	,187	,719	,482	,356	,176	,105	,663		,730	,258	,844	,510	,465	,603	,399
3q loss	,647	,204	,658	,523	,456	,141	,141	,417	,259		,284	,663	,482	,617	,481	,358
6p gain	,921	,060	,341	,805	,167	,028	,008	,354	,001	,002		,571	,694	,122	,027	,185
6q loss	,885	,053	,269	,873	,058	,021	,010	,274	,002	,003	,379		,651	,124	,029	,251
8p gain	,872	,060	,295	,830	,117	,017	,010	,347	,002	,003	,455	,741		,084	,021	,251
8p loss	,787	,074	,389	,756	,150	,074	,021	,213	,002	,006	,426	,712	,348		,068	,219
8q gain	,456	,033	,280	,676	,251	,051	,038	,628	,026	,043	,430	,949	,528	,546		,490
16q loss	,589	,067	,248	,702	,126	,014	,015	,505	,002	,004	,342	,770	,674	,114	,041	

*Log-rank test (for categorical variables) or cox proportional hazard analysis (for continuous variables)
†Multivariate analysis using Cox proportional hazard analysis
‡Likelihood ratio test, p-value represented by color: >.10 =white, <.10 and >.05 = light shaded, < .05 = dark shaded

Table 3. **Relation between different histopathological, clinical and chromosomal aberations**

	m/v	involv. ciliary body	8p gain	16q loss	1p loss	cell type	epithelioid present	vascular pattern	3p loss	3q loss	8q gain	8p loss	6q loss	6p gain
m/v		0.167	0.953	0.042	0.283	0.196	0.218	0.862	0.118	0.099	0.079	0.600	0.903	0.077
involv. ciliary body	0.343		0.562	0.481	0.365	0.259	na	0.185	0.566	0.684	0.559	0.962	0.313	0.692
8p gain	1.038	1.667		0.010	0.929	0.208	0.565	0.071	0.650	0.816	na	na	0.378	0.322
16q loss	0.256	1.867	0.927		0.005	0.756	0.766	0.575	0.178	0.255	0.103	0.430	0.271	0.129
1p loss	0.556	2.040	5.303	6.491		0.088	0.426	0.086	0.012	0.084	0.063	0.389	0.948	0.155
cell type	0.440	0.409	3.913	0.796	6.220		na	0.136	0.049	0.085	0.153	0.002	0.774	0.550
epithelioid present	1.889	na	1.508	0.818	1.658	na		0.003	0.048	0.076	0.418	0.173	0.324	0.161
vascular pattern	0.915	3.200	3.758	1.500	2.900	0.367	6.500		0.016	0.004	0.003	0.153	0.268	0.163
3p loss	0.467	1.538	1.321	2.374	4.222	0.316	3.148	3.600		0.000	0.000	0.000	0.798	0.866
3q loss	0.448	1.357	1.154	2.072	2.619	0.367	2.667	4.800	396.333		0.000	0.000	0.966	0.702
8q gain	0.421	1.569	na	3.200	3.000	0.424	1.513	5.042	24.000	19.727		0.002	0.047	0.603
8p loss	0.750	1.042	na	1.714	0.547	0.143	2.568	2.333	24.000	20.000	11.478		0.593	0.553
6q loss	1.066	0.329	1.758	2.054	0.962	0.838	0.583	0.523	1.143	0.978	3.021	1.367		0.126
6p gain	0.328	0.643	0.340	2.944	0.216	1.638	0.415	0.361	0.900	0.787	0.727	1.492	2.629	

na = not available
The odds ratios are given below the black boxes and the corresponding p-values above. The shaded areas represent p-value <. 05.

Other tumors, s.a. breast cancer and neuro-ectodermal tumors have also shown deletion on 16q. [14,15] In these tumors candidate genes have not yet been identified. Since UM cells are derived from neuro-ectodermal tissue this might be of potential interest. In many reports outcome was correlated with tumor location. [7,16] Since we had limited sample size in the group tumors located in the ciliary body we were not able to make reliable assumptions on association of outcome with tumor location. Largest tumor diameter in our study was histopathologically measured. This parameter may be used non-invasively in a clinical setting (measurement on ultrasound) and may be the most reliable non-invasive prognostic parameter. However, there is a variation between clinical and histopathological measurements. The tumor size measured on ultrasound is in general larger than the histopathological measurement. In contrast, the detection of specific chromosomal aberrations by routine FISH, CGH and karyotyping provides a more objective measurement of potential tumor behavior. Identification of monosomy 3 in a tumor sample is widely accepted as the most reliable prognostic parameter. [5-7] Monosomy of chromosome 3 is considered as an early event, occurring before alterations of chromosome 8, 1 and 6. [5-7] Moreover, it may cause isochromosome formation of especially isochromosome 6p and 8q. [8,9] Table 3 may also support this hypothesis, since the odds ratios for loss of chromosome 3p or 3q and gain of chromosome 8q or loss of 8p were higher than the combination of loss of

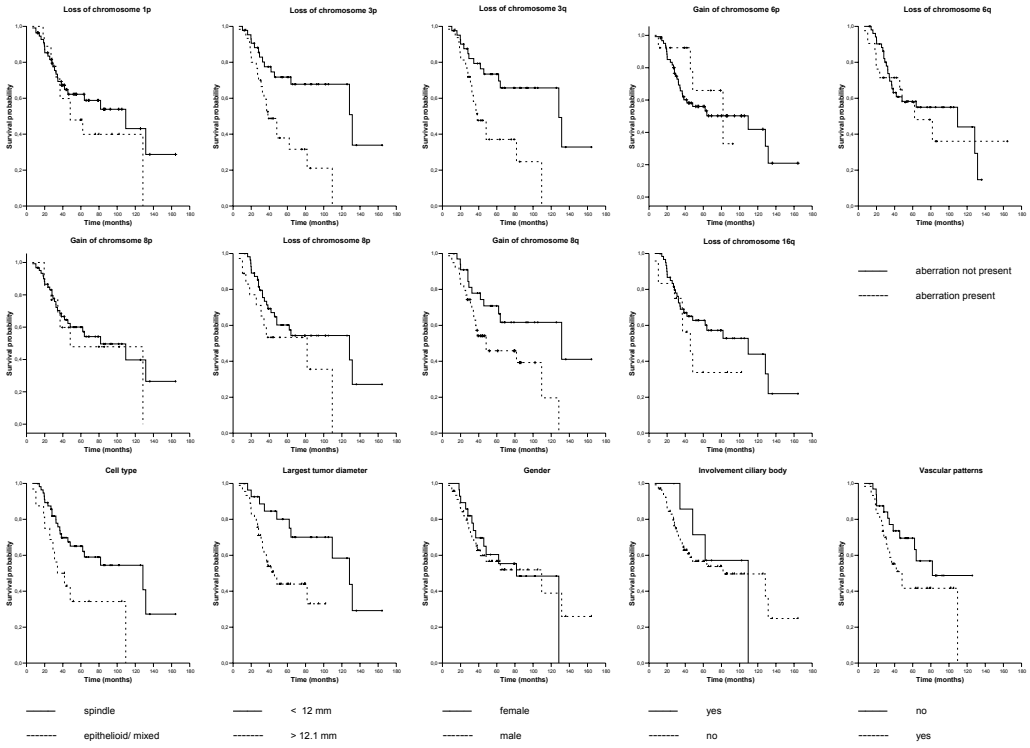


Figure 2. **Kaplan-Meier survival curves for clinical, histopathological and chromosomal aberrations.**

chromosome 3p or 3q and gain of chromosome 8p. However, in our series we cannot conclude the same for isochromosome 6p. In addition, gain of chromosome 8q was significantly associated with survival in the univariate analysis (Table 2), but when corrected for confounding variables, s.a. vascular pattern, cell type, LTD and chromosome 3p or 3q loss, significance was absent implying that gain of chromosome 8q occurs together with at least one of those other variables. On the contrary, when this same procedure was followed for chromosome 3p or 3q loss we observed that the significance remained. In table 3 the odds ratios were shown for different chromosomal parameters. If we put the odds ratios in following order, chromosome 8q gain, and consequently 8p loss, follows monosomy 3, and loss of chromosome 1p and 16q occur thereafter. This is consistent with the findings observed by Hoglund *et al*. [10] Moreover, tumor diameter is associated with most of the chromosomal aberrations, implying that larger tumors have more aberrations. Our study involves patient samples from relatively large tumors that were treated by enucleation. Considering monosomy 3 as an early event [17] it is likely that it would be observed in even the smallest amount of tissue despite the heterogeneity of UM. Though, there are no studies to date that confirm the uniform distribution of cytogenetic abnormalities in UM, and it is at least theoretically possible that small amounts of tissue (s.a. used for karyotyping, FISH and CGH) do not contain the cytogenetic markers of interest.

References

1. Heim S, Mitelman, F. Cancer Cytogenetics. New York: Wiley-Liss, 1995.
2. Aalto Y, Eriksson L, Seregard S, Larsson O, Knuutila S. Concomitant loss of chromosome 3 and whole arm losses and gains of chromosome 1, 6, or 8 in metastasizing primary uveal melanoma. Invest Ophthalmol Vis Sci 2001;42:313-7.
3. Kilic E, Naus NC, van Gils W, Klaver CC, van Til ME, Verbiest MM, Stijnen T, Mooy CM, Paridaens D, Beverloo HB, Luyten GP, de Klein A. Concurrent loss of chromosome arm 1p and chromosome 3 predicts a decreased disease-free survival in uveal melanoma patients. Invest Ophthalmol Vis Sci 2005;46:2253-7.
4. Hausler T, Stang A, Anastassiou G, Jockel KH, Mrzyk S, Horsthemke B, Lohmann DR, Zeschnigk M. Loss of heterozygosity of 1p in uveal melanomas with monosomy 3. Int J Cancer 2005;116:909-13.
5. Prescher G, Bornfeld N, Hirche H, Horsthemke B, Jockel KH, Becher R. Prognostic implications of monosomy 3 in uveal melanoma. Lancet 1996;347:1222-5.
6. Sisley K, Rennie IG, Parsons MA, Jacques R, Hammond DW, Bell SM, Potter AM, Rees RC. Abnormalities of chromosomes 3 and 8 in posterior uveal melanoma correlate with prognosis. Genes Chromosomes Cancer 1997;19:22-8.
7. White VA, Chambers JD, Courtright PD, Chang WY, Horsman DE. Correlation of cytogenetic abnormalities with the outcome of patients with uveal melanoma. Cancer 1998;83:354-9.
8. Prescher G, Bornfeld N, Becher R. Two subclones in a case of uveal melanoma. Relevance of monosomy 3 and multiplication of chromosome 8q. Cancer Genet Cytogenet 1994;77:144-6.
9. Prescher G, Bornfeld N, Friedrichs W, Seeber S, Becher R. Cytogenetics of twelve cases of uveal melanoma and patterns of nonrandom anomalies and isochromosome formation. Cancer Genet Cytogenet 1995;80:40-6.
10. Hoglund M, Gisselsson D, Hansen GB, White VA, Sall T, Mitelman F, Horsman D. Dissecting karyotypic patterns in malignant melanomas: temporal clustering of losses and gains in melanoma karyotypic evolution. Int J Cancer 2004;108:57-65.
11. Mitelman F. ISCN (1995): An International Sytem for Human Cytogenetic Nomenclature. Basel: S. Karger, 1995.
12. Foss AJ, Alexander RA, Hungerford JL, Harris AL, Cree IA, Lightman S. Reassessment of the PAS patterns in uveal melanoma. Br J Ophthalmol 1997;81:240-6; discussion 247-8.
13. Sisley K, Parsons MA, Garnham J, Potter AM, Curtis D, Rees RC, Rennie IG. Association of specific chromosome alterations with tumour phenotype in posterior uveal melanoma. Br J Cancer 2000;82:330-8.

14. Dallas PB, Terry PA, Kees UR. Genomic deletions in cell lines derived from primitive neuroectodermal tumors of the central nervous system. Cancer Genet Cytogenet 2005;159:105-13.
15. Rakha EA, Green AR, Powe DG, Roylance R, Ellis IO. Chromosome 16 tumor-suppressor genes in breast cancer. Genes Chromosomes Cancer 2006.
16. Sisley K, Cottam DW, Rennie IG, Parsons MA, Potter AM, Potter CW, Rees RC. Non-random abnormalities of chromosomes 3, 6, and 8 associated with posterior uveal melanoma. Genes Chromosomes Cancer 1992;5:197-200.
17. Naus NC, Verhoeven AC, van Drunen E, Slater R, Mooy CM, Paridaens DA, Luyten GP, de Klein A. Detection of genetic prognostic markers in uveal melanoma biopsies using fluorescence in situ hybridization. Clin Cancer Res 2002;8:534-9.

Acknowledgements

Prof. Dr. A. Hagemeijer, Dr. R. Slater and E. van Drunen performed the majority of the cytogenetic analyses during the early years of the study.

Supplementary information

EOM	gender	age	localisation	cell type	Largest tumour diameter	vascular pattern	metastasis	Karyotype
3	m	62	choroid	mixed	12	ND	no*	46,X,-Y,+5,-6,+18
9	m	62	choroid	epithelioid	15	ND	yes	46,XY (6/11)/ 46,X,-Y,i(6)(p10),i(8)(q10),+i(8)(q10),+i(8)(q10),der(13)t(13;16?)(p12;p11?),-16 (4/11)
27	m	86	ciliary body	epithelioid	8	ND	no*	46,XY normal
31	m	65	choroid	epithelioid	7	yes	yes	44,X,-Y,der(1)t(1;6)(p11;p11),add(2)(q22),-3,-6,+8
36	m	27	ciliary body	epithelioid	10.5	yes	yes	45,XY,-3 (37,5%)/45,XY,-3,i(8q) (62,5%) 1/16 46,XY,-3,i(8q),+8
37	m	71	choroid	mixed	7	ND	yes	46, XY,del(11)(q22q25),add(15)
38	m	49	choroid	mixed	5.5	ND	no	45,X,-Y (79%)/46,XY (21%)
40	m	33	choroid	mixed	13	ND	no	47,XY,del(1)(p22),add(7)(q36),+i(8q)? (18%)/47,XY,del(1)(p22),del(6)(q13q24),add(7)(q36),+i(8q) (82%).
42	f	47	choroid	mixed	14	yes	yes	46,XX normal
43	m	80	choroid	mixed	13	yes	no*	45,X,-Y,del(1)(p22p32),+der(2;8)(q10;q10),+i(2)(q10),-3,+4,der(6)t(6;15)(p22;q21),7?,-8,-8,del(9)(p13),add(21)(p11)
45	m	61	choroid	epithelioid	11	ND	yes	46,XY (56%)/ 45,X,-Y (44%)
48	f	55	ciliary body	spindle	10	no	yes	43,XX,i(1)(q10),-3,-4,der(6)del(6)(q1?5q2?5)ins(6;?3)(q1?5;?p12p22),der(13;22)(q10;q10),t(14;19)(q22;?p13),der(16)t(3;16)(q21;p12),-18,+20
50	m	57	choroid	epithelioid	6	no	yes	45,X,-Y (18%)/ 45,X,-Y,add(13)(q14)(70%)/ 45,X,-Y,der(10;15)(q10;q10),add(13)(q14)(12%)
53	m	68	choroid	mixed	13	ND	yes	46,X,-Y,dic(1;16)(p11;q11),inv(2)(p11p13),-3,+8[16]
55	f	38	choroid	spindle	11	ND	yes	46,XX(20%)/ 45,X,-X(20%)/ 88,XXXX,der(1)t(1;6)(p12;p11)x2,+der(1)t(1;6)[1],add(2)(q36 of q37)x2,-6,-6,+8,+8,-14,-15,-16,-21
62	f	21	choroid	spindle	7	no	no	46,XX,t(11;15)(q13.2;q13)(87.5%)/46,XX (12.5%)
63	f	73	choroid	epithelioid	12	ND	no*	46-48,XX,-3,i(6)(p10)[5],i(8)(q10),+i(8)(q10),+i(8)(q10)x2[4],del(16)(q21)[5]
71	m	66	choroid	mixed	10	no	yes	45,X,-Y
97	m	73	choroid	spindle	8	no	no*	45,X,-Y(57%).46,XY(43%)
102	m	76	choroid	spindle	12	no	no*	46,XY,add(9)(q34),add(22)(p11)[3]/46,XY,add(9)(q34),add(9)(p24),add(22)(p11)[15]
107	m	76	choroid	mixed	19	yes	no*	43,X,-Y,dic(3;19)(q11;q13.2),-13,der(17)t(13;17)(q13;p12),-21,add(22)(p11) [4]/43,X,-Y,dic(3;19)(q11;q13.2),-13,der(17)t(13;17)(q13;p12),add(22)(p11) [6]
121	m	49	choroid	mixed	14	yes	yes	46-47,XY,del(1)(p31p36),-3,der(4)t(1;4)(q12;q21),+8,+21[cp3]/45,X,-Y[3]/46,XY[8]
123	m	58	choroid	epithelioid	11	yes	no	46,XY[22]
125	f	74	choroid	mixed	18	yes	no	72-76,XXX,dic(1;7)(p10;p14),+dic(1;7)(p10;p14),-3,+4,+6,-7,+i(8)(q10),+9,-11,-15,+16,+18,+20,+?21,+22[cp12]/49-54,idem[cp2]
130	f	57	ciliary body	mixed	16	yes	no	41-48,XX,der(1)t(1;6)(p11;p1?2),add(4)(q1?2),-5,-6,+7,+8,+8,-9,add(11)(q13~14),+add(11)(q13~14),-13,-16,+22,+mar.ish der(6)(wcp6+),+mar.ish der(16)t(6;16)(wcp6+,wcp16+)[cp6]
131	f	60	choroid	mixed	8	yes	no	46,XX[11]
136	f	85	choroid	mixed	15	no	yes	41-44,XX,der(1;8)(q10;q10).ish der(1;8)(wcp8+,2053b3+,p1.164+,D8Z2+,puc1.77+,wcp1+),-3,+der(8).ish der(8)ins(8)(p?21q?23q?24.1)del(8)(q22q22)(wcp8+,114C11+,105H8+,p1.164-,2053b3+),-15,del(16)(q11q1?3).ish del(16)(wcp16+,pHUR195-)[cp15]
141	m	54	choroid	mixed	11	no	no	46,XY,+2,dic(6;13)(q12;p10),dic(6;14)(q12;p10)[16]
147	m	56	choroid	spindle	13	no	no	44~47,XY,del(1)(p2?)[5],add(7)(p?2)[4],+9[2],-15[4],add(19)(q1?3[3],+mar[2][cp6]/46,XY[3]

EOM	gender	age	localisation	cell type	Largest tumour diameter	vascular pattern	metastasis	Karyotype
148	m	59	choroid	mixed	13	yes	yes	45,X,-Y[3]/46,XY[10]/47,XY,+?der(2)[2]/46,XY,add(8)(p),der(15)t(1;15)(q11;p11)[1]
150	m	42	choroid	spindle	12	no	no	46,XY[4]/47,XY,+9[3]/47,XY,+9,der(10)t(6;10)(p12;q26)[4]
151	f	48	choroid	spindle	12	no	no	46,XX[3]/46,XX,der(20)t(6;20)(p12;p12)[5]/47,XX,idem,+8[4]/47,XX,idem,+8,psudic(17;15)(p13;p11)[3]
152	f	76	choroid	epithelioid	15	yes	yes	45~48,XX,-3,i(8)(q10),+i(8)(q10),+i(8)(q10)[cp5]/46,XX[4]/47~49,XX,+3[3],+5[2],+6[2][cp4]
157	f	72	choroid	mixed	13	yes	no*	46,XX[10]/47,XX,+8[7]
158	m	61	choroid	spindle	11	no	yes	45,X,-Y[10]/46,XY[4]
159	f	37	choroid	spindle	12	no	no	40~46,XX,add(2)(q3?4).ish der(2)t(2;6)[6],der(5)t(5;6)(q34;?)[9],del(6)(q?)[3],der(7)t(7;8)(p21;q?),add(10)(p1?4).ish der(10)t(8;10)[2],add(11)(q1?4).ish der(11)t(8;11)[9],der(16)t(8;16)(q?;q24)[7], add(18)(q23)[1] [cp19][19]/46,XX[1]
160	f	72	choroid	mixed	14	yes	yes	46,XX[10]/40~42,XX,del(1)(p21),-3,-6,i(8)(q10)[1],-12,-18[cp5]/47,XX,del(1q),der(1)t(1;8)(p;q),+7,-8,+9,del(11p)[1]
165	m	42	choroid	spindle	12	no	no	47,XY,add(6)(q21),der(7)t(1;7)(q12;q36),+8[20]
166	f	49	choroid	mixed	18	no	no	47,X,-X,-3,+7,i(8)(q10),+i(8)(q10)[1]/46,XX[4]
174	f	59	choroid	spindle	19	no	yes	75,XXY,1p+,-3,+i(6)(p),i(8)(q),9p+
177	m	59	choroid	spindle	8	yes	yes	45~47,XY,-5[2],+8[2],add(8)(p22),+add(8)(p22)[4],-9[2],del(13)(?q14q21),der(17)ins(17;13)(q12;?q14q21)del(17)(q22q23),-19[2],-22,+mar[cp7]
178	f	73	choroid	mixed	17	yes	no	45~47,XX,del(1)(p21),add(7)(q36),+8[2],-15,add(17)(p12),+ring[1][cp5]/46,XX,del(1)(p21),der(5)t(5;14),add(7)(q36),+8,-14,-15,add(17)(p12),+ring[5]
179	m	60	choroid	spindle	11	no	no	46,XY[15]
180	f	79	choroid	epithelioid	16	no	yes	43~45,X,-X,-3,i(8)(q10),+i(8)(q10),+i(8)(q10)[2],der(16;21)(q10;q10),der(22)[1][cp5][5]
182	f	52	choroid	spindle	14	no	no	46,XX,der(6)t(6;6)(q16;p12),add(22)(p11),add(22)(q13)[16]
187	m	65	choroid	epithelioid	15	yes	yes	45,X,-Y,-3,-4,i(8)(q10),+i(8)(q10),+mar
189	m	80	choroid	mixed	14	yes	yes	44,XY,-1,-2,-3,dic(1;6)(q10;q10),+ring[5]/45,XY,-1,-2,-3,dic(1;6)(q10;q10),+8,+ring[12]/90,XXYY,idem,+8,+8[1]
191	f	46	choroid	spindle	14	no	yes	46,XX,add(1)(q42),add(4)(q3?2),del(6)(q1?4q2?5),der(8)t(6;8)(p12;q24),-16,add(16)(q?2),add(17)(p13),add(21)(p11),+mar1,+mar2 [19]
193	m	44	choroid	epithelioid	16	yes	yes	46, XY[16]
195	m	68	choroid	epithelioid	16	no	no	46, X,-Y,-3,+i(8)(q10),+i(8)(q10)[4]/47,idem,+add8(p?)[12]
199	f	64	choroid	spindle	17	yes	yes	44~45,XX,der(1)t(1;6)(p12;p11),-3,+i(8)(q10),+i(8)(q10)[1],der(14)t(14;22)(q13;q11),-16,-22[cp19]
205	m	46	choroid	spindle	7	yes	no	44,X,-X,der(22)t(12;22)(q12;p12) [3]/45,X,-X,I(12)(q10),idic(22)(p12) [2]/46 XX [26]
207	m	76	choroid	spindle	15	yes	no	45,X,-Y,-3,i(6)(p10),+8[8]/46,XY[7]
211	f	66	choroid	spindle	14	no	yes	73,X,-X,-X,add(1)(q43),+2,-3,+7,+8,+i(8)(q10),+i(8)(q10),-9,add(9)(q?13),-10,+11,+12,+13,+14,+15,-17,+19,-21[1]/149,idemx2[1]
218	m	60	choroid	spindle	17	no	no	41,XY,-1,der(11)t(1;11)(q12;p15),add(12)(p13),-15,-16,-21,-22[16]
219	m	69	choroid	mixed	18	yes	yes	45,XY,-3,i(8)(q10)[14]
226	m	27	choroid	mixed	13	yes	no	46,XY,-3,i(8)(q10),+i(8)(q10)[6]/47,XY,-3,i(8)(q10),+i(8)(q10)[3]/46,XY[2]
237	m	77	choroid	spindle	14	no	yes	45,X,-Y,add(1)(p2),-3,+i(8)(q10)[4]

EOM	gender	age	localisation	cell type	Largest tumour diameter	vascular pattern	metastasis	Karyotype
240	m	52	choroid	spindle	15	no	yes	46,XY,-3,+i(8)(q10)[17]
241	f	84	choroid	spindle	14	no	no	46,XX[22]
242	f	47	choroid	spindle	16	no	no	46,XX,der(3)t(3;8)(q2?9;q11),der(6)add(6)(p?22)t(6;8)(q16;q?21),+der(6)add(6)(p?22)t(6;13)(q11;q11),-13,add(17)(p13)[10]
246	m	57	choroid	epithelioid	15	no	no	40-47,X,-Y,-3.?add(6)(q13),i(8)(q10),+i(8)(q10),+i(8)(q10)[2],+i(8)(q10)[1][cp3][3]
253	m	74	choroid	epithelioid	19	yes	yes	77-86,XXYY,i(1)(q10),+der(1)i(1)(q10)t(1;17)(q21;p12) or der(1)i(1)(q10)t(1;17)(q21;24)x3,-3[3],i(4)(p10),+i(4)(p10)[3],-6[3],i(6)(p10),i(8)(q10)x2,-9,-9,-10,add(11)(p14)x2,-13,-14[3],-15,-15,-16[3],
								add(20)(q13)x2[cp4][4]/79-86,XXYY,i(1)(q10),+i(1)(q10),+i(1)(q10)[6],+2[5],-3[5],-3[4],-4[4]i(4)(p10),+i(4)(p10)[7],-5[8],-6,i(6)(p10)[8],-7[3],+7[3],i(8)(q10)x2,-9,-10[8],-11[7],-12[6],-13[6],-14[5],-15[6],-15[3],-16[7],-17[3],+18[3],-19[3],add(20)(q13)x2,+mar[2][cp9][9]
254	f	74	choroid	mixed	18	yes	yes	45,XX,der(1)t(1;16)(p22;p11),-3,+i(8)(q10),-16[16]
256	m	40	choroid	spindle	12	no	no	46,XY,der(19)t(6;19)(p23;p13.3)[18]
257	m	87	choroid	spindle	12	no	no*	46,XY,der(8)t(8;18)(p12;q12),der(21)t(6;21)(p11;p11)[12]/46,XY,del(6)(q15q26),der(8)t(8;8)(p12;q12),der(21)t(6;21)(p11;p11)[3]
261	m	40	choroid	spindle	9	no	no	55,XY,+2,+add(5)(q12),+der(5;17)(q10;q10),del(6)(q12q27),+8,+8,+der(9)t(8;9)(q11;p12),+11,+20[16]
262	f	64	choroid	epithelioid	13	yes	yes	45-91,X,-X,-3,+i(8)(q10),inc.[6]/46,XX[8]
265	m	40	choroid	spindle	13	no	yes	46,XY,del(6)(q15q22)[2]/46,XY,del(6)(q15q22),add(14)(q32)[1]/ 46,XY,del(6)q15q22),der(17)t(7;17)(q11.2;p13)[5]/ 46,XY,del(6)(q15q22),add(14)(q32),der(17)t(7;17)(q11.2;p13)[8]
270	m	65	choroid	spindle	14.5	yes	yes	46,XY,add(6)(q21),add(7)(q21),?del(9)(p13p23),dup(10)(q26q23),del(11)(q22),add(14)(p11),add(19)(13.4)[1]/46,XY,add(6)(q21),add(7)(q21),?del(9)(p13p23),dup(10)(q26q23),del(11)(q22),
								add(14)(p11),add(19)(13.4),der922)t(8;22)9q21;p10)[13]/43,XY,add(6)(q21),add(7)(q21),add(8)(p11),?del(9)(p13p23),-10,dup(10)(q26q23),del(11)(q22),add(14)(p11),der(15;15)(q10;q10),add(19)(q13.4)[2]
271	f	55	choroid	spindle	13	yes	no	45,XX,-3,der(8)t(8;8)(p23;q13)[14]
272	f	52	choroid	spindle	15	yes	no*	46,XX,der(1)t(1;16)(p2?1;p11),-3,+(8)(q10),-16[16]
274	m	42	choroid	spindle	17	no	no	46,XY,del(11)(q2?2),der(18)t(6;18)(p21;q23),add(19)(p13.3)[4]/ 46,XY,add(9)(q34),del(11)(q2?2),der(18)t(6;18)(p21;q23),add(19)(p13.3)[19]
281	m	69	choroid	spindle	16	no	no	46,XY,der(7)t(6;7)(p22;p22)[20]

*Patients without metastases, death due to other causes

Chapter 4

Concurrent loss of chromosome arm 1p and chromosome 3 predicts a decreased disease-free survival in uveal melanoma patients

Chapter 4

Concurrent loss of chromosome arm 1p and chromosome 3 predicts a decreased disease-free survival in uveal melanoma patients

Emine Kilic*, Nicole C. Naus*, Walter van Gils, Caroline C. Klaver, Marjan E. van Til, Michael M. Verbiest, Theo Stijnen, Cornelia M. Mooy, Dion Paridaens, H. Berna Beverloo, Gregorius P. Luyten, Annelies de Klein

*Both authors contributed equally

Invest Ophthalmol Vis Sci. 2005 Jul;46(7):2253-7.

Abstract

Purpose: Uveal melanoma is a highly malignant disease with a mortality of 50% at 10-15 years. Previous studies have shown that chromosomal changes are associated with a decreased survival of the patient. However, these studies analyzed small numbers of tumors that did not allow robust statistical analysis. Here we assess the independent value of numerical changes of chromosomes 1, 3, 6 and 8 on the disease free survival (DFS) in a large series of uveal melanoma patients.

Patients and methods: 120 tumors from uveal melanoma patients were analyzed for numerical changes of chromosomes 1, 3, 6 and 8 with cytogenetic analysis, fluorescent in situ hybridization and/or comparative genomic hybridization. Data were correlated with disease outcome in univariate and multivariate analyses using Kaplan-Meier and Cox regression analyses.

Results: At a mean follow-up time of 45 months, 42 patients had died or were suffering from metastatic disease. In the univariate analysis, loss of chromosome 3, gain of 8q, largest tumor diameter or the presence of epithelioid cells was associated with a decreased disease free survival. In the multivariate analysis, the effect of monosomy 3 on survival was largely modified by changes in chromosome 1p36. We found that, regarding all chromosomal changes, only concurrent loss of chromosome 1p and 3 was an independent prognostic parameter for DFS ($p<0.001$).

Conclusions: In uveal melanoma, concurrent loss of chromosomes 1p and 3 is an independent predictor of decreased disease free survival.

Introduction

Uveal melanoma is the most common form of primary eye cancer in adults with a mortality rate of fifty percent after ten to fifteen years. [1] Metastases occur predominantly in the liver. Early identification of patients at high risk of metastases may allow detection of metastases at a stage in which adjuvant therapy can be justified. Several prognostic factors based on clinical and histological features are known, for instance gender, age at time of diagnosis, largest tumor diameter (LTD), involvement of ciliary body and presence of epithelioid cells.[2] Nevertheless, none of these prognostic factors is specific enough for identification of patients at risk of metastatic disease.

Cytogenetic abnormalities are correlated with the clinical outcome of patients with leukemia and lymphoma. [3] Uveal melanomas are highly amenable for cytogenetic analysis and show mostly simple karyotypes in contrast to most other solid tumors. Non random chromosomal abnormalities, such as variation in chromosomes 1p, 3, 6 and 8 were detected in these tumors. [4, 5] Loss of chromosome 3 and gain of chromosome 8q have been associated with a high mortality rate, whereas abnormalities of chromosome 6 were found to correlate with a good prognosis. [6] However, these data were obtained from relatively small studies. Furthermore, the independent value of these chromosomal changes and the effect of chromosome 1p loss on survival remain to be determined.

The purpose of this present study was to investigate the association between chromosomal changes and clinical and histological variables. Furthermore, we aimed to examine the independent effect and interactions of numerical changes of chromosomes 1, 3, 6 and 8 on disease free survival (DFS) of uveal melanoma patients.

Patients and methods

Patients and tumor samples

From March 1992 to April 2003, we collected tumor material of 152 consecutive patients who underwent enucleation for ciliary body or choroidal melanoma. 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 obtained within 1 hour after enucleation according to a standardized protocol; incision is made through the tumor leaving the optic nerve intact. The quantity of obtained tissue (5-8 mm^3) depended on tumor size. A sample was taken from the side opposed to the optic nerve and divided into two; one part was processed for cytogenetic analysis and/or fluorescent in situ hybridization (FISH), whereas the other part was stored in liquid nitrogen. Until January 1995 only cytogenetic analysis was performed which was successful in 15 out of 46 cases. From that time on tumors (n=106) were analyzed with FISH and, if metaphases could be obtained, with cytogenetic analysis. In the latter ones comparative genomic hybridization (CGH) analysis (n=30) was performed on tumor material that could not be completely analyzed by these two techniques. Conventional histopathologic examination was performed on all tumors and confirmed the origin of the tumor. Cytogenetic studies were also carried out on stimulated peripheral blood samples of each patient to exclude the presence of congenital chromosome abnormalities. Follow-up data from time of diagnosis till the end of the study in April 2004 were

obtained by reviewing each patient's charts and contacting their general physician. Three patients were at that time lost to follow-up. From two of these patients, however, a late date of follow-up was obtained and they were therefore also included in the study. From the 120 patients included in the survival analysis, there were 67 men and 53 women. The age at time of diagnosis ranged from 21 to 87 years (mean 61). The mean duration of follow-up, from diagnosis to presence of metastases or end of study, was 45 months (range 6-142 months).

Histological findings

The mean and median tumor diameter and thickness were 12.7 and 13.0 mm (SD 3.3; range 4.5-19), and 7.8 and 8.0 mm (SD 3.7; range 1.5-22), respectively. Twenty tumors showed involvement of the ciliary body and 100 were located in the choroid. Cell type was classified as mixed/epithelioid in 69 tumors and spindle cell type in 51 tumors.

Cytogenetic, FISH and CGH analysis

Cytogenetic analysis

Chromosome preparations were made according to standard procedures and stained with acridine orange or atebrine to obtain R or Q banding. Cytogenetic abnormalities were described in accordance with the ISCN (1995).[7]

FISH analysis

Dual color FISH on uncultured tumor material using centromeric and locus specific cosmid, P1 or YAC probes for chromosome 1, 3, 6 and 8 was performed as described previously. [8] Seven probes were used: p1-79 (mapped to chromosome band 1p36), Pα 3.5 (centromere 3), YAC 827D3 (3q24), cos85 (6p21) and cos52 (6q23) (Prof. Y Nakamura, Tokyo, Japan), D8Z2 (centromere 8) and ETO (8q22). The probes were validated on normal peripheral blood cell metaphase spreads and ten metaphases were analyzed for each probe. Cut-off limits were less than three percent. The concentration for centromeric probes was 5 ng per slide; for cosmids, P1 and YAC probes 50 to 75 ng per slide were used. After hybridization and washing, slides were counterstained with 4', 6-diamidino-2-phenylindole and mounted in anti-fade solution (Dabco-Vectashield 1:1). Signals were counted in 300 interphase nuclei according to the criteria of Hopman *et al*. [9] Scoring for deletion (>15% of the nuclei with one signal) or amplification (>10% of the nuclei with 3 or more signals) were adapted from the available literature. [10]

CGH analysis

DNA from formalin-fixed paraffin-embedded tumor material was isolated from 40 10μm sections. The pigmented tumor was scraped off from the glass slides using a fine scalpel. Excised material was deparaffinized in xylene and ethanol and air-dried. Isolation of DNA was performed using the DNA tissue kit (Qiagen, Hilden, Germany). Concentration was determined using a fluorometer (Biorad, Veenendaal, The Netherlands), whereas molecular weight was estimated on ethidium-bromide stained agarose gels. Tumor DNA and reference DNA (0.5 μg) was labeled using the Bio-prime DNA labeling kit (Invitrogen, Breda, The Netherlands) with Spectrum Green (Vysis, Hoofddorp, The Netherlands) or Alexa 594 (Molecular Probes, Leiden, The Netherlands)

respectively. Equal probe mixture of tumor and reference DNA was denatured and hybridized in the presence of human cot-1 DNA to normal male metaphase chromosomes for three days at 37ºC. Samples were counterstained with DAPI in anti-fade solution. Images were acquired with a Zeiss axioplan microscope equipped with Isis software from Metasystems (Metasystems, Altlussheim, Germany). For each case ten metaphases were analyzed. Loss of DNA sequences was defined as chromosomal regions where the mean green: red ratio was below 0.8, while gain was defined as chromosomal regions where the ratio was above 1.2. Threshold levels were determined on basis of analysis of known chromosomal aberrations.

Data classification

We subdivided the variation in chromosomes 1p, 3, 6p, 6q and 8q using cytogenetic and FISH analysis into 3 categories: loss of one copy, normal copy numbers (two copies) and gain of one or more copies. Monosomy 3 was defined when, using FISH, there was only one signal seen for both the centromere 3 and 3q24 probe. Gains of 6p and 8q were scored when more than 2 signals were found for both the 6p21 and 8q22 probe, and loss of 1p and 6q when the probes for 1p36 and 6q23 showed only one signal.
When different subclones were identified, only the FISH findings of the largest clone were classified. Cytogenetic and CGH results were classified for those regions studied with FISH analysis. All major chromosomal changes detected by cytogenetic analysis could also be detected by FISH analysis.

Statistical analysis

The primary end point for DFS was the time to development of metastatic disease from time of enucleation, whereby death due to other causes was treated as censored. The influence of single prognostic factors on DFS was assessed using the log rank test (for categorical variables) or Cox proportional hazard analysis (for continuous variables) and Kaplan-Meier curves were made to illustrate the differences in survival. Comparisons of the distributions of clinical and chromosomal variables were performed with Fisher's exact test (for categorical variables) and the Mann-Whitney test (for continuous variables). To identify the independent value of the prognostic factors on disease-free survival we used a multivariate Cox proportional hazard analysis and the likelihood ratio test. Possible prognostic factors were age at time of diagnosis, cell type (spindle cell vs. mixed / epithelioid cell), largest tumor diameter, mutual loss of chromosome 1p36 and 3, and gain of 8q. All tests were two-sided. An effect was considered significant if the p-value was 0.05 or less. The statistical analyses were performed with the SPSS-11 software.

Results

Of the 152 uveal melanoma, 32 cases we could not be analyzed for chromosome 1p, 3, 6 and 8 abnormalities. A total of 120 uveal melanoma cases were analyzed for chromosomal changes using cytogenetic, FISH and/or CGH analyses. Cytogenetic analysis was successful in 69 out of 120 tumors. For 55 tumors cytogenetic and FISH data were available, while for 47 tumors only FISH was performed. Additionally, in 30 tumors CGH analysis was performed. Not all probes could be tested on all tumors because of lack of material. The mean number of probes successfully

used for FISH was 5.5. Combining cytogenetic, FISH and CGH data genomic abnormalities were found in 88 percent of the 120 tumors. Results for all chromosome regions (1p, 3, 6p, 6q and 8q) were obtained for 108 tumors (varying from 108-118 successful analyses per region, table 1). Thirty-eight patients had died from metastatic disease and four were suffering from metastases at time of evaluation.

Table 1. **Univariate analysis of prognostic markers on disease free survival in uveal melanoma**

Variable	**Mean**	**p-value***
Age at time of diagnosis (yrs)	61	0.079†
Largest tumor diameter (mm)	12.7	**0.011**†
Tumor thickness (mm)	7.8	0.293†
Variable	**no. of patients (%)**	**p-value***
Mixed/epithelioid cell type	69 (58)	**0.003**
Involvement ciliary body	20 (17)	0.521
Male gender	67 (56)	0.978
loss of 1p36	41/118 (35)	0.081
loss of chromosome 3	55/109 (50)	**<0.001**
gain of chromosome 6p	34/111 (27)	0.497
loss of chromosome 6q	33/108 (31)	0.319
gain of chromosome 8q	69/110 (63)	**<0.001**

* Log-rank test
† Cox-regression analysis

* Chromosome locus at which the abnormality is absent (-) or present (+)
† The p-value is for the comparison among different subgroups within a chromosome aberration group and was calculated by Fisher's exact test
‡ The p-value is for the comparison of means among different subgroups within a chromosome aberration group and was calculated by Mann-Whitney test
Significant p-values are indicated in bold

Univariate analysis of the single prognostic factors showed significantly lower DFS for patients with loss of chromosome 3, gain of 8q and with a mixed/epithelioid cell type in the tumor compared to patients without these chromosomal changes or with a spindle cell type (table 1). The largest tumor diameter was also significant in the univariate analysis. Other potential prognostic factors such as gender, age at time of diagnosis, tumor thickness and tumor location (i.e. involvement of ciliary body) did not reach significance. Also chromosomal changes such as loss of chromosome band 1p36, gain of chromosome 6p and loss of chromosome 6q were not significantly associated with disease free survival.

To examine the possibility that other chromosomal variations may affect the prognosis of the monosomy 3 patients, we constructed Kaplan-Meier curves of chromosome 3 changes stratified for the other chromosomal changes and performed log rank tests (results not shown). We found

that the effect of monosomy 3 on DFS was substantially modified by changes in copy number of chromosome 1p36. In tumors with normal copy numbers of chromosome 1p36, a small difference in DFS was observed between those patients with and without loss of chromosome 3 ($p=0.064$) whereas this difference was highly significant in patients with tumors with also loss of chromosome 1p36 ($p<0.001$). The interaction term between tumors with loss of chromosome 1p36 and 3 and the remaining patients (i.e. patients with tumors with normal copies of chromosome 1p36 and 3 or with either 1p36 or 3 loss) was highly suggestive (HR= 3.61), but did not reach significance ($p=0.155$). In addition, we compared the DFS of patients with a concurrent loss of chromosome 1p36 and chromosome 3 with the remaining patients using the log rank test. The difference in survival was found to be highly significant ($p<0.001$) (figure 1). Remarkably, gain of chromosome 1p36 occurred in 5 patients, but this number was too small to perform statistical analysis.

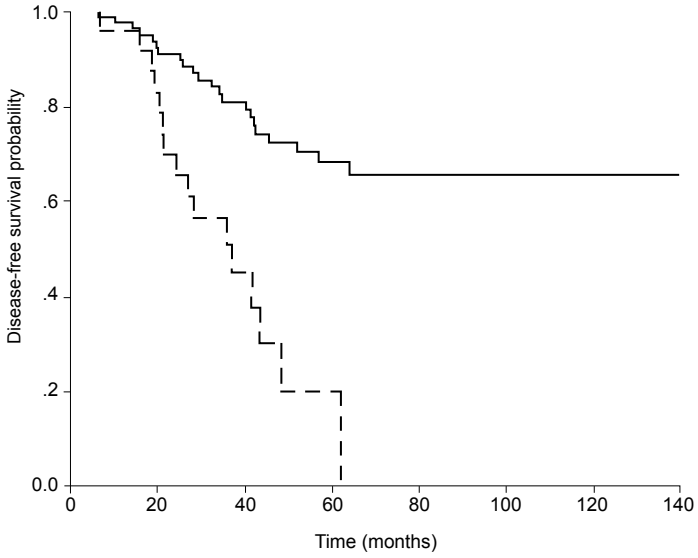


Figure 1. **Kaplan-Meier survival curve of loss of chromosome 1p36 and/or 3**
The dashed line represents the tumors with concurrent loss of chromosomes 1p36 and 3; the solid line represents the tumors with loss of only chromosome 1p36, only monosomy 3 or loss of neither chromosomes 1p36 nor 3 ($p<0.001$).

Considering the strong interaction between chromosome 1p and 3 losses, we validated whether this concurrent loss is an independent parameter for DFS. Significant correlations between age at time of diagnosis, tumor diameter, cell type, chromosome 6p and 8q gains using Mann-Whitney and Fisher's exact tests (table 2) were observed. Monosomy 3 was associated with age at time of diagnosis ($p=0.050$), cell type ($p=0.013$) and mean tumor diameter ($p=0.002$). Gain of chromosome 6p was correlated with cell type ($p=0.008$) and gain of chromosome 8q with mean tumor diameter ($p<0.001$). These chromosomal changes and confounding variables were analyzed in a multivariate model. After correcting for these variables, we found that patients with tumors with concurrent loss of chromosomes 1p36 and 3 have an almost 7.8 times higher chance of developing metastases compared to those without these losses or with either 1p36 or 3 loss ($p=0.039$) (table 3). Gain of chromosome 8q (HR=2.43, $p=0.054$) and mixed/epithelioid cell type (HR=2.24, $p=0.077$) almost reached significance and the other variables (gain of chromosome 6p, largest tumor diameter and age at time of diagnosis) were not significant.

Furthermore, the interaction term between monosomy 3 and gain of chromosome 8q was not significant (HR=0.53; p=0.469). When analyzed in a multivariate model with the confounding variables, such as age at time of diagnosis, gain of chromosome 6p, cell type and mean tumor diameter, we found a hazard ratio of 0.67 with a p-value of 0.676 (data not shown).

Discussion

Previous studies already demonstrated the non-random occurrence of cytogenetic abnormalities of chromosome 1, 3, 6 and 8 in uveal melanoma. Monosomy 3 and gain of 8q have been shown to be associated with poor survival after treatment for uveal melanoma. [6, 11, 12] Using univariate analysis we confirmed these findings. In addition, we demonstrated in the present study, which is the largest series described so far therefore allowing multivariate statistical analysis, that tumors with a concurrent loss of chromosomes 3 and 1p36 are at high risk of metastasizing (HR=7.81, table 3). The molecular genetic changes that underlie these chromosomal changes have not yet been determined.

Chromosome 1p loss occurs frequently in many solid tumors like skin melanoma and neuroblastoma. In the latter tumor type, loss of chromosome 1p is known to be a predictor of unfavorable outcome of the patient. [13, 14] In uveal melanoma loss of chromosome 1p has been described, but any prognostic significance had not been determined up to now. Contrary to Sisley *et al*. [15] in our study loss of material of 1p36 was not associated with large ciliary body melanomas, but was rather detected in metastasizing tumors in agreement with Aalto *et al*. [16] Eighty-nine percent of the metastasized tumors with chromosome 1p36 loss had concurrent monosomy 3. Concurrent loss affecting survival suggests an interaction of proteins encoded by genes located on these chromosomes, which may promote tumorigenesis, metastatic disease and consequently reduce survival. However, we cannot exclude that these sites encode for proteins that might independently promote tumorigenesis and metastasis.

Gain of chromosome 8q was a significant predictor of survival in the univariate analysis. In the multivariate analysis it did not reach statistical significance as an independent prognostic marker. Previous studies already suggested that acquisition of isochromosome 8q is a secondary event and that gain of additional copies is related to tumor size. [11, 17, 18] Moreover, monosomy 3 seemed to predispose to isochromosome formation. [4] This may explain the correlation of gain of chromosome 8q with survival as observed in other studies. [6, 11] We demonstrated a strong correlation between the largest tumor diameter and the presence of chromosome 8q abnormalities, suggesting that acquisition of additional copies of 8q may result in a growth advantage of the tumor.

Similarly, the abnormalities of chromosome 6 were not independently associated with survival, in contrast to previous claims. [6] We found a strong correlation between the gain of chromosome 6p and spindle cell type. Sisley and White and coworkers associated chromosomal changes, such as loss of chromosome 3, gain of chromosome 8q and abnormalities of chromosome 6 with prognosis. [6, 15] However, as far as we know their findings were not corrected for tumor diameter or cell type as in the present study. This could have influenced their findings, leading to contradictory observations. Another known prognostic marker for a poor outcome of uveal melanoma patients is the presence of epithelioid cells. We found a strong correlation between

chromosomal aberrations (chromosomes 3, 6 and 8) and cell type (table 2). Even though epithelioid or mixed cell type was significantly associated with decreased DFS in the univariate analysis, it was not in the multivariate analysis.

Although loss of an entire chromosome is a common change in uveal melanoma, partial deletions of chromosome 3 have been reported leading to the hypothesis that two regions, one on the p-arm and one on the q-arm, might be involved in metastasis. [19]

Table 2. **Correlation between chromosomal abnormalities and clinical data**

		Chromosome 1p36 loss*			Chromosome 3 loss*			Chromosome 6p gain*			Chromosome 8q gain*		
Clinical data		-	+	P-VALUE	-	+	P-VALUE	-	+	P-VALUE	-	+	P-VALUE
Gender	male	42	20	0.193†	32	29	0.311†	45	19	0.481†	24	39	0.498†
	female	29	21		22	26		32	15		17	30	
Mean age (yrs)		61	61	0.345‡	57	64	**0.050**‡	61	59	0.298‡	59	61	0.223‡
Cell type	spindle	32	17	0.432†	24	17	**0.013**†	27	21	**0.008**†	23	24	**0.024**†
	mixed/epithelioid	39	24		25	38		50	13		18	45	
Mean tumor thickness (mm)		7.8	8.0	0.355‡	7.7	8.5	0.133‡	7.8	8.6	0.135‡	7.5	8.4	0.127‡
Mean tumor diameter (mm)		12.5	13.0	0.186‡	12.1	13.9	**0.002**‡	12.6	13.6	0.059‡	11.3	13.9	**<0.001**‡
Involvement of ciliary body	no	61	31	0.133†	48	43	0.106†	62	30	0.239†	34	57	0.592†
	yes	10	10		6	12		15	4		7	12	

* Chromosome locus at which the abnormality is absent (-) or present (+)
† The p-value is for the comparison among different subgroups within a chromosome aberration group and was calculated by Fisher's exact test
‡ The p-value is for the comparison of means among different subgroups within a chromosome aberration group and was calculated by Mann-Whitney test
Significant p-values are indicated in bold

Table 3. **Prognostic markers for metastasis in 120 uveal melanoma patients***

Variable	Hazard ratio	p-value†
Loss of chromosome 1p36 with loss of 3	7.81	0.039
Gain of chromosome 8q	2.43	0.054
Mixed/epithelioid cell type	2.24	0.077
Gain of chromosome 6p	1.33	0.558
Largest tumor diameter	1.03	0.588
Age at time of diagnosis	1.00	0.900

* Multivariate analysis using Cox proportional hazard analysis
† Likelihood ratio test

Seven patients in our study had a partial deletion of chromosome 3 (either one copy of the centromeric region or 3q-region) from whom 2 patients had died due to metastatic disease. In five tumors two signals for the centromere and only one signal for the 3q probe were observed, whereas two tumors had one copy of the centromere and two of the 3q-probe. Since these changes were observed with FISH analysis and karyograms of these tumors were not available, we were not able to identify any breakpoints. These and more subtle structural aberrations can be resolved with techniques with higher resolution, such as genomic arrays or LOH. However, changes such as base substitutions, very small deletions or insertions will still be missed.

Our study on chromosomal abnormalities in uveal melanoma is, to our knowledge, the largest series reported in the literature. Our study may be biased because we examined only tumors from patients treated by enucleation, as no tumor material is available from patients treated with radiotherapy protocols. There is a need to stratify patients prospectively into low and high risk groups for metastases. Our findings suggest that chromosomal abnormalities may be useful in identifying patients at high risk of metastases. Previous studies by Sisley *et al*. have shown a correspondence between major clonal alterations in FNAB's and the main tumor using cytogenetic techniques. [20] Furthermore, they showed that with short-term cultures of FNAB's conventional cytogenetic analysis was possible in 60% of the cases. In addition, Naus *et al*. indicated that application of FISH on FNAB's is a reliable method for assaying genetic prognostic parameters. [8] Only in 0.8% a small variation that have could lead to a misclassification was found.

There are at least two potential challenges involved in the application of our data to patients on a prospective basis. First, our study involves patient samples from relatively large tumors that were treated by enucleation. It remains to be seen that our data can be applied to smaller tumors that will be treated by radiation therapy. Second, despite correspondence between chromosomal abnormalities detected from FNAB samples and tissue retrieved at enucleation, there are no studies to date that confirm the uniform distribution of cytogenetic abnormalities in uveal melanoma, and it is at least theoretically possible that an FNAB might capture tissue that does not contain the cytogenetic markers of interest. Nevertheless, data from our study, the largest cohort of patients studied to date for cytogenetic abnormalities in primary uveal melanoma, suggests the feasibility of studying patients with uveal melanoma in prospective trials using samples retrieved by FNAB.

Acknowledgements

Prof. Dr. A. Hagemeijer, Dr. R. Slater and E. van Drunen performed the majority of the cytogenetic analyses during the early years of the study.

References

1. Egan KM, Seddon JM, Glynn RJ, Gragoudas ES, Albert DM. Epidemiologic aspects of uveal melanoma. *Surv Ophthalmol*. 1988;32:239-251
2. Mooy CM, De Jong PT. Prognostic parameters in uveal melanoma: a review. *Surv Ophthalmol*. 1996;41:215-228
3. Le Beau MM, Rowley JD. Chromosomal abnormalities in leukemia and lymphoma: clinical and biological significance. *Adv Hum Genet*. 1986;15:1-54
4. Prescher G, Bornfeld N, Friedrichs W, Seeber S, Becher R. Cytogenetics of twelve cases of uveal melanoma and patterns of nonrandom anomalies and isochromosome formation. *Cancer Genet Cytogenet*. 1995;80:40-46

5. Sisley K, Rennie IG, Cottam DW *et al.* Cytogenetic findings in six posterior uveal melanomas: involvement of chromosomes 3, 6, and 8. *Genes Chromosomes Cancer*. 1990;2:205-209
6. White VA, Chambers JD, Courtright PD, Chang WY, Horsman DE. Correlation of cytogenetic abnormalities with the outcome of patients with uveal melanoma. *Cancer*. 1998;83:354-359
7. Mitelman Fe. ISCN (1995): An International Sytem for Human Cytogenetic Nomenclature. Basel: S. Karger, 1995
8. Naus NC, Verhoeven AC, van Drunen E *et al.* Detection of genetic prognostic markers in uveal melanoma biopsies using fluorescence in situ hybridization. *Clin Cancer Res*. 2002;8:534-539
9. Hopman AH, Ramaekers FC, Raap AK *et al.* In situ hybridization as a tool to study numerical chromosome aberrations in solid bladder tumors. *Histochemistry*. 1988;89:307-316
10. van Dekken H, Pizzolo JG, Reuter VE, Melamed MR. Cytogenetic analysis of human solid tumors by in situ hybridization with a set of 12 chromosome-specific DNA probes. *Cytogenet Cell Genet*. 1990;54:103-107
11. Sisley K, Rennie IG, Parsons MA *et al.* Abnormalities of chromosomes 3 and 8 in posterior uveal melanoma correlate with prognosis. *Genes Chromosomes Cancer*. 1997;19:22-28.
12. Prescher G, Bornfeld N, Hirche H *et al.* Prognostic implications of monosomy 3 in uveal melanoma. *Lancet*. 1996;347:1222-1225
13. Caron H, van Sluis P, de Kraker J *et al.* Allelic loss of chromosome 1p as a predictor of unfavorable outcome in patients with neuroblastoma. *N Engl J Med*. 1996;334:225-230
14. Knuutila S, Aalto Y, Autio K *et al.* DNA copy number losses in human neoplasms. *Am J Pathol*. 1999;155:683-694
15. Sisley K, Parsons MA, Garnham J *et al.* Association of specific chromosome alterations with tumour phenotype in posterior uveal melanoma. *Br J Cancer*. 2000;82:330-338
16. Aalto Y, Eriksson L, Seregard S, Larsson O, Knuutila S. Concomitant loss of chromosome 3 and whole arm losses and gains of chromosome 1, 6, or 8 in metastasizing primary uveal melanoma. *Invest Ophthalmol Vis Sci*. 2001;42:313-317
17. Prescher G, Bornfeld N, Becher R. Two subclones in a case of uveal melanoma. Relevance of monosomy 3 and multiplication of chromosome 8q. *Cancer Genet Cytogenet*. 1994;77:144-146
18. Gordon KB, Thompson CT, Char DH *et al.* Comparative genomic hybridization in the detection of DNA copy number abnormalities in uveal melanoma. *Cancer Res*. 1994;54:4764-4768
19. Tschentscher F, Prescher G, Horsman DE *et al.* Partial deletions of the long and short arm of chromosome 3 point to two tumor suppressor genes in uveal melanoma. *Cancer Res*. 2001;61:3439-3442
20. Sisley K, Nichols C, Parsons MA *et al.* Clinical applications of chromosome analysis, from fine needle aspiration biopsies, of posterior uveal melanomas. *Eye*. 1998;12 (Pt 2):203-207

Part IV

Critical regions and genes

Chapter 5

Expression of *APITD1* is not related to copy number changes of chromosomal region 1p36 or the prognosis of uveal melanoma

Chapter 5

Expression of *APITD1* is not related to copy number changes of chromosomal region 1p36 or the prognosis of uveal melanoma

Walter van Gils, Hanneke W. Mensink, Emine Kilic, Jolanda Vaarwater, Michael M. Verbiest, Dion Paridaens, Gregorius P. Luyten, Annelies de Klein and Hennie T. Brüggenwirth

Invest Ophthalmol Vis Sci. 2007 Nov;48(11):4919-23.

Abstract

Purpose: Concurrent loss of chromosome 1p36 and chromosome 3 leads to decreased disease-free survival in uveal melanoma patients. A candidate tumor suppressor gene *APITD1* is located on the critical region on chromosome-arm 1p and it was therefore hypothesized that lower expression levels of this gene could lead to decreased survival in patients with concurrent loss of a region on chromosome-arm1p and chromosome 3. Using neuroblastoma cells, which, like uveal melanoma, originate from neural crest cells, Krona *et al.* showed that *APITD1* has cell growth and/or cell death properties. In this study we analyzed if *APITD1* expression corresponds with DNA copy number and is related with survival in uveal melanoma.

Methods: To detect whether copy number loss of *APITD1* results in lowered mRNA expression of the gene, we combined FISH analysis with real-time PCR. In addition, the effect of *APITD1* expression on survival was studied using Kaplan-Meier survival analysis.

Results: Expression of *APITD1* mRNA was not related to DNA copy number ($p=0.956$) or chromosome 3 status ($p=0.958$). Kaplan-Meier survival analysis showed very similar survival curves for tumors with high and low *APITD1* expression with a log-rank significance value of $p=0.9682$.

Conclusions: These results indicate that *APITD1* is not the tumor suppressor gene on 1p36 responsible for the negative prognostic effect in uveal melanoma with concurrent loss of chromosomes 1p36 and 3.

Introduction

Uveal melanoma (UM) is the most common primary malignant intra-ocular tumor in the Western world, with a yearly incidence of 6 per million.[1] Cytogenetic and molecular genetic studies revealed that over 80% of the UMs from sporadic cases have a nearly diploid character with simple non-random chromosomal aberrations, of which amplification of chromosome regions 8q and 6p and loss of chromosome 3 and the chromosomal regions 6q and the distal part of 1p are the most frequent.[2,3] Loss of the chromosomal region 1p36 is also frequently observed in various other tumor types, including neuroblastoma and pheochromocytoma, which also originate from neural crest-derived cells. In neuroblastoma, loss of chromosome-arm 1p is known to be a predictor of unfavorable clinical outcome.[4,5] In UM, loss of the tip of 1p, as was identified with FISH-probe RP11-48E9 located on 1p36, has been detected in metastasizing tumors[3]. Furthermore, concurrent loss of this region and chromosome 3 is associated with decreased survival of UM patients.[2] This suggests that a tumor suppressor gene involved in UM is located on the distal region of 1p. In our own tumor set, we could not identify losses of the telomeric part of chromosome-arm 1p that were smaller than 1p34-pter and Hughes *et al*. identified a smallest region of overlap (SRO) ranging from 1p34-pter using array-CGH.[6] This region is still considerably large and is very gene-dense, which makes it hard to identify candidate genes. However, in neuroblastoma a 500kb region on 1p36.2-1p36.3 was reported,[7] which includes the promising candidate tumor suppressor gene *APITD1* (APoptosis-Inducing, TAF9-like Domain 1) positioned at chromosome band 1p36.22. The protein, encoded by this gene contains a domain which is similar to the human TATA box binding protein-associated factor, TAFII31 (locus name TAF9). TAFII31 has been identified as a critical protein required for p53-mediated transcription activation.[8] As p53 is associated with apoptotic cell death and growth arrest, APITD1 might be important in tumor suppression. Krona *et al.* showed that addition of *APITD1* mRNA to neuroblastoma cells, results in a reduction of cell growth (up to 90%) compared to non-treated cells, suggesting that APITD1 does indeed have a role in the cell death pathway of neuroblastoma.[9] Loss of function or downregulation of APITD1 can thus be a way for tumor cells to overcome the cell growth-regulating properties of the p53 pathway. In UM, the p53 pathway is not affected through alterations in p53 protein levels.[10] Therefore, decreased expression of *APITD1* could be implicated in UM by interfering with the p53 pathway. We have analyzed whether loss of chromosomal region 1p36 leads to decreased expression of *APITD1* in UM. Furthermore, we evaluated whether lower expression levels of *APITD1* were associated with a decreased patient survival. A relation between 1p36 loss and decreased expression would indicate *APITD1* as a possible candidate tumor suppressor gene responsible for poor prognosis in UMs with concurrent loss of chromosomal region 1p36 and chromosome 3.

Methods

Patient samples and cell lines

Fresh tumor material was obtained from patients without prior radiation or chemotherapy within 1 hour after primary enucleation. Informed consent was given prior to enucleation and the study was performed according to the tenets of the Declaration of Helsinki. Tumors were

processed for FISH and cytogenetic analysis as described previously.[11] Part of the tumor was snap-frozen and stored in liquid nitrogen. In addition, eleven UM derived cell lines were used. Mel270, Mel 202, EOM 3, OCM1 and 92.1 are cell lines derived from primary tumors. OMM 1, OMM 2 and OMM 3 were established from metastases from different UM patients and OMM 2.2, OMM 2.3 and OMM 2.6 are all cell lines derived from different metastases of the same patient of whom also Mel 270 was derived.[12-15] Also 2 cell lines obtained from normal eye melanocytes (MC), EMC 1 and EMC 4, were included as a control for expression in normal melanocytic cells.

Fluorescence In Situ Hybridization (FISH)

Dual color FISH on uncultured tumor material, using centromeric and locus specific cosmid, P1 or YAC probes for chromosome 1p, 3, 6 and 8, was performed as described previously.[16] Twenty tumors were selected from our UM database based on the FISH scores on chromosome 1p, ten with loss of 1p36.33 and ten with normal copy numbers of this region. All tumors were further analyzed with BAC probe RP11-199O1 mapping to the *APITD1* gene sequence at 1p36.22, combined with BAC probe RP11-48E9, mapping to 1p36.33, as reference probe. Both probes were selected from the human genome browsers of UCSC (http://genome.ucsc.edu/cgi-bin/hgGateway) and NCBI (http://www.ncbi.nlm.nih.gov/mapview/map_search.cgi). The probes were validated on normal peripheral blood cell metaphase spreads and interphase nuclei. For each probe ten metaphases and 100 interphases were analyzed. Abnormal copy numbers were detected in less than 3 percent of the scored interphase nuclei. In the tumors, signals were counted in 200 interphase nuclei according to the criteria of Hopman *et al.*[17] Cut-off limits for deletion (15% of the nuclei with one signal) or amplification (>10% of the nuclei with 3 or more signals) were adapted from the available literature.[18]

RNA purification, cDNA synthesis and TaqMan assay

Five to eight sections with a thickness of 50 μm were made from fresh frozen tumor material, depending on the size of the tumor. RNA was isolated from the sections using RNA-Bee (TelTest Inc, Friendsweed, Texas USA) and cell line RNA was isolated with the RNeasy Mini Kit (Qiagen, Venlo, the Netherlands) according to the manufacturer's protocol. RNA quantity was measured using the NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware USA) and the quality was assayed on the Bioanalyzer 2100 (Agilent, Palo Alto, California USA). From 1 μg of total RNA cDNA was synthesized using 2μl of a random hexamer primer (0,5 μg/μl) and 10 U of super RT (HT Biotechnology LTD, Cambridge, England) according to the manufacturers instructions. Solutions were diluted to 10 ng/μl for cDNA synthesis.

For the TaqMan assay the TaqMan® Universal PCR Master Mix Kit (Applied Biosystems) was used. TaqMan Gene Expression Assays were selected for *APITD1* and 3 reference genes, beta-2-microglobulin (*B2M*; 15q21-q22), beta-glucuronidase (*GUSB*; 7q21.11) and hypoxanthine phosphoribosyltransferase (*HPRT1*; Xq26.1). Each of these assays consists of two unlabelled primers and a probe labeled with the 5´ reporter dye FAM and the 3´ quencher TAMRA (ordered from the Applied Biosystems assays-on-demand platform). Every reaction contained 3.375 μl H_2O, 12.5 μl Master Mix (2x) (without AmpErase® UNG), 2.5 μl TaqMan Gene Expression

Assay and 1 μl of cDNA. The reactions were run on the ABI PRISM™ 7700 Sequence Detection System (Applied Biosystems). The solution was subjected to a protocol of subsequently 50 °C for 2 min., 95 °C for 10 min. and 45 cycles of 95 °C for 15 s, followed by 60 °C for 1 min.
The efficiency of the TaqMan assay was determined by assaying a control pool of human RNA extracted from three lymphoblastic cell lines in dilutions of 10, 20, 100, 200 and 1000 times. With the ΔRn (threshold) set at 0.1, a standard curve of mean Ct for three replicates at each dilution versus log10 amount of cDNA was determined. The efficiency of the reaction was calculated from the slope of this standard curve using the formula $E^{target} = 10^{-1/slope}$. These efficiencies were 1.9991, 1.9956 and 1.9996 for the reference gene TaqMan assays *B2M*, *GUSB* and *HPRT1* respectively. *APITD1* had an efficiency of 1.9833. Of the three endogenous control genes tested, *GUSB* and *HPRT1* showed the least intra-tumor variation and *GUSB* expression levels came closest to *APITD1* expression (data not shown). Therefore *GUSB* was chosen as the endogenous control in *APITD1* relative expression measurements. To estimate the relative expression of *APITD1* the difference in Ct value of *APITD1* and the chosen endogenous control gene, ΔCt, was determined for each sample. This ΔCt value was transformed by $2^{-\Delta Ct \times 1000}$ to correct for the logarithmic nature of the Ct value. The differences in amplification efficiency between the assays, approximately 2 in all assays, were insignificant and therefore not taken into account in the calculations.

Statistical analysis

Relative expression of *APITD1* mRNA in a group of 10 tumors with loss of one copy of the *APITD1* region was compared with relative expression in a group of 10 tumors without loss of this region in a two-sample t-test. The relative expression of *APITD1* was also compared between groups of tumors with and without monosomy of chromosome 3, consisting of 10 and 9 samples respectively (the chromosome 3 status of one patient was uncertain). The tumors were divided into a group with high and low expression to analyze the influence of *APITD1* expression levels on disease-free survival, with 12 (the geometric mean of all samples) chosen as the highest value for $2^{-\Delta Ct \times 1000}$ in the group of low expression, thereby dividing the patients in two groups of ten each. Kaplan-Meier survival analysis and the log rank test were performed to determine the influence of *APITD1* expression on survival. Disease-free survival is the time from enucleation to the development of metastatic disease or disease-related death. All tests were two-sided. Statistical analyses were performed using SPSS-11 software.

Results

FISH

Twenty tumors were selected from our UM database on the basis of routine FISH scores on chromosomal region 1p36. Ten showed loss of one copy of 1p36.33 and ten had normal copy numbers of this region. All tumors were analyzed using FISH with BAC probes RP11-199O1 (1p36.22) and RP11-48E9 (1p36.33). Results are presented in table 1. The results found with the diagnostic probe RP11-48E9 were not different from the results obtained with the *APITD1* probe RP11-199O1, indicating that the region of loss detected in routine FISH encompassed at least the *APITD1* gene in all cases.

Real-time PCR

APITD1 expression was analyzed using Quantitative Real-Time PCR. *APITD1* was not differentially expressed in melanocytic and UM derived cell lines. Independent sample t-test showed a 4.1 times higher expression in cell lines compared to expression in the primary tumors ($p<0.001$) (fig.1). There was no significant difference in *APITD1* expression levels between tumors with and without loss of 1p36, irrespective of chromosome 3 status ($p= 0.956$). Irrespective of chromosomal 1p36 status *APITD1* expression levels were also not significantly different between tumors with and without loss of chromosome 3 ($p=0.958$) and between tumors with concurrent loss of 1p36 and chromosome 3 and tumors without loss of chromosome-arm 1p and chromosome 3 ($p=0.764$). To test for a significant difference between *APITD1* expression and patient survival, the samples were separated into groups of high and low *APITD1* expression. Kaplan Meier survival analysis showed very similar patterns of patient survival in both groups, with a resulting log rank p-value close to 1 (0.9682) (fig.2).

Table 1. **Relative expression of APITD1 compared to GUSB with DNA copy number and follow-up data**

Tumor	Relative expression of APITD1 ($2^{-\Delta Ct}$ x 1000)	DNA Copy number[1]			Survival (years)	Event[2]
		RP11-48E9 1p36.33	RP11-199O1 APITD1	RP11-64F6 Chromosome 3		
1	3.01	1	1	1	3.14	0
2	2.52	1	1	1	1.31	1
3	7.71	1	1	1	1.62	1
4	13.33	1	1	1	6.02	0
5	8.28	1	1	1	3.05	1
6	38.01	1	1	1	0.43	1
7	16.08	1	1	2	7.83	0
8	17.03	1	1	2	4.03	1
9	11.54	1	1	2	5.97	0
10	13.17	1	1	2	4.97	0
11	9.58	2	2	1	8.41	0
12	15.13	2	2	1	2.13	1
13	22.82	2	2	1	7.31	0
14	11.90	2	2	1	3.42	1
15	18.24	2	2	2	8.74	0
16	13.97	2	2	2	2.26	0
17	12.62	2	2	2	2.87	1
18	5.98	2	2	2	6.56	0
19	8.66	2	2	2	4.05	0
20	9.80	2	2	NA	5.63	0

[1]The reported copy numbers were scored in 72% of the counted nuclei in one case and over 80% in all other cases; NA= data not available. [2]0: no event has occurred; 1: event (melanoma-related death).

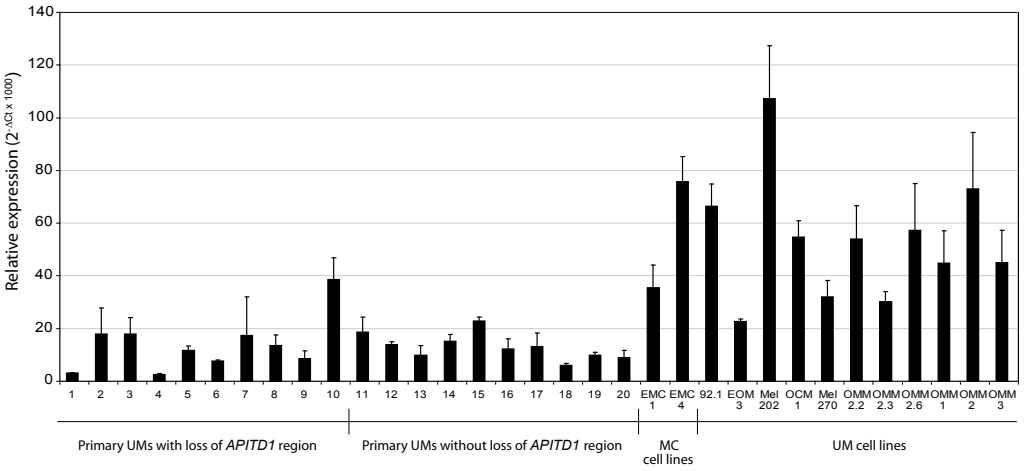


Figure 1. ***APITD1* expression in UM and MC cell lines and primary UMs.** Results of the primary UMs are divided into two groups, based on the number of *APITD1* DNA copies. The Y-axis indicates the relative expression of *APITD1* compared to the *GUSB* housekeeping gene, calculated with $2^{-\Delta Ct \times 1000}$. The identity of each sample is indicated along the X-axis.

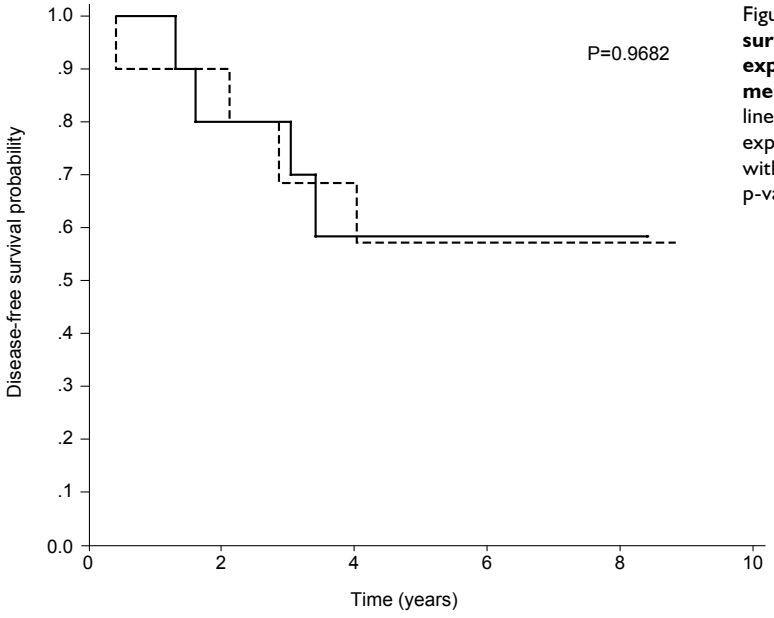


Figure 2. **Kaplan-Meier survival analysis of APITD1 expression in 20 uveal melanoma patients.** Dashed line: tumors with high *APITD1* expression; solid line: tumors with low *APITD1* expression. p-value is indicated.

Discussion

A frequent characteristic of UM is deletion of the distal part of chromosome 1p. In combination with the loss of chromosome 3, loss of 1p36 leads to decreased disease free survival.[2] This implicates 1p36 as a location of a UM prognosis-related related suppressor gene. In neurblastoma, also originating from neural crest-derived cells, a small cluster of genes in a 500kb SRO was reported. The genes in this cluster, *APITD1, UBE4B*/*UFD2*, *KIF1B*, *PGD*, *DFFA* and *PEX14*, are all downregulated in high-stage neuroblastomas and are all candidate tumor suppressor genes. For *APITD1, UBE4B*/*UFD2* and *DFFA* a relation with high stage neuroblastoma was reported. Besides a lower expression in high-stage neuroblastomas, a splice site mutation was detected in *UBE4B*/*UFD2* in a high-stage neuroblastoma with a fatal outcome and there are also some coding mutations found in the *DFFA* gene in neuroblastoma. The *DFFA* gene has essential functions in the final stage of apoptosis. This altogether suggests a role of these genes at least in high-stage neuroblastoma. [7,10,19-22] In UM, the p53 pathway is not affected through alterations in p53 protein levels.[9] Therefore, interference of the p53 pathway could be caused by another mechanism. Kilic *et al.* suggested that p73, a p53 homologue located on 1p36, might be a possible prognosis-related suppressor gene (unpublished data). Another candidate gene, located on 1p36 is *CHD5*, which encodes a protein that functions in the p53 pathway, was recently shown to function as a tumor suppressor *in vivo.*[23] The *APITD1* gene in the reported neuroblastoma gene cluster is associated with p53 activity and has been shown to inhibit cell growth.[7,10] Since expression of *APITD1* is almost absent in a variety of tumors[10] and because of its is relation with p53 activity, down-regulation of *APITD1* could provide an alternative way to interfere with the p53-mediated pathway in tumors without alterations in p53 protein levels. Therefore, we characterized expression and copy number of the *APITD1* gene in UM.

We combined FISH analysis with real-time PCR to assess if copy number loss of *APITD1* results in lowered expression of the gene. A Kaplan-Meier survival analysis was performed with *APITD1* expression as a discriminator to study the effect of *APITD1* expression on survival. We analyzed *APITD1* expression in 10 tumors with and 10 tumors without chromosomal loss of 1p36, using a FISH probe mapping to 1p36.33. In all cases, loss of this region concurred with the loss of one copy of the *APITD1* region, whereas retention of two copies of 1p36.33 was always combined with two copies of the *APITD1* region. There was no difference found in *APITD1* expression between tumors with and without loss of 1p36. Similar results were obtained for tumors with and without loss of chromosome 3 and for tumors with and without concurrent loss of 1p36 and chromosome 3. In addition, in Kaplan-Meier survival analysis based on high and low expression of *APITD1,* the two groups showed very similar survival curves with a log-rank significance value of 0.9682 (fig.2). This indicated that downregulation of *APITD1* is probably not the mechanism for immortality of those cell lines We showed that expression of the *APITD1* transcript is generally elevated in both melanocyte and UM derived cell lines, compared to primary UMs. This finding is in concordance with the earlier report on *APITD1* expression in neuroblastoma in which *APITD1* expression was also considerably lower in primary tumors compared to neuroblastoma-derived cell lines.[10]

The negative effect on prognosis of loss of chromosome-band 1p36 in tumors with monosomy of chromosome 3 is most probably caused by decreased expression of a tumor suppressor gene located on this region as a result of the chromosomal loss. Following the data in table 1

we could verify for this group of cases that concurrent loss of chromosomal region 1p36 and chromosome 3 has an adverse effect on patient outcome (Log Rank significance p-value of 0.0259), which shows that that this cohort has the correct size and composition to evaluate this adverse survival effect. In the present study, *APITD1* mRNA levels alone are not associated with survival. Furthermore, we could not find differential expression for *APITD1* in tumors with and without loss of the 1p36 region. From these results we conclude that *APITD1* is not the suppressor gene on 1p36 responsible for the poor prognosis in UMs with concurrent loss of chromosome 1p36 and chromosome 3.

Acknowledgements

We would like to thank Ms. Eline van Meel for excellent technical assistance. We would also like to thank Ms. Ruchi Saxena for critically reviewing the manuscript and helpful comments.

References

1 Singh AD, Topham A. Incidence of uveal melanoma in the United States: 1973-1997. *Ophthalmology*. 2003;110:956-961.

2 Kilic E, Naus NC, van Gils W *et al*. Concurrent loss of chromosome arm 1p and chromosome 3 predicts a decreased disease-free survival in uveal melanoma patients. *Invest Ophthalmol Vis Sci*. 2005;46:2253-2257.

3 Aalto Y, Eriksson L, Seregard S, Larsson O, Knuutila S. Concomitant loss of chromosome 3 and whole arm losses and gains of chromosome 1, 6, or 8 in metastasizing primary uveal melanoma. *Invest Ophthalmol Vis Sci*. 2001;42:313-317.

4 Caron H, van Sluis P, de Kraker J *et al*. Allelic loss of chromosome 1p as a predictor of unfavorable outcome in patients with neuroblastoma. *N Engl J Med*. 1996;334:225-230.

5 Casciano I, Mazzocco K, Boni L *et al*. Expression of DeltaNp73 is a molecular marker for adverse outcome in neuroblastoma patients. *Cell Death Differ*. 2002;9:246-251.

6 Hughes S, Damato BE, Giddings I *et al*. Microarray comparative genomic hybridisation analysis of intraocular uveal melanomas identifies distinctive imbalances associated with loss of chromosome 3. *Br J Cancer*. 2005;93:1191-1196.

7 Ejeskar K, Sjoberg RM, Abel F *et al*. Fine mapping of a tumour suppressor candidate gene region in 1p36.2-3, commonly deleted in neuroblastomas and germ cell tumours. *Med Pediatr Oncol*. 2001;36:61-66.

8 Lu H, Levine AJ. Human TAFII31 protein is a transcriptional coactivator of the p53 protein. *Proc Natl Acad Sci U S A*. 1995;92:5154-5158.

9 Krona C, Ejeskar K, Caren H, Abel F, Sjoberg RM, Martinsson T. A novel 1p36.2 located gene, APITD1, with tumour-suppressive properties and a putative p53-binding domain, shows low expression in neuroblastoma tumours. *Br J Cancer*. 2004;91:1119-1130.

10 Brantley MA, Jr., Harbour JW. Deregulation of the Rb and p53 pathways in uveal melanoma. *Am J Pathol*. 2000;157:1795-1801.

11 Naus NC, Zuidervaart W, Rayman N *et al*. Mutation analysis of the PTEN gene in uveal melanoma cell lines. *Int J Cancer*. 2000;87:151-153.

12 Verbik DJ, Murray TG, Tran JM, Ksander BR. Melanomas that develop within the eye inhibit lymphocyte proliferation. *Int J Cancer*.1997;73:470-478.

13 Luyten GP, Naus NC, Mooy CM *et al*. Establishment and characterization of primary and metastatic uveal melanoma cell lines. *Int J Cancer*. 1996;66:380-387.

14 Kan-Mitchell J, Mitchell MS, Rao N, Liggett PE. Characterization of uveal melanoma cell lines that grow as xenografts in rabbit eyes. *Invest Ophthalmol Vis Sci*. 1989;30:829-834.

15 De Waard-Siebinga I, Blom DJ, Griffioen M *et al*. Establishment and characterization of an uveal-melanoma cell line. *Int J Cancer*. 1995;62:155-161.

16 Hagemeijer A, de Klein A, Wijsman J, van Meerten E, de Greef GE, Sacchi N. Development of an interphase fluorescent in situ hybridization (FISH) test to detect t(8;21) in AML patients. *Leukemia*. 1998;12:96-101.

17 Hopman AH, Ramaekers FC, Raap AK *et al*. In situ hybridization as a tool to study numerical chromosome aberrations in solid bladder tumors. *Histochemistry*. 1988;89:307-316.

18 van Dekken H, Pizzolo JG, Reuter VE, Melamed MR. Cytogenetic analysis of human solid tumors by in situ hybridization with a set of 12 chromosome-specific DNA probes. *Cytogenet Cell Genet*. 1990;54:103-107.
19 Caren H, Ejeskar K, Fransson S *et al*. A cluster of genes located in 1p36 are down-regulated in neuroblastomas with poor prognosis, but not due to CpG island methylation. *Mol Cancer*. 2005;4:10.
20 Abel F, Sjoberg RM, Krona C, Nilsson S, Martinsson T. Mutations in the N-terminal domain of DFF45 in a primary germ cell tumor and in neuroblastoma tumors. *Int J Oncol*. 2004;25:1297-1302.
21 Abel F, Sjoberg RM, Ejeskar, Krona C, Martinsson T. Analyses of apoptotic regulators CASP9 and DFFA at 1P36.2, reveal rare allele variants in human neuroblastoma tumours. *Br J Cancer*. 2002;86:596-604.
22 Krona C, Ejeskar K, Abel F *et al*. Screening for gene mutations in a 500 kb neuroblastoma tumor suppressor candidate region in chromosome 1p; mutation and stage-specific expression in UBE4B/UFD2. *Oncogene*. 2003;22:2343-2351.
23 Bagchi A, Papazoglu C, Wu Y *et al*. CHD5 is a tumor suppressor at human 1p36. *Cell*. 2007;128:459-475.

Chapter 6

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

Chapter 6

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

Walter van Gils, Emine Kiliç, Michael M.P.J. Verbiest, Bert Eussen, Nicole C. Naus, Bruce R. Ksander, Gregorius P.M. Luyten, Annelies de Klein and Hennie T. Brüggenwirth

Submitted for publication

Abstract

Purpose: Loss of chromosome 3 is frequently observed in uveal melanoma and is associated with poor prognosis. In about 50% of the uveal melanomas one copy of chromosome 3 is lost. Using comparative genomic hybridization (CGH) we have detected a chromosome 3q13-3q21 deletion in a uveal melanoma cell line, Mel270, which is derived from a primary tumor. The aim of the present study is to demarcate this region, which could harbor a tumor suppressor gene (TSG).
Methods: Genomic DNA was extracted from four uveal melanoma cell lines, established from one primary tumor, Mel270, and its metastases. Subsequently, loss of heterozygosity (LOH) analysis was performed. LOH studies were complemented with fluorescent *in situ* hybridization (FISH).
Results: LOH and FISH studies revealed a chromosome 3q deletion ranging from 3q21.2-3q24 in two cell lines, Mel270 and OMM2.3, derived from the primary tumor and one of its metastases, respectively. In addition, a region of allelic loss, mapping to 3p24, was found in these cell lines. In contrast, FISH probes mapping to 3p24 revealed the presence of two copies. In OMM2.2, established from a different metastasis that originated from the same primary tumor from which OMM2.3 was also derived, LOH was detected at most of the loci that were analyzed. This finding is consistent with isodisomy of chromosome 3 in OMM2.2.
Conclusions: We have fine-mapped structural deletions located at chromosome 3q and a hemizygous region at chromosome 3p in uveal melanoma cell lines. These results contribute to a further demarcation of a candidate region for tumor suppressor genes.

Introduction

Uveal melanoma, the most common malignant intra-ocular tumor in adults, affects 6 per million adults of the Western population yearly. [1] Uveal melanomas originate from neural crest derived melanocytes of the uvea and can be located posterior in the choroid and anterior in the ciliary body or in the iris. In the past the only treatment available was enucleation of the eye, while nowadays eye-conservative treatments like brachytherapy, external beam irradiation and stereotactic radiotherapy have become the first choice of treatment. Only 2% of the cases have clinically detectable metastasis at presentation and despite improved primary tumor treatment protocols, 50% of the patients die from distant metastasis that most often disseminate to the liver (90% of all cases with metastasis). [2]

Cytogenetic and molecular genetic studies revealed that the larger part of the uveal melanomas from sporadic cases have a nearly diploid character with simple non-random chromosomal aberrations. Loss of chromosomes 1 and 3, structural abnormalities of chromosome 6, and gain of chromosome 8q are most frequently observed. [3-8] Chromosome 3 loss is a prognostic marker for decreased survival of the patient [4-6, 9] and several studies indicated gain of chromosome 8 as an independent prognostic marker of poor survival. [5, 6] Furthermore, loss of chromosome 1p was observed in primary tumors that had metastasized and in metastases. [8, 10]

Involvement of chromosome 3 is considered a primary event. [3, 11] In uveal melanomas that are characterized by different sub-populations, loss of chromosome 3 is a constant event, whereas a variable number of copies of the long arm of chromosome 8 can be observed. In many uveal melanomas an entire chromosome 3 is lost [8, 12, 13] and in some cases with two apparently normal chromosomes 3, acquired isodisomy has been observed. [12] It is generally believed that loss of a tumor suppressor gene (TSG) located at chromosome 3 plays a role in uveal melanoma development. Only a few melanomas with structural abnormalities of chromosome 3 or translocations involving chromosome 3 have been reported up to now, which complicates mapping of putative TSGs. However, a study by Tschentscher *et al.* [14], who investigated uveal melanomas with structural abnormalities of chromosome 3, revealed two regions of allelic loss on chromosome 3, *i.e.* 3p25 and 3q24-3q26. Moreover, they concluded that tumors that metastasized showed loss of both regions. Parrella *et al.* defined a minimal region of allelic loss, ranging from 3p25.1-3p25.2 in a set of uveal melanomas. [15]

The present study aimed at further delineation of a chromosome 3q deletion, previously detected with conventional comparative genomic hybridization (CGH) in Mel270, a uveal melanoma cell line established from a primary tumor that metastasized. [16] LOH analyses were performed on Mel270 and a series of metastasis-derived cell lines, obtained from liver metastasis, originating from the primary tumor from which Mel270 was derived. These analyses were complemented by FISH analyses, allowing identification of chromosomal aberrations on a single cell level. In addition, we have analyzed the p-arm of chromosome 3 for the presence of allelic loss. Our results are discussed in the context of other published structural chromosome 3 deletions found in uveal melanomas.

Materials and methods

Cell Lines

For the present study uveal melanoma cell lines were used. Mel270 was established from a primary uveal melanoma. [17] OMM2.2, OMM2.3 and OMM2.6 are metastatic cell lines that originated from three different liver metastases of the same patient from whose primary tumor Mel270 was established. [18] All cell lines were grown in HEPES and glutamate containing RPMI 1640 culture medium, supplemented with 10% fetal calf serum, 1% penicillin and 1% streptomycin. Cells were passaged, depending on growth rate.

Comparative Genomic Hybridization

Comparative genomic hybridization on metaphases was performed according to Naus *et al*. [16]

Loss of Heterozygosity

Polymorphic microsatellites for LOH studies were selected using the UCSC Genome Bioinformatics website (http://www.genome.cse.ucsc.edu) and synthesized by Life Technologies (Breda, The Netherlands). An overview of the markers that we used is given in Table 1 (chromosome 3q) and Table 2 (chromosome 3p). Primer sequences and locations are available upon request. Genomic DNA was isolated from the cell lines using standard techniques. Amplification reactions were performed in a 50 μl mixture, containing 50 pmoles of each oligonucleotide, 10 mM of each dNTP, 0.25 units Supertaq polymerase (HT Biotechnology Ltd., Cambridge, England), Supertaq buffer and about 100 ng genomic DNA. Reactions were denatured at 95°C and subjected to 30 cycles of denaturation at 95°C for 1 minute, annealing for 1.5 minutes at 55°C (except for marker *D3S1580*: annealing temperature 57°C) and elongation at 72°C for 2 minutes, followed by 10 minutes final extension at 72°C. Obtained polymerase chain reaction (PCR) products were purified using Qiaquick PCR purification system (Qiagen, Westburg, Leusden, The Netherlands). Subsequently, 1 μl purified PCR product was radioactively end-labeled, using 5 U polynucleotide kinase (Roche Molecular Biochemicals, Almere, The Netherlands) and 2 μCi [γ-32P] ATP (Amersham Pharmacia Biotech UK Ltd., Little Chalfont, United Kingdom). Denaturing stop mix (95% deionized formamide, 20 mM EDTA, 0.02% xylene cyanol FF, 0.02% bromophenol blue) was added in an equal volume. After heating for 5 minutes, the samples were quickly chilled and 3 μl samples were loaded on a 6% denaturing polyacrylamide gel, which was run at 60 W. After electrophoresis the gel was dried on Whattman paper and a Fuji super RX film was exposed. Autoradiograms were visually analyzed. Since cell lines were used, marker patterns specific for the uveal melanoma cell lines could not be compared with that of a corresponding control. Therefore, results obtained for Mel270 were compared to those achieved for the corresponding metastases. Furthermore, DNA samples extracted from other uveal melanoma cell lines and human placenta DNA from a healthy individual were taken along.

Table 1. **Results of chromosome 3q analysis of Mel270, using FISH and LOH**

Position#	BAC	Copy number	Marker	Number of alleles
3q21.1			D3S1267	2
3q21.2	RP11-95H16	2	D3S1269	2
3q21.2			D3S1589	1
3q21.2	RP11-205A6	1		
3q21.3	RP11-59J16	?	D3S3606	1
3q22.1	-		D3S1587	1
3q22.1	-		D3S1292	1
3q22.1	-		D3S1273	1
3q22.2	RP11-220J13	?	D3S1615	1
3q22.3			D3S3528	1
3q22.3	RP11-162J10	1	D3S1576	1
3q23	RP11-166D18	1	D3S3554	1
3q23	-		D3S1309	1*
3q23	-		D3S3694	1
3q23	RP11-160A13	1	D3S3546	1
3q24	RP11-165M11	?	D3S1569	2
3q24	RP11-72E23	1	D3S1557	1
3q24	-		D3S1593	1
3q24	RP11-88H10	1	D3S1608	1*
3q24	-		D3S3627	1
3q24	-		D3S196	1*
3q24	-		D3S2440	1
3q24	-		D3S3618	1*
3q24	-		D3S1306	1
3q24	-		D3S3626	2
3q24	RP11-229G6	2	-	
3q25.1	RP11-145F16	?	D3S1299	1
3q25.1	RP11-64F6	2	D3S1279	2
3q25.1	RP11-65L11	2	-	
3q25.2	RP11-80I14	2	D3S1280	2
3q26.1	-		D3S3702	2
3q26.31	-		D3S2421	1
3q28	-		D3S1580	1*
3q28	-		D3S1294	2
3q28	-		D3S1601	2
3q29	-		D3S1272	1

Corresponding FISH clones and markers are presented at the same line. ?: presence of a subclone, *: presence of a weak second allele.
position according to the Humane Genome Browser (March 2006) (http://genome.cse.ucsc.edu/).

Fluorescent in Situ Hybridization

Dual color interphase FISH was performed on cultured Mel270 cells as described previously by Naus *et al.* [19] The probes that we used are locus-specific bacterial artificial chromosome (BAC) clones, selected from the Roswell Park Cancer Institute database (http://genomics.roswellpark.org/human/overview.html) and obtained from CHORI-BACPAC Resources (Oakland, CA, United States). An overview of the BAC probes that we used is given in Tables 1 and 2. Some of them

correspond to the markers that we selected for LOH analysis. Five ng of centromeric probe Pα3.5 was used per slide, 100 ng of telomeric probe B47A2 (kind gift of L. Kearney and J. Flint) [20] and 75 ng probe in case of BAC clones. The probes were validated on normal peripheral blood cell metaphase spreads and ten metaphases were analyzed for each probe. Cut-off limits were less than three percent. For deletion mapping, signals in 300 interphase nuclei were counted according to the criteria of Hopman *et al*. [21] The cut-off values used for monosomy (only one signal in more than 15% of the nuclei) or polysomy (more than 10% of the nuclei with 3 or more signals) were adapted from available literature. [22] In case subclones were identified, only findings concerning the largest clone were used for analysis.

Table 2. **Results of chromosome 3p analysis of Mel270, using FISH and LOH.**

Position#	BAC	Copy number	Marker	Number of alleles
3p26.3			D3S3050	2
3p26.1	RP11-28P14	2	D3S1304	1*
3p26.1	-		D3S3728	2
3p26.1	-		D3S3591	1
3p26.1	-		D3S1537	2
3p26.1	-		D3S4545	2
3p25.3	RP11-128A5	2	D3S3691	2
3p25.3	-		D3S1597	2
3p25.1	-		D3S3693	1
3p25.1	-		D3S3608	2
3p24.3	RP11-255O19	2	D3S1286	2
3p24.3	-		D3S1293	1
3p24.3	RP11-208G16	2	-	
3p24.3-24.2	RP11-41F5	2	-	
3p24.1	-		D3S1266	1
3p24.1	RP11-11L6	2	-	
3p24.1			D3S3727	1
3p22.3			D3S2432	2
3p22.3			D3S3518	2
3p22.3			D3S1619	2
3p22.2	RP11-209O16	2	-	
3p21.32-3p21.31	RP11-189H19	2	-	
3p13			D3S2406	2

Corresponding FISH clones and markers are presented at the same line. *: presence of a weak second allele.
Position according to the Humane Genome Browser (March 2006) (http://genome.cse.ucsc.edu/).

Results

Loss of Heterozygosity

We have previously shown a deletion of chromosome 3q13-3q21 in Mel270, using conventional CGH (fig. 1). [16] For a further demarcation of this region of loss, LOH studies were performed. For the LOH analyses microsatellite markers were selected in and around the deletion region 3q13-3q21 in Mel270. Using this set of markers, loss of heterozygosity was identified for Mel270

but only with markers, mapping to chromosome 3q22.1. Therefore, we extended our marker set with markers mapping between chromosome band 3q22 and the 3q telomere. Cell lines from corresponding metastases, *i.e.* OMM2.2, OMM2.3, and OMM2.6 were analyzed along with Mel270. Mel270 and OMM2.3 showed LOH with consecutive markers, mapping to loci at 3q21.2-3q23 (fig. 1 and fig. 2). In OMM2.6 our results were indicative of loss at loci from 3q13.31 to the 3q telomere, whereas OMM2.2 showed loss at most loci tested along the q-arm.
In uveal melanomas that metastasized, another region of LOH, mapping to chromosome 3p25, has been observed .[14, 15] To establish whether chromosome 3p deletions could also be detected in Mel270 and related cell lines OMM2.2, OMM2.3 and OMM2.6, eighteen different markers, mapping between chromosome band 3p26.2 and 3p13, were analyzed (fig. 1). In Mel270, OMM2.3 and OMM2.6 loss of heterozygosity was detected with successive markers *D3S1293*, *D3S1266* and *D3S3727*, representing loci at 3p24.1-3p24.3. Results obtained for OMM2.2 were again indicative of isodisomy.

Fluorescent In Situ Hybridization

Complementary to the LOH studies, interphase FISH was performed on Mel270 cells, using fifteen BAC clones mapping to 3q21.1-3q25.2. Twelve of these clones corresponded to the polymorphic markers we used (see Table 1). Results obtained from LOH analysis and FISH studies were not in complete accordance. While FISH clone *RP11-88H10* showed loss of one copy, the corresponding microsatellite marker *D3S1608* showed the presence of a weak second allele (fig 3A and Table 1). With FISH clone RP11-165M11 unclear results were obtained and the corresponding marker *D3S1569* showed presence of 2 alleles. Markers *D3S1309*, *D3S196* and *D3S3618* also revealed a weak second allele while flanking FISH probes and markers were indicative of loss. Marker *D3S3626* showed no allelic loss and could demarcate the telomeric border of the deletion. Results obtained with markers *D3S1299*, *D3S2421* and *D3S1272*, located at the telomeric site of the deletion border, showed the presence of one allele and marker *D3S1580* the presence of a weak second allele.
The p-arm of chromosome 3 was studied with a panel of eight FISH clones, mapping from 3p21.32-3p26.1. Although microsatellite analysis was indicative of allelic loss at four consecutive loci ranging from 3p24.1-3p24.3, interphase FISH revealed the presence of two copies of chromosome 3p at all the loci studied (table 2 and fig. 3B). Signals obtained with probe *RP11-11L6* were not specific enough, using interphase FISH. Therefore, metaphases were analyzed; in 33 out of 36 metaphases two copies were detected.

Discussion

Cytogenetic studies revealed that Mel270 exhibits the most important chromosomal aberrations, *i.e.* loss of chromosome 3 and gain of chromosome 8, which are consistently observed in uveal melanoma.[16] Therefore, we believe that Mel270 can safely be used as a model system, guaranteeing an unlimited supply of material, which is very helpful in the search of putative TSGs. Loss of heterozygosity analysis on cell lines is complicated by the fact that corresponding normal DNA of the patient is not available. In case only one allele is observed it is not possible to discriminate between loss of one allele and lack of heterozygosity of the used microsatellite.

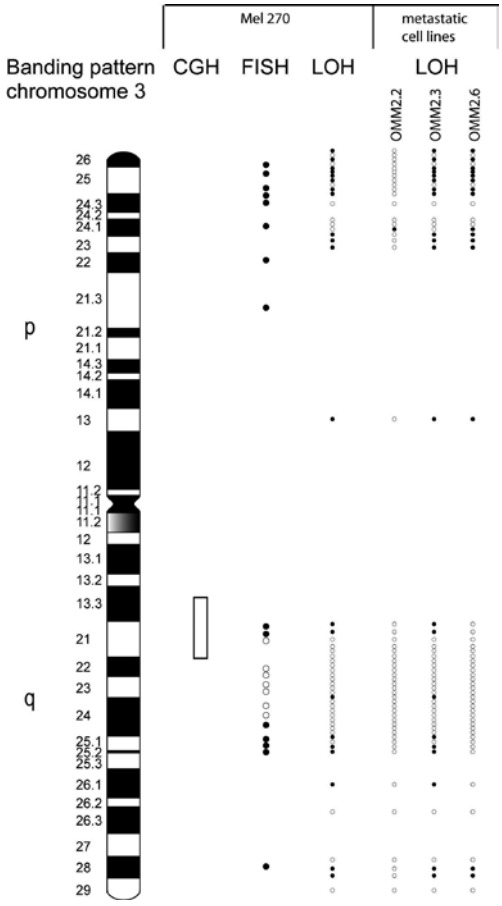


Figure 1. **CGH, FISH and microsatellite analyses on Mel270 and 3 metastatic cell lines.** On the right of the chromosome 3 ideogram the results of respectively, CGH, FISH and microsatellite instability analyses are shown. The deleted region in CGH on nine metaphases is indicated with an open bar. For both FISH and microsatellite analysis the positions and results are indicated with circles. Filled circle: no copy number change was observed; open circle: copy number loss (FISH); loss of heterozygosity (LOH). The LOH analysis results of the three metastatic cell lines OMM2.2, OMM2.3 and OMM2.6 are shown next to the LOH data of Mel270.

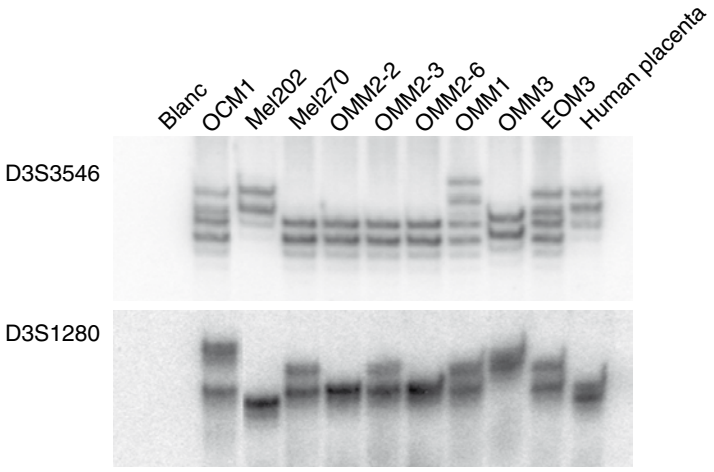


Figure 2. **Microsatellite analysis of several cell lines with different primer sets.** Representative results obtained with chromosome 3q markers *D3S3546* and *D3S1280* are shown.

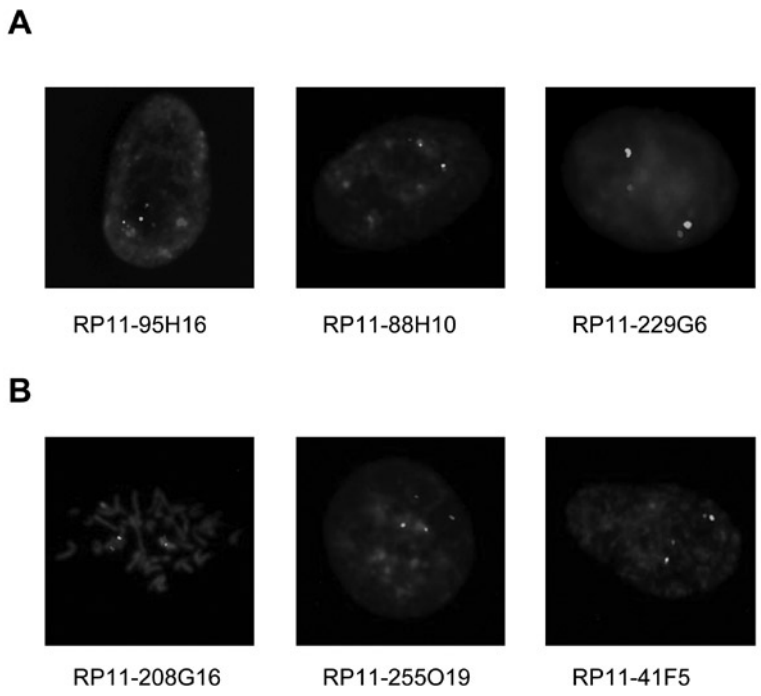


Figure 3. **FISH analysis of Mel270.**
A: representative results of dual color FISH on Mel270 cells, hybridized with a combination of a chromosome 3q probe (red) and a centromere 3 probe (Pα3.5) (#3) (green), except for probe *RP11-95H16* (green) that was combined with a 3pter probe (B47A2) (red). Panel A: 3pter probe and *RP11-95H16*; #3 and *RP11-88H10;* and #3 and *RP11-229G6.*
B: representative results of dual color FISH on Mel270 cells, hybridized with a combination of a chromosome 3p probe (red) and a centromere 3 probe (Pα3.5) (#3) (green). Panel B: #3 and *RP11-208G16*, #3 and *RP11-255O19*, and #3 and *RP11-41F5*.
Color figure can be found on page 144.

However, since we were able to compare a cell line derived from a primary tumor (Mel270) and three metastatic cell lines (OMM2.2, OMM2.3 and OMM2.6), obtained from three different liver metastases from this same primary tumor, we were able to differentiate possible LOH. Furthermore, in case of Mel270, FISH was performed, complementary to LOH analysis.
Combining the data obtained with microsatellite analysis and FISH revealed allelic loss at chromosome 3q21.2-3q24 in Mel270 (Table 1). Cell line OMM2.3 showed LOH at the same loci. Results obtained with microsatellite analysis and FISH were not always corresponding. Markers *D3S1309*, *D3S1608*, *D3S196* and *D3S3618* showed the presence of a weak second allele. This might be the result of the presence of an extra homologous binding site in the genome, giving rise to an extra product, since FISH data obtained with probe *RP11-88H10*, corresponding to *D3S1608*, clearly showed loss of one allele. Markers *D3S1309* and *D3S1608*, for example, are both attributed to two STSs, as was found with the web-based NCBI e-PCR tool. Unknown second target sites could also be present for the other markers. Weak extra alleles might also be explained by the presence of a subclone in the tumor. Marker D3S3626, showing two

alleles, marks the end of LOH region. But also at the telomeric site of this deletion border some inconsistencies were observed. Markers *D3S1299*, *D3S2421* and *D3S1272* showed the presence of one allele and marker *D3S1580*, which is also attributed to two STSs, showed only a weak second allele. Inconsistencies like this can also be explained by lack of heterozygosity for these markers. Furthermore, it is also known from literature that LOH analysis is not always a reliable technique. [23]
While CGH analysis previously revealed a deletion at 3q13-3q21 [16], our fine-mapping studies pointed to a deletion ranging from 3q21.2-3q24. This discrepancy can be explained by the fact that the resolution of conventional CGH on metaphases is limited. [24]
Along the p-arm allelic loss was detected in Mel270 and corresponding cell lines OMM2.3 and OMM2.6 with three consecutive markers, mapping to 3p24.1-3p24.3. Since FISH analysis revealed two copies in this region, this could point to a hemizygous region. However, false positive results, due to lack of heterozygosity, cannot be excluded. CGH analysis and karyotyping (results not shown) were not indicative of any chromosome 3 loss in OMM2.2 and OMM2.6, but the LOH analysis points to isodisomy of whole chromosome 3 in OMM2.2, and a large part of the long arm of chromosome 3 in OMM2.6.

Although in a large part of the uveal melanomas monosomy of chromosome 3 is found [13], several studies revealed structural abnormalities of chromosome 3p and or 3q in uveal melanomas. A partial duplication, involving the long arm of chromosome 3 with a breakpoint at 3q25 has been described by Prescher *et al*. [25] Scholes *et al*. [13], who performed LOH studies, reported a 3q deletion, ranging from a region between marker *D3S1589* (3q21.2) and *D3S1605* (3q25.32) to the telomere. A LOH study by Tschentscher *et al*. [14], performed on uveal melanoma with structural abnormalities, allowed definition of a smallest region of overlap (SRO) at chromosome 3q24-3q26. *(D3S196-D3S1763*). At the telomeric site the SRO is flanked by marker *D3S1763*, mapping to 3q26.1, and marker *D3S2425* located at 3q26.31, showing loss and retention, respectively. At the centromeric site the SRO is flanked by marker *D3S196* (3q24) showing LOH and marker *Mdf2* at the RHO locus (3q21.3) showing retention of both alleles. In the present study, a deletion, starting at marker *D3S1589* at 3q21.2 and ending at marker *D3S3626* (3q24*)* at the telomeric site, was found. Combining our data with data from Tschentscher *et al*. [14] yielded an overall SRO, ranging from 3q21.3-3q24. Furthermore, these data corroborate with a study by Dahlenfors *et al*. pointing to the 3q23 region as a possible TSG location, based on a rearrangement found in one UM. [26] As far as we know, UM specific TSGs mapping to 3q21.3-3q24 have not been identified to date. A candidate tumor suppressor gene in uveal melanoma, *tp63*, is located outside this region, on 3q27. [27] In other tumor types loss of chromosome 3 has also been described. Loss of chromosome 3q seems to be an early event in pheochromocytomas as well. [28]
Aberrations of the p-arm of chromosome 3 have also been described. In one uveal melanoma a translocation with a breakpoint at 3p13 was found. [29] Tschentscher *et al*. suggested that in metastasized uveal melanoma two regions on chromosome 3 (a region on the q-arm and a region on the p-arm) harbor TSGs. [14] This could explain the frequently observed loss of an entire chromosome 3 in those tumors. Although our CGH and FISH analysis did not reveal a chromosome 3p deletion in Mel270, microsatellite analysis pointed to a region of allelic loss, ranging from 3p24.1-3p24.3. This possible hemizygous region is flanked by marker *D3S1293*

(3p24.3) showing LOH and marker *D3S1286* (3p24.3) showing retention of two alleles at the telomeric site. The border of the centromeric site is defined by marker D3S3727 (3p24.1) and *D3S2432* (3p22.3), showing allelic loss and retention of two alleles, respectively. This region does not overlap with the SRO's defined by Tschentscher *et al*. (3p25.3-3p26.1) and Parrella *et al*. (3p25.1-3p25.2) (14, 15). However, in an earlier study by Sisley *et al*. allelic loss of the *Thyroid Hormone Receptor B* (*THRB*) locus was observed in 60% of the uveal melanoma investigated. [30] The human *THRB* locus, which maps to chromosome 3p24.2 (human genome draft, version May 2004), acts as a transcriptional activator and silencer. Aberrant expression and/or mutations in *THR* genes could be associated with carcinogenesis. [31] Another candidate TSG that is located in the hemizygous region is *Retinoic Acid Receptor β2* (*RARβ2*) (3p24.2). Decreased levels of this receptor are associated with malignancies, like breast tumors, lung cancer and squamous cell cancer of head and neck. [31] Other possible TSGs on 3p like the *Von Hippel Lindau* (*VHL*) gene (3p25.3), *Ras association domain family 1* (*RASSF1*) (3p21.31) and the *Fragile Histidine Triad* (*FHIT*) (3p14.2) map to regions outside the hemizygous region, detected in the present study. [31] Interestingly, gene expression profiling studies of Tschentscher *et al*. (2003) and Onken *et al*. (2004) described gene expression classifiers predictive for tumor class, monosomy of chromosome 3 and prognosis, that harbor a number of genes that are located on the regions on chromosome 3 delineated in this study. The respective genes are *NR1D2* and *RPL15* on the 3p region and *PIK3R4*, *EIF2A* and *KIAA0678* on the 3q region. [32,33] This supports our statement that it is likely that one or more genes on those regions function as tumor suppressor genes in poor prognostic uveal melanoma. For further reduction and localization of putative TSG loci, high-resolution analysis, focusing on those regions in a set of tumors with a short DFS is required.

Acknowledgements

We would like to thank AnetteVerhoeven for excellent technical assistance.

References

1. Egan KM, Seddon JM, Glynn RJ, Gragoudas ES, Albert DM. Epidemiologic aspects of uveal melanoma. *Surv Ophthalmol*. 1988;32:239-251.
2. Diener West M, Hawkins BS, Markowitz JA, Schachat AP. A review of mortality from choroidal melanoma. II A Meta-analysis of 5-year mortality rates following enucleation, 1996 through 1988. *Ach. Ophthalmol*. 1992:110:245-250.
3. Horsman DE, White VA. Cytogenetic analysis of uveal melanoma. Consistent occurrence of monosomy 3 and trisomy 8q. Cancer. 1993;71:811-819.
4. Prescher G, Bornfeld N, Hirche H, Horsthemke B, Jöckel KH, Becher R. Prognostic implications of monosomy 3 in uveal melanoma. *Lancet*. 1996; 347:1222-1225.
5. Sisley K, Rennie IG, Parsons MA, *et al*. Abnormalities of chromosomes 3 and 8 in posterior uveal melanoma correlate with prognosis. *Genes Chromosomes Cancer*. 1997;19:22-28.
6. White VA, Chambers JD, Courtright PD, Chang WY, Horsman DE. Correlation of cytogenetic abnormalities with the outcome of patients with uveal melanoma. *Cancer*. 1998;83:354-359.
7. Parrella P, Sidransky D, Merbs SL. Allelotype of posterior uveal melanoma: implications for a bifurcated tumor progression pathway. *Cancer Res*. 1999;59:3032-3037.
8. Aalto Y, Eriksson L, Seregard S, Larsson O, Knuutila, S. Concomitant loss of chromosome 3 and whole arm losses and gains of chromosome 1, 6, or 8 in metastasizing primary uveal melanoma. *Invest Ophthalmol Vis Sci*. 2001;42:313-317.
9. Sisley K, Parsons MA, Garnham J, *et al*. Association of specific chromosome alterations with tumour phenotype in posterior uveal melanoma. Br J Cancer. 2000;82:330-338.

10. Kiliç E, Naus NC, van Gils W, Klaver CC, *et al*. Concurrent loss of chromosome arm 1p and chromosome 3 predicts a decreased disease-free survival in uveal melanoma patients. Invest Ophthalmol Vis Sci. 2005;46:2253-2257
11. Prescher G, Bornfeld N, Becher R. Two subclones in a case of uveal melanoma. Relevance of monosomy 3 and multiplication of chromosome 8q. *Cancer Genet Cytogenet*. 1994;77:144-146.
12. White VA, McNeil BK, Horsman DE. Acquired homozygosity (isodisomy) of chromosome 3 in uveal melanoma. *Cancer Genet Cytogenet*. 1998;102:40-45.
13. Scholes AGM, Liloglou T, Maloney P, *et al*. Loss of heterozygosity on chromosomes 3, 9, 13, and 17, including the retinoblastoma locus, in uveal melanoma. *Invest Ophthalmol Vis Sci*. 2001;42:2472-2477.
14. Tschentscher F, Prescher G, Horsman DE, *et al*. Partial deletions of the long and short arm of chromosome 3 point to two tumor suppressor genes in uveal melanoma. *Cancer Res*. 2001;61:3439-3442.
15. Parella P, Fazio VM, Gallo AP, Sidransky D, Merbs SL. Fine mapping of chromosome 3 in uveal melanoma: identification of a minimal region of deletion on chromosome arm 3p25.1-3p25.2. *Cancer Res*. 2003;63:8507-8510.
16. Naus NC, van Drunen E, de Klein A, *et al*. Characterization of complex chromosomal abnormalities in uveal melanoma by fluorescence in situ hybridization, spectral karyotyping, and comparative genomic hybridization. *Genes Chromosomes Cancer*. 2001;30:267-273.
17. Verbik DJ, Murray TG, Tran JM, Ksander BR. Melanomas that develop within the eye inhibit lymphocyte proliferation. Int J Cancer . 1997;73:470-478.
18. Luyten GP, Naus NC, Mooij CM, *et al*. Establishment and characterization of primary and metastatic uveal melanoma cell lines. *Int J Cancer*. 1996;66:380-387.
19. Naus NC, Verhoeven AC, van Drunen E, *et al*. Detection of genetic prognostic markers in uveal melanoma biopsies using fluorescence in situ hybridization. *Clin Cancer Res*. 2002;8:534-539.
20. Ning Y, Roschke A, Smith ACM, *et al*. A complete set of human telomeric probes and their clinical application. *Nat. Genet*. 1996;14:86-89.
21. Hopman AH, Ramaekers FCS, Raap AK, *et al*. In situ hybridization as a tool to study numerical chromosome aberrations in solid bladder tumors. *Histochemistry*. 1988;89:307-316.
22. van Dekken H, Pizzolo JG, Reuter VE, Melamed MR. Cytogenetic analysis of human solid tumors by in situ hybridization with a set of 12 chromosome-specific DNA probes. *Cytogenet Cell Genet*. 1990;54:103-107.
23. Tomlinson IPM, Lambros MBK, Roylance RR. Loss of heterozygosity analysis: practically and conceptually flawed? *Genes, Chromosomes Cancer*. 2002;34:349-353.
24. Pinkel D, Segraves R, Sudar D, *et al*. High resolution analysis of DNA copy number variation using comparative genomic hybridization to microarrays. *Nature Genet*. 1998;20:207-211.
25. Prescher G, Bornfeld N, Friedrichs W, Seeber S, Becher R. Cytogenetics of twelve cases of uveal melanoma and patterns of nonrandom anomalies and isochromosome formation. *Cancer Genet Cytogenet*. 1995;80:40-46.
26. Dahlenfors R, Tornqvist G, Wettrell K, Mark J. Cytogenetical observations in nine ocular malignant melanomas. *Anticancer Res*. 1993;13:1415-1420.
27. Flores ER. The roles of p63 in cancer. *Cell Cycle*. 2007;6:300-304.
28. Dannenberg H, Speel EJM, Zhao J, *et al*. Losses of chromosomes 1p and 3q are early genetic events in the development of sporadic pheochromocytomas. *Am J Pathol*. 2000;157:353-359.
29. Blasi MA, Roccella F, Balestrazzi E, *et al*. 3p13 region: a possible location of a tumor suppressor gene involved in uveal melanoma. *Cancer Genet Cytogenet*. 1999;108:81-83.
30. Sisley K, Curtis D, Rennie IG, Rees RC. Loss of heterozygosity of the thyroid hormone receptor B in posterior uveal melanoma. *Melanoma Res*. 1993; 3: 457-461.
31. Yang Q, Yoshimura G, Mori I, Sakurai T, Kakudo K. Chromosome 3p and breast cancer. *J Hum Genet*. 2002;47:453-459.
32. Tschentscher, F., *et al*., Tumor classification based on gene expression profiling shows that uveal melanomas with and without monosomy 3 represent two distinct entities. *Cancer Res*. 2003;63:2578-2584.
33. Onken, M.D., *et al*., Gene expression profiling in uveal melanoma reveals two molecular classes and predicts metastatic death. *Cancer Res*. 2004;64:7205-7209.

Chapter 7

Demarcation of a deleted region at chromosome 3 in uveal melanoma

Chapter 7

Demarcation of a deleted region at chromosome 3 in uveal melanoma

Walter van Gils, Emine Kilic, Eline H. van Meel, Michael M. Verbiest, Berna Beverloo, Hennie T. Brüggenwirth, Dion Paridaens, Gregorius P. Luyten, Annelies de Klein

Submitted for publication

Abstract

Objectives: Uveal Melanoma (UM) is the most common primary intraocular malignancy of the eye in adults. Certain chromosomal aberrations serve as excellent prognostic markers for UM. Monosomy of chromosome 3 is the most frequently found non-random chromosomal aberration in UM strongly correlating with poor prognosis. This loss is considered a primary event that is not commonly seen in other tumor types. Chromosome 3 is believed to harbor tumor suppressor genes (TSGs) that are important in UM development. Until now, only a few UMs with partial aberrations on chromosome 3 or translocations involving chromosome 3 have been described. We have searched UMs for a common deleted region on chromosome 3 to reduce the candidate TSG region.

Methods: Our database, consisting of 120 UMs with cytogenetic, pathological and follow-up data, contained two patients with a deletion of the entire long arm of chromosome 3 and only one UM with a partial 3q deletion. We demarcated this deletion using Comparative Genomic Hybridization and Fluorescence In Situ Hybridization.

Results: The delineated region spans from the centromere to approximately 3q26.2. The patients with either 3q or partial 3q deletions did not suffer from metastatic disease during 11 years of follow-up.

Discussion: Apparently, these partial chromosome 3 deletions do not lead to the development of metastases. The hypothesis that loss of both a region on 3p and 3q leads to decreased survival in UM and that loss of only 3q is not the sole event leading to a poor prognosis is supported by these findings.

Introduction

Uveal melanoma (UM) is the most common primary intraocular malignancy. Several prognostic parameters, among which some are cytogenetic, are available to select patients at risk of developing metastases. Those parameters have been selected from the non-random chromosomal aberrations occurring in uveal melanoma. Amplification of chromosome 8q, amplification of 6p, loss of 6q and loss of 1p36 frequently occur in UM. [1-6] However, monosomy of chromosome 3 is the most frequently found non-random chromosomal aberration that highly correlates with poor prognosis. This effect becomes even stronger when besides 3 also 1p36 is lost. [7] Involvement of chromosome 3 is considered to be a primary event; loss of chromosome 3 is a constant event in UMs that are characterized by different sub-populations, in contrast to the variable number of copies of chromosome 8q that can be observed. [1, 8] In many uveal melanomas an entire chromosome 3 is lost, or isodisomy of this chromosome is acquired. [4, 6, 9] Monosomy of chromosome 3 is not commonly observed in other tumor types and it is widely believed that chromosome 3 harbors tumor suppressor genes that play an important role typically for uveal melanoma development. Until now, only a few melanomas with partial aberrations on chromosome 3 or translocations involving chromosome 3 have been described, which obviously makes it difficult to map the putative tumor suppressor genes. In a loss of heterozygosity (LOH) study on UM, Tschentscher *et al.* found two regions of allelic loss on chromosome 3, one located at 3p25 and one ranging from 3q24-q26. [10] In all metastasized tumors both regions were lost. In the UM cell line Mel270, we have also demarcated two candidate regions for tumor suppressor genes on chromosome 3 (van Gils *et al.*, manuscript submitted for publication). The results suggested a region of LOH at 3p24, and on the long arm of chromosome 3 a deletion was observed from 3q21.3 until 3q24, overlapping with the region found by Tschentscher *et al.* [10]. To determine whether loss of a specific region on chromosome 3q is involved in UM development and progression, and to further delineate this region, we searched for more UMs with partial 3 deletions. In our patient cohort we identified two patients with a deletion of 3q and one case with a partial deletion of 3q. We have subsequently demarcated the borders of this deletion, using Fluorescence In Situ Hybridization (FISH) and Comparative Genomic Hybridization (CGH).

Methods

Patient material

UMs from patients who underwent enucleation of the tumor-containing eye for ciliary body or choroidal melanoma, at the Erasmus MC Rotterdam and Rotterdam Eye Hospital were collected. Informed consent was given prior to enucleation and the study was performed according to the tenets of the Declaration of Helsinki. Fresh tumor material was obtained within 1 hour after enucleation and processed for FISH and cytogenetic analysis as described. [11]

The remainder of the eye was embedded in paraffin immediately after enucleation at the department of Pathology. A set of 120 tumors were selected from our database, and included in this study, based on the availability of cytogenetic, pathological and follow-up data.

Selection of patients

From these 120 tumors, we selected those tumors suspect of partial losses of chromosome 3, based on the outcome of CGH or routine FISH analysis using probes on centromere 3 and 3q24. In total 5 patients with a partial loss of chromosome 3 could be identified. Three tumors were included in this study because routine FISH showed two copies of centromere 3 and only one copy of 3q (Case 1, 3 and 4). Tumor 2 was included based on cytogenetic findings indicating loss of 3q (FISH on 3q was not informative). For tumor 5 routine FISH showed one copy of centromere 3 and two copies of 3q.

CGH

CGH was performed as described by Kiliç *et al*. [7] Images were acquired with a Zeiss axioplan microscope equipped with Isis version 5 software, MetaSystems (Ohio, USA). Ten metaphases were analyzed. Loss of DNA sequences was defined as chromosomal regions where the mean green: red ratio was below 0.8, while gain was defined as chromosomal regions where the ratio was above 1.2. Threshold levels were determined on basis of analysis of known chromosomal aberrations.

FISH

Dual-color FISH was performed on uncultured tumor material fixed directly after enucleation as described previously. [13] If directly fixed material was not available, nuclei isolated from paraffin embedded UMs were used. Tumor cells were deparaffinized and the nuclei were isolated and fixed with ethanol/acetic acid (3:1). Nuclei were spotted on slides using the Cytofuge (Nordic Immunological Laboratories B.V., Tilburg, The Netherlands) and subsequently treated according to the procedure described by Van Dekken *et al.* [14] The concentration for centromeric probes was 5 ng per slide; for Bacterial Artificial Chromosome (BAC) probes 50 to 75 ng per slide was used. When nuclei isolated from paraffin embedded UMs were used, the samples were analyzed with the routine FISH probes as previously described by Naus *et al*. (2000). [15] For additional FISH, BAC probes were selected from the human genome browsers of UCSC (http://genome.ucsc.edu/cgi-bin/hgGateway) and NCBI (http://www.ncbi.nlm.nih.gov/mapview/map_search.cgi). CTD-probes were obtained from Invitrogen (Carlsbad, USA) and RP-probes from CHORI-BACPAC Resources (Oakland, USA). Signals were counted in 300 interphase nuclei according to the criteria of Hopman *et al.* [16] Cut-off limits for deletion and amplification were adapted from the available literature.

Results

All enucleated UMs were karyotyped if metaphase spreads could be obtained. Karyotyping results from 3 of the 5 selected tumors were available (Table 1).

Material of all five cases was analyzed with CGH and, if possible, with FISH using diagnostic probes hybridizing to chromosomes 1, 3, 6 and 8 (data not shown). To control for ploïdy status and to confirm regions of loss, additional FISH was performed on material of four of the included cases. Data of additional FISH analyses are summarized in Table 2.

CGH showed that the tumor from case 3 had two copies of chromosome 3. Since diagnostic

FISH with probe RP11-64F6 (3q25.1) showed that only 15.5% of the nuclei contained one signal, the subclone of nuclei containing the deletion is probably too small to be detected with CGH. No additional FISH was performed on this tumor. The tumor derived from case 5 showed complete monosomy of chromosome 3, with differences in the extent of the loss for certain regions on both the p and q arm in its CGH profile. To find out whether this pattern was specific and not due to label preference, fluorescent labels of patient and control DNA were swapped during the random prime labeling and CGH was repeated. The same pattern was obtained, suggestive of a specific signal, probably caused by more than one region of loss along the q-arm of chromosome 3 in this tumor. However, all FISH probes analyzed along chromosome 3, indicated a loss. A partial loss of chromosome 3 was observed in the tumors derived from cases 1, 2 and 4 using CGH analysis. The tumors of cases 2 and 4 showed deletion of the entire 3q-arm, whereas the tumor derived from case 1 showed a partial deletion of the long arm of chromosome 3. CGH revealed a deletion, ranging from the centromeric region of chromosome 3 to approximately 3q26.2 (fig. 1A and B). Thus, the tumor, obtained from case 1, was the only tumor with a clear partial deletion on chromosome 3q in this study. This tumor had two copies of 1p36, gain of chromosome 6p, loss of 6q and normal copies of chromosome 8. The deleted region on chromosome 3q was further defined using FISH. Probes were selected in both border regions. At the telomeric border of the deletion, loss of one signal was found with probe RP11-478O19 (3q26.1), whereas two signals were found at 3q26.2 with RP11-816J6 (fig. 1C). In routine FISH, two copies of the centromere were detected. To find out whether the breakpoint of the deletion was at the centromere or whether it was located in a region beneath it, FISH was performed with probes, mapping to 3q11. RP11-449F7 showed one signal with a percentage of 78% (fig. 1D). Two other probes, CTD-2007O24 and CTD-2005N19 revealed loss of one copy at 3q11 as well. In conclusion, the 3q deletion in the tumor of this patient ranges from the centromere until at least 3q26.1, possibly until 3q26.2 (Figure 2).

Discussion

It is generally thought that loss of chromosome 3 would result in loss of two or more distinct TSGs located on the short and long arms of chromosome 3. Concurrent loss of the candidate regions on 3p and 3q seems to be essential in uveal melanoma development, as was hypothesized by Tschentscher *et al.* [10] Loss of an entire chromosome 3 would therefore be favorable and could explain the high frequency of monosomy 3 observed in UM. The finding that loss of chromosome 3 is an early event in UM development is also in support of the hypothesis that this chromosome harbors on both its arms TSGs that are involved in UM. This explains why partial abnormalities on chromosome 3 are rare and in most cases of UM with chromosome 3 aberrations a complete monosomy is found. CGH and micro-satellite analysis has been used to delineate these regions further and the different studies are summarized in figure 2. Aalto and coworkers used CGH analysis on 29 primary tumors and revealed one tumor with a partial deletion on chromosome 3q, ranging from 3q25 to 3q26.3. [6] In one study, a chromosome 3q translocation (t(3;14) (q23;q32)) was reported. [18] With microsatellite analysis with a low-resolution marker set Scholes *et al.* identified a region of LOH from at least 3q25.1 and possibly from 3q21 until the telomere. [9] Parrella *et al.* reported three partial deletions with LOH of more than one marker, in their LOH

study on 21 UMs that did not show monosomy of chromosome 3 in earlier allelotyping. Two of these tumors had a common region of loss ranging at most from 3q13.31 to 3q22.2. [19] Tschentscher *et al.* applied CGH and LOH on UMs and UM derived cell lines and rapport a common deleted region from 3q24 to 3q26.[10] This shortest region of overlap (SRO) overlaps with the 3q21.3-3q24 region deleted in the UM cell line Mel 270 and its metastasis OMM2.3. as we observed using FISH and LOH analysis (W.van Gils *et al.*, manuscript submitted).

When combining the data of the different studies with our data, we find a new SRO with its proximal border at 3q24 and a distal border at 3q26.2. This candidate region on chromosome 3q in UM harbors many possible TSGs. One of those genes in the breakpoint region is the ectopic viral integration site 1 (*EVI1*) gene that is frequently deregulated in hematological malignancies, often through translocations. [20, 21] A second candidate in this region is telomerase RNA component (*TERC*), required as the template in telomeric repeat synthesis. [22] Deregulation of telomere synthesis might contribute to tumorigenesis by circumventing terminal growth arrest and apoptosis following unacceptable short telomeres. A third candidate gene is Programmed Cell Death 10 (*PDCD10*). Mutations within this apoptosis related gene cause cerebral cavernous malformations. Intriguingly, 5% of patients with familial cerebral cavernomas develop retinal cavernoma. [23]

Whether loss of this particular region of chromosome 3 contributes to the development of metastasis is not clear. The three patients in our database of which the UMs only showed a (partial) loss of the q-arm of chromosome 3, did not develop metastatic disease during 5 to 11 years of follow-up. Deletion of this region is apparently not enough for UMs to develop metastases, which also supports the hypothesis that loss of both a region on 3p and 3q leads to a poor signature in UM. Previously, we have observed that concurrent loss of chromosome 1p and chromosome 3 significantly predicts for decreased disease-free survival in uveal melanomas patients. [7] Thus in addition to chromosome 3 genes, genes on other chromosomal regions could contribute towards the development of metastasis.

Examination of more uveal melanomas with partial 3q deletions could possibly further narrow down the region of interest. To increase chances to further demarcate the SRO, it would be interesting to include uveal melanomas from patients with a short survival, for which no deletions were observed on chromosome 3 in diagnostic cytogenetics and classical CGH, and to analyze those tumors with high resolution techniques like array-CGH and SNP analysis.

Table 1. **Tumor selection indicative of partial losses with corresponding karyotype***

Uveal melanoma	Karyotype (ISCN 2005)	Initial FISH results on chr. 3 (copy nr.)**	
		#3	3q
Case 1	NA*	2	1
Case 2	43,X,-Y,dic(3;19)(q11;q13.2),-13, der(17)t(13;17)(q13;p12),add(22) (p11)[6]/43,idem,-21[4]	2	NA
Case 3	NA*	2	1
Case 4	46,XX,der(20)t(6;20)(p12;p12) [5]/47,idem,+8[4]/ 47,idem,+8,psu dic(17;15)(p13;p11)[3]/46,XX[3]	2	1
Case 5	45~48,XX,-3,i(8)(q10),+i(8) (q10),+i(8)(q10)[cp5]/ 47~49,XX,+3[3],+5[2],+6[2] [cp4]/46,XX[4]	1	2

*NA = data not available

** Cut-off limits for deletion (>10% of counted nuclei with one copy) were adapted from the available literature.[17]

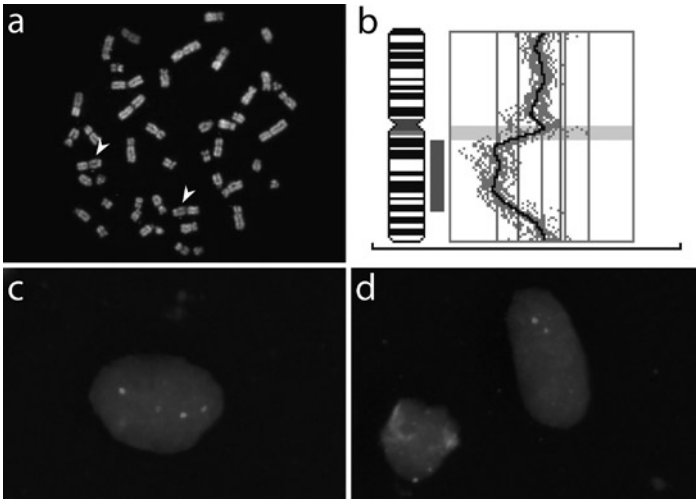
Table 2. **FISH results of chromosome 3 deletion analysis**

Tumor	Probe	Chromosomal position*	Copy number (Percentage)**
Case 1	CTD-2005N19	3q11.2	1 (70.0)
	CTD-2007O24	3q11.2	1 (82.3)
	RP11-449F7	3q11.2	1 (77.7)
	RP11-64F6	3q25.1	1 (78.7)
	RP11-117L15	3q25.31	1 (83.7)
	RP11-90M7	3q26.1	1 (68.5)
	RP11-478O19	3q26.1	1 (67.5)
	RP11-816J6	3q26.2	2 (95.3)
	RP11-54L9	3q28	2 (96.3)
Case 2	RP11-632N21	3p24	2 (87.0)
	Pα 3.5	Centromere 3	2 (97.0)
	CTD-2005N19	3q11.2	1 (83.5)
	RP11-64F6	3q25.1	1 (67.5)
	RP11-147L6	3q26	1 (91.5)
	RP11-54L9	3q28	1 (84.5)
Case 3***	---	---	---
Case 4	RP11-384L8	3p22.3	3 (15.0)
	Pα 3.5	Centromere 3	3 (25.0)
	RP11-64F6	3q25.1	1 (93.0)
Case 5	RP11-632N21	3p24	1 (82.5)
	Pα 3.5	Centromere 3	1 (78.5)
	CTD-2005N19	3q11.2	1 (77.5)
	RP11-64F6	3q25.1	1 (81.5)

* UCSC Genome Browser on Human March 2006 Assembly.

** The copy number of the largest clone (percentage between brackets) is given.

*** No FISH chromosome 3 deletion analysis was performed on Case 3.



Color figure can be found on page 144

Figure 1. **CGH and FISH on case 1. A)** Metaphase spread after comparative genomic hybridization with tumor DNA labeled in green, reference DNA labeled in red. The arrowheads point to the red regions on the q-arm of chromosomes 3 indicating a loss. **B)** CGH results on chromosome 3 of ten metaphase spreads processed with Isis version 5 software. The thick line represents the average signal of the ten metaphases. The lower limit was set as green-to-red ratio 0.8, the upper limit as a ratio of 1.2. The partial deletion is indicated with a red bar alongside of the chromosome image. **C)** and **D)** are images of FISH. **C)** FISH on isolated nuclei with probes RP11-816J6 (labeled in red) and RP11-54L9 (labeled in green) showing the presence of two copies of regions 3q26.2 and 3q28, respectively. **D)** Probes RP11-449F7 (red) and RP11-64F6 (green) showed loss of one copy of chromosome 3 in the regions 3q11.2 and 3q25.1 respectively.

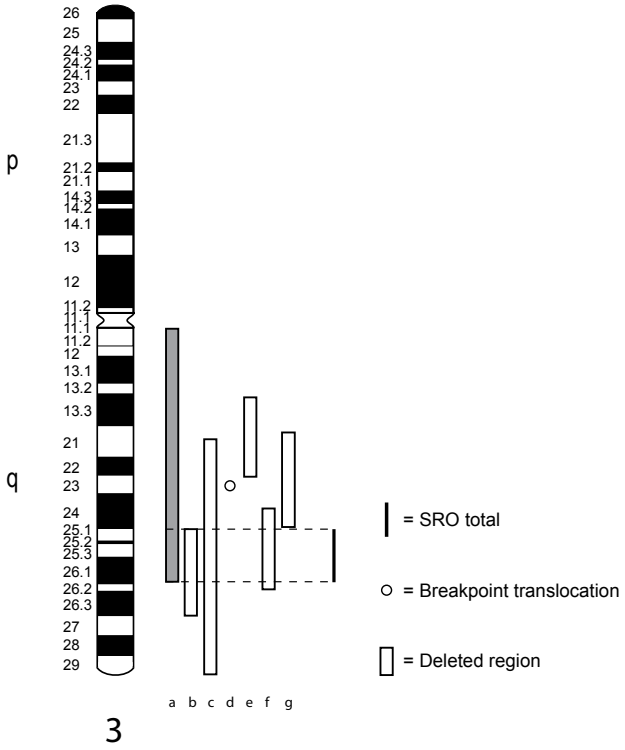


Figure 2. **Summary of previously described partial deletions in UM on one of the arms of chromosome 3 together with the partial deletion found in patient 1.** Chromosome banding pattern is designated on the left according to ISCN (2005). On the right of the ideogram, regions of deletion found in different studies are represented by the vertical bars, translocation regions by open circles. The region identified in this study is designated by a grey bar. The different studies are indicated by letters under the respective bar. **A)** Partial deletion found in this study in patient 1; **B)** Deletion found with CGH by Aalto *et. al.* [6].; **C)** Deletion identified by Scholes *et al.* [9] with microsatellite markers; **D)** Translocation (t(3;14)(q23;q32)) identified in UM derived cell line by Dahlenfors *et al.* [18].; **E)** Common region of deletion in microsatellite analysis by Parrella *et al.* [5].; **F)** Smallest region of overlap identified by Tschentscher *et al.* [10] with microsatellite analysis; **G)** Partial deletion found in an LOH study by Van Gils *et al.* on UM derived cell line Mel 270.

References

1. Horsman DE, White VA. Cytogenetic analysis of uveal melanoma. Consistent occurrence of monosomy 3 and trisomy 8q. *Cancer*. 1993;71:811-819
2. Prescher G, Bornfeld N, Hirche H *et al.* Prognostic implications of monosomy 3 in uveal melanoma. *Lancet*. 1996;347:1222-1225
3. Sisley K, Rennie IG, Parsons MA *et al.* Abnormalities of chromosomes 3 and 8 in posterior uveal melanoma correlate with prognosis. *Genes Chromosomes Cancer*. 1997;19:22-28.
4. White VA, Chambers JD, Courtright PD, Chang WY, Horsman DE. Correlation of cytogenetic abnormalities with the outcome of patients with uveal melanoma. *Cancer*. 1998;83:354-359
5. Parrella P, Sidransky D, Merbs SL. Allelotype of posterior uveal melanoma: implications for a bifurcated tumor progression pathway. *Cancer Res*. 1999;59:3032-3037
6. Aalto Y, Eriksson L, Seregard S, Larsson O, Knuutila S. Concomitant loss of chromosome 3 and whole arm losses and gains of chromosome 1, 6, or 8 in metastasizing primary uveal melanoma. *Invest Ophthalmol Vis Sci*. 2001;42:313-317
7. Kilic E, Naus NC, van Gils W *et al.* Concurrent loss of chromosome arm 1p and chromosome 3 predicts a decreased disease-free survival in uveal melanoma patients. *Invest Ophthalmol Vis Sci*. 2005;46:2253-2257
8. Prescher G, Bornfeld N, Becher R. Two subclones in a case of uveal melanoma. Relevance of monosomy 3 and multiplication of chromosome 8q. *Cancer Genet Cytogenet*. 1994;77:144-146
9. Scholes AG, Liloglou T, Maloney P *et al.* Loss of heterozygosity on chromosomes 3, 9, 13, and 17, including the retinoblastoma locus, in uveal melanoma. *Invest Ophthalmol Vis Sci*. 2001;42:2472-2477
10. Tschentscher F, Prescher G, Horsman DE *et al.* Partial deletions of the long and short arm of chromosome 3 point to two tumor suppressor genes in uveal melanoma. *Cancer Res*. 2001;61:3439-3442
11. Naus NC, Zuidervaart W, Rayman N *et al.* Mutation analysis of the PTEN gene in uveal melanoma cell lines. *Int J Cancer*. 2000;87:151-153.
12. Lapierre JM, Tachdjian G. Detection of chromosomal abnormalities by comparative genomic hybridization. *Curr Opin Obstet Gynecol*. 2005;17:171-177
13. Hagemeijer A, de Klein A, Wijsman J *et al.* Development of an interphase fluorescent in situ hybridization (FISH) test to detect t(8;21) in AML patients. *Leukemia*. 1998;12:96-101.
14. Van Dekken H, Krijtenburg PJ, Alers JC. DNA in situ hybridization (interphase cytogenetics) versus comparative genomic hybridization (CGH) in human cancer: detection of numerical and structural chromosome aberrations. *Acta Histochem*. 2000;102:85-94
15. Naus NC, Verhoeven AC, van Drunen E *et al.* Detection of genetic prognostic markers in uveal melanoma biopsies using fluorescence in situ hybridization. *Clin Cancer Res*. 2002;8:534-539
16. Hopman AH, Ramaekers FC, Raap AK *et al.* In situ hybridization as a tool to study numerical chromosome aberrations in solid bladder tumors. *Histochemistry*. 1988;89:307-316
17. van Dekken H, Pizzolo JG, Reuter VE, Melamed MR. Cytogenetic analysis of human solid tumors by in situ hybridization with a set of 12 chromosome-specific DNA probes. *Cytogenet Cell Genet*. 1990;54:103-107
18. Dahlenfors R, Tornqvist G, Wettrell K, Mark J. Cytogenetical observations in nine ocular malignant melanomas. *Anticancer Res*. 1993;13:1415-1420
19. Parrella P, Fazio VM, Gallo AP, Sidransky D, Merbs SL. Fine mapping of chromosome 3 in uveal melanoma: identification of a minimal region of deletion on chromosomal arm 3p25.1-p25.2. *Cancer Res*. 2003;63:8507-8510
20. Nucifora G, Laricchia-Robbio L, Senyuk V. EVI1 and hematopoietic disorders: history and perspectives. *Gene*. 2006;368:1-11
21. Morishita K, Parganas E, William CL *et al.* Activation of EVI1 gene expression in human acute myelogenous leukemias by translocations spanning 300-400 kilobases on chromosome band 3q26. *Proc Natl Acad Sci U S A*. 1992;89:3937-3941
22. Bryan TM, Cech TR. Telomerase and the maintenance of chromosome ends. *Curr Opin Cell Biol*. 1999;11:318-324
23. Labauge P, Krivosic V, Denier C, Tournier-Lasserve E, Gaudric A. Frequency of retinal cavernomas in 60 patients with familial cerebral cavernomas: a clinical and genetic study. *Arch Ophthalmol*. 2006;124:885-886

Chapter 8

Regional deletion and amplification on chromosome 6 in a uveal melanoma case without abnormalities on chromosomes 1p, 3 and 8

Chapter 8

Regional deletion and amplification on chromosome 6 in a uveal melanoma case without abnormalities on chromosomes 1p, 3 and 8

Walter van Gils, Emine Kilic, Hennie T. Brüggenwirth, Jolanda Vaarwater, Michael M. Verbiest, Berna Beverloo, Marjan E. van Til-Berg, Dion Paridaens, Gregorius P. Luyten and Annelies de Klein

Melanoma Research, in press

Abstract

Objectives: 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 at 6q that are involved in UM development. However, both regions have not been well defined yet. Also in other neoplasms gain of 6p and loss of 6q are frequently occurring events. In this case report, we describe the delineation of a partial gain on chromosome 6p and a partial deletion on 6q in a UM with the objective to pinpoint smaller candidate regions on chromosome 6 involved in UM development.

Methods: Conventional cytogenetics, CGH and FISH were used to delineate regions of loss and gain on chromosome 6 in this UM patient.

Results: With conventional cytogenetics a deleted region was found on chromosome 6q that was further delineated to a region ranging from 6q16.1 to 6q22 using CGH and FISH. A region of gain from 6pter to 6p21.2 was also demarcated with CGH and FISH. No other deletions or amplifications on recurrently involved chromosomes were found in this patient.

Conclusions: This study indicates the presence of one or more tumor suppressor genes on chromosomal region 6q16.1-6q22 and the presence of one or more oncogenes on chromosomal region 6pter-6p21.2, which are likely to be important in uveal melanoma and other tumors.

Introduction

Uveal melanoma (UM) is the most common primary malignancy in the eye with an incidence of about 6 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. 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. [2-7] 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] Deletion of the long arm of chromosome 6 also occurs in many neoplasms, including carcinomas of the prostate and breast and melanomas. [10-13] 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] This suggests the presence of a tumor-suppressor gene or genes at 6q. In UM, aberrations of chromosome 6 also occur frequently and a correlation with increased survival has been described. [16] In contrast to this, Aalto *et al*. showed that loss of chromosome 6q is associated with decreased survival. [17] Although these 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] Although in certain tumor types a smaller candidate region or regions for oncogenes on 6p and tumor suppressor genes on 6q have been identified, in UM the regions of interest have not been well defined yet. In this report, a UM case with gain of chromosome 6p and loss of chromosome 6q and without numerical aberrations on chromosomes 1, 3 and 8 is investigated in detail. The fact that not the complete chromosome arms of chromosome 6, but only a smaller region of both the q-arm and the p-arm are involved, offers the opportunity to demarcate the regions of loss and amplification on chromosome 6, involved with UM development, further.

Methods

Case history

A 39-year-old male without a relevant medical history, no use of any medication nor any intoxication, presented with painful irritation and decreased visual acuity in his left eye starting six weeks before his initial visit. The right eye had a visual acuity of 1.0 without correction, whereas the left eye had a visual acuity of 0.6 without correction and stenopeic 1.0. On fundoscopic examination the patient appeared to have a large pigmented tumor with small superficial hemorrhages located in the temporal posterior pole of his left eye with an exsudative retinal detachment. Fluorescence angiography showed tumor vascularization with minimal leakage in the late phase. Ultrasound examination showed a solid homogeneous tumor with a largest tumor diameter of 13.2 mm, a sereous retinal detachment, no choroidal excavation, and low to

middlehigh internal reflectivity. Subsequently the affected eye was enucleated. After 48 months of follow-up the patient is still alive without signs of metastatic disease. Family history was negative for UM or other tumors.

Patient sample

Informed consent was given prior to enucleation and the study was performed according to the tenets of the Declaration of Helsinki. Permits were granted by the local medical ethical committee. Fresh tumor material (designated EOM 265) was obtained within 1 hour after enucleation and processed for FISH and cytogenetic analysis as described. [19]

Cytogenetic analysis

Chromosome preparations were made following standard cytogenetic procedures and stained for R and Q banding. Cytogenetic abnormalities were described according to the International System for Human Cytogenetic Nomenclature (ISCN). [20]

Fluorescence In Situ Hybridization (FISH)

Dual color FISH on uncultured tumor material using centromeric and locus specific cosmid, P1 or YAC probes for chromosome 1, 3, 6 and 8 was performed as described previously. [21] For further analysis, suitable bacterial artificial chromosome (BAC) probes were selected from the human genome browsers of UCSC (http://genome.ucsc.edu/cgi-bin/hgGateway) and NCBI (http://www.ncbi.nlm.nih.gov/mapview/map_search.cgi).

Comparative Genomic Hybridization (CGH)

CGH was performed as described by Kilic *et al*. [21] Images were acquired with a Zeiss axioplan microscope equipped with Isis version 5 software, MetaSystems (Ohio, USA). Ten metaphases were analysed. Loss of DNA sequences was defined as chromosomal regions where the mean green: red ratio was below 0.8, while gain was defined as chromosomal regions where the ratio was above 1.2. Threshold levels were determined on basis of analysis of known chromosomal aberrations. [22]

Results

Histopathological examination

The pigmented tumor originated from the choroid with a spindle cell morphology. The histopathological largest tumor diameter was 13 mm with a prominence of 12 mm. The tumor showed no invasion through Bruchs' membrane or into the retina. No invasion of the sclera was seen and also the optic nerve was free of tumor. Vascular loops could not be identified and the mitotic figures were rather low with 2/15 HPF.

Cytogenetic and FISH *analysis*

EOM 265 presented with karyotype: 46,XY,del(6)(q15q22)[2]/46,XY,del(6)(q15q22),add(14)(q32)[1]/ 46, XY, del(6)(q15q22), der(17)t(7;17)(q11.2;p13)[5]/ 46,XY,del(6)(q15q22),add(14)(q32), der(17)t(7;17)(q11.2;p13)[8] (fig. 1).

FISH analysis showed no copy number abnormalities on chromosomes 1, 3, 6 and 8 other than gain of a region of chromosome 6p (two extra signals). Probe cos52 on 6q23 showed normal copy numbers.

CGH analysis of EOM 265 showed a deletion was on chromosome 6 between 6q14 and 6q23 with signals close to the threshold level with a significant deleted region on 6q21-6q22.1. CGH also indicated amplification of 6p22-6p25 (fig. 2). No abnormalities of chromosome 14 were found in CGH. It could well be that the extra material on this chromosome as seen in the karyotype was caused by chromosome 6p material translocated to the tip of chromosome 14q (fig. 1). This translocation could however not be confirmed by FISH due to scarcity of metaphase spreads in the sample. A just significant gain of chromosome 7q was also seen. this gain is probably the result of extra material of chromosome 7q on the derivative chromosome 17 in subclones of the tumor. These subclones are less numerous present in the karyotype (13 of the 16 methaphases) compared to the abnormalities on chromosme 6q. However the finding in the karyotype do not necessary reflect the situation in vivo since in vitro specific clones could have a growth advantage. CGH analysis did not show copy number abnormalities on chromosomes 1, 3 and 8. (fig. 1). Disomy of chromosome 3 was confirmed in MLPA with kits P070 and P036B that cover all subtelomeric regions, revealing normal copy numbers for both distal regions of chromosome 3 (data not shown).

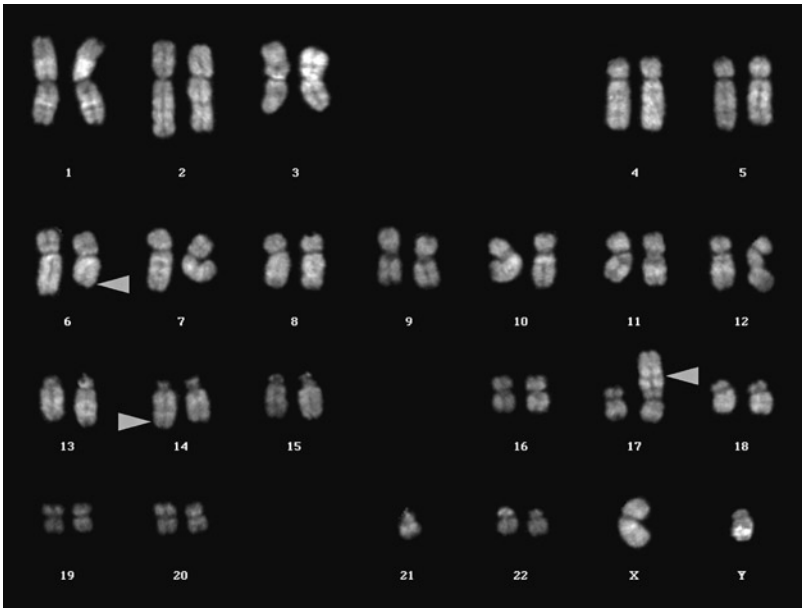


Figure 1. **Representative karyogram for EOM 265.** The karyogram (QFQ banding) shows the following karyotype: 46,XY,del(6)(q15q22),add(14)(q32), der(17)t(7;17)(q11.2;p13). Structural abnormalities are indicated by arrowheads. Loss of 21 is a non-clonal event, and therefore not indicated in the karyotype.

The regions found on chromosome 6 in CGH were further investigated using FISH with the probes presented in figure 2, covering regions from 6pter to 6p21.2 and from 6q14 to 6q22.33. Probe RP11-215O12 on 6p21.3 and all probes located more distal on the chromosome showed presence of 3 (28-31%) to 4 copies (10-52%) of this region, whereas normal copy numbers were found with probe RP11-375E1, located more proximal on 6p21.3. This results in an amplified region of 35 Mb ranging from 6p21.3 to 6pter. The presence of a subclone with 4 copies of this region explains the strong amplification signal of the region as was observed in CGH.Probe RP1-154G14 mapping to 6q16.1 and all probes mapping to more proximal region on 6q, showed presence of two copies, while all probes in this region located more distal, showed loss of one signal in almost all nuclei. Of the probes surrounding the distal border of the deleted region, RP11-358H7 located on 6q22.32 as well as the probes located more proximal all showed clear losses in more than 40% of the interphase nuclei, whereas probes located distal from this probe showed no loss. These experiments confirmed the deletion and further delineated the deletion to a region of 32 Mb ranging from 6q16.1 to 6q22, to which interesting candidate tumor suppressor genes are mapped (fig. 2).

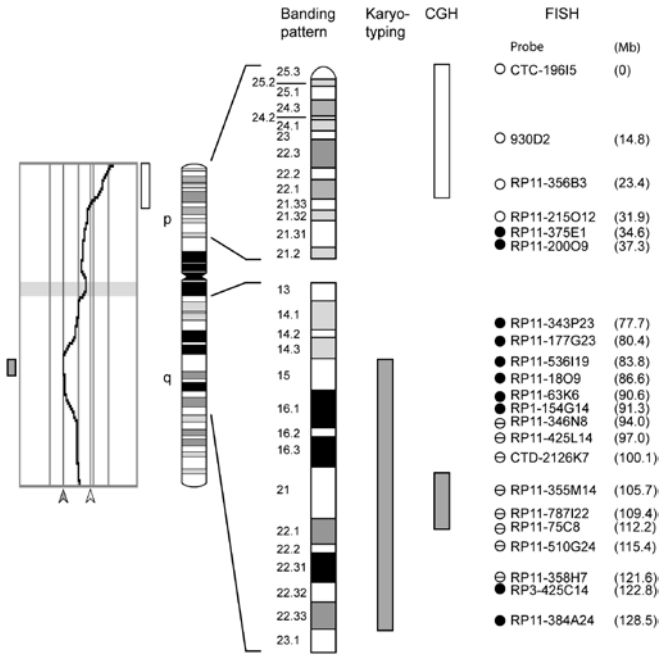


Figure 2. **Regions of deletion and amplification on chromosome 6 in EOM 265.** On the left of the chromome 6 ideogram a CGH plot of the complete chromosome 6 is shown, with the thick black line representing the average signal of ten metaphases. The lower limit (the vertical line indicated by a gray arrow) was set as green-to-red ratio 0.8, the upper limit (indicated with an open arrow) has a ratio of 1.2. Deletions are indicated with a gray bar on the left and gains are indicated with an open bar on the right of the plot. Right to the ideogram of chromosome 6 the results of respectively karyotyping, CGH and FISH are shown. Losses found in karyotyping and CGH are indicated with grey boxes, gain in a white box. FISH probes with increased copy numbers are indicated with open dots, FISH probes that showed loss are indicated with open dots with a horizontal line. FISH probes that showed normal copy numbers are indicated with black dots. The chromosomal location in megabasepairs of each probe is indicated between brackets.

Discussion

Inactivation of tumor suppressor genes by homozygous deletions, mutations or epigenetic events such as methylation of promoter regions is a common event in human cancers. However, in UM the molecular pathogenesis is largely unknown. Several chromosomal aberrations do occur in these tumors, like monosomy 3, loss of 1p and 6q, and gain of 8q and 6p. In this patient gain 6p21.3-6pter and loss of 6q16.1-6q22 were observed as the only recurrent aberrations in those regions. Although Aalto *et al*. found that loss of chromosome 6q is associated with decreased survival [17], it is described that abnormalities on chromosome 6 resulting in a relative increase of 6p material are associated with a better prognosis. [16] Also the clinical characteristics indicated a relatively good prognosis. Choroidal localization, spindle cell type, absence of vascular patterns and low mitotic activity all correlate with a better prognosis. [23-25] Only gender of the patient and the relatively large size of the tumor contribute negatively to prognosis. This is, overall, in agreement with the fact that the patient is still alive after 4 years of follow-up whereas UM mortality peaks at 2-3 years after treatment. [26] Furthermore, in gene expression profiling, the tumor showed a gene expression profile corresponding to Class 1, described by Onken *et al.* and Tschentscher *et al.* [27,28] to be the UM class related with a better prognosis and disomy of chromosome 3 (data not shown). It is very likely that an oncogene involved in UM development is located on the demarcated region on 6p ranging from 6p21.3 to the telomeric region and that one or more tumor suppressor genes likely to be important in UM development are located in the region of 6q16.1 to 6q22. Apparently, genes on the both regions are involved in tumor intitiation and development rather than with progression. This possibility is supported by the findings on three other tumors in our archive. These UMs, described by Naus *et al.* (2002) as EOM 141, EOM 150 and EOM 182, have gains and losses of considerably larger regions of chromosome 6 compared to the subject of this study, with no numerical abnormalities on chromosomes 1, 3 and 8 and until now, none of these patients did develop metastatic disease. [29] Also in other studies UMs with large structural abnormalities on chromosome 6 without abnormalities on chromosomes 1p, 3 and 8 are described. [30-32]

The region on 6p spans approximately 35 megabasepairs and is therefore too large to point out candidate oncogenes. HLA genes, which are located on this region of chromosome 6p, have been described to play a role in UM. [33,34] In addition, the gene expression signature reported by Tschentscher *et al.* (2003), that was based on classification of UMs in classes with and without monosomy of chromosome 3 respectively, contains 3 genes located in of the region on 6p that we describe in this report, namely *HCGIV.8*, *ALDH5A1* and *TFAP2A,* all with a higher expression value in tumors with disomy of chromosome 3. [27] Interestingly, the developmentally regulated activator of transcription *TFAP2A* (transcription factor AP2-Alpha), was also present in the micro array classifier created by Onken *et al.* (2004). [28] Gain of 6p in tumors has not previously been described to correlate with increased survival. In other malignancies gain of 6p correlates with decreased survival. [35] In UM, the frequently occurring event of 6p gain most likely contributes to tumorigenesis and not progression.

This study indicates that one or more tumor suppressor genes likely to be important in UM development are located in the region of 6q16.1 to 6q22. Partial or complete deletions of

chromosome 6q are also frequently found in other types of tumors. The region found in this study partially overlaps with smaller regions found in prostate cancer, ovarian cancer and more than one region found in breast cancer and lymphoproliferative disorders. [12,13,36-41] In malignant cutaneous melanoma a large region with highest frequency of LOH ranging from 6q22 to 6q27 was identified and this loss is significantly associated with poor prognosis. [11,14] In contrast, in prostate cancer, ovarian cancer and breast cancer 6q loss occurs with a high frequency, but no relation with prognosis is observed, indicating a role in tumor development but not in tumor progression. The region of 32 Mb demarcated in this study is still considerably large and contains numerous genes, for example *EphA7*, *AIM1* and *CCNC* that could function as tumor suppressor genes. The Eph tyrosine kinase receptor *Epha7* is found to be downregulated in colorectal cancers [42] and is shown to be part of a regulatory system for apoptosis in neural progenitors. Loss of *Epha7* expression caused a reduction of apoptosis *in vivo*. [43] *AIM1*, absent in melanoma 1, was found not to be expressed in tumorigenic cell lines, but highly expressed in suppressed melanoma cell lines. [44] *CCNC*, the cyclin C gene active in the G1-phase, is frequently deleted in leukemias and described to have decreased expression in papillary carcinoma. [45,46]

In addition to the even smaller regions of overlap on chromosome 3p identified by Cross *et al*. (2006) [47], the partial deletion on chromosome 6q is the smallest demarcated region on chromosome 6 in UM until now and will be very helpful in the search for candidate tumor suppressor genes involved in development and progression of UM, which also accounts for candidate oncogenes in the amplified region on chromosome 6p. Both regions will help to get a better understanding of UM development.

In addition to the even smaller regions of overlap on chromosome 3p identified by Cross *et al*. (2006) [47], the partial deletion on chromosome 6q is the smallest demarcated region on chromosome 6 in UM until now and will be very helpful in the search for candidate tumor suppressor genes involved in development and progression of UM, which also accounts for candidate oncogenes in the amplified region on chromosome 6p. Both regions will help to get a better understanding of UM development.

References

1 Singh AD, Topham A. Incidence of uveal melanoma in the United States: 1973-1997. *Ophthalmology* 2003; **110**: 956-961.

2 Lau CC, Harris CP, Lu XY *et al*. Frequent amplification and rearrangement of chromosomal bands 6p12-p21 and 17p11.2 in osteosarcoma. *Genes Chromosomes Cancer* 2004; **39**: 11-21.

3 Ozaki T, Schaefer KL, Wai D *et al*. Genetic imbalances revealed by comparative genomic hybridization in osteosarcomas. *Int J Cancer* 2002; **102**: 355-365.

4 Aalto Y, Nordling S, Kivioja AH *et al*. Among numerous DNA copy number changes, losses of chromosome 13 are highly recurrent in plasmacytoma. *Genes Chromosomes Cancer* 1999; **25**: 104-107.

5 Kuchiki H, Saino M, Nobukuni T *et al*. Detection of amplification of a chromosomal fragment at 6p21 including the cyclin D3 gene in a glioblastoma cell line by arbitrarily primed polymerase chain reaction. *Int J Cancer* 2000; **85**: 113-116.

6 Bastian BC, Olshen AB, LeBoit PE *et al*. Classifying melanocytic tumors based on DNA copy number changes. *Am J Pathol* 2003; **163**: 1765-1770.

7 Zielinski B, Gratias S, Toedt G *et al*. Detection of chromosomal imbalances in retinoblastoma by matrix-based comparative genomic hybridization. *Genes Chromosomes Cancer* 2005; **43**: 294-301.

8 Tarkkanen M, Kiuru-Kuhlefelt S, Blomqvist C *et al*. Clinical correlations of genetic changes by comparative genomic hybridization in Ewing sarcoma and related tumors. *Cancer Genet Cytogenet* 1999; **114**: 35-41.

9 Namiki T, Yanagawa S, Izumo T *et al*. Genomic alterations in primary cutaneous melanomas detected by metaphase comparative genomic hybridization with laser capture or manual microdissection: 6p gains may predict poor outcome. *Cancer Genet Cytogenet* 2005; **157**: 1-11.

10 Hyytinen ER, Saadut R, Chen C *et al*. Defining the region(s) of deletion at 6q16-q22 in human prostate cancer. *Genes Chromosomes Cancer* 2002; **34**: 306-312.

11 Millikin D, Meese E, Vogelstein B *et al*. Loss of heterozygosity for loci on the long arm of chromosome 6 in human malignant melanoma. *Cancer Res* 1991; **51**: 5449-5453.

12 Orphanos V, McGown G, Hey Y *et al*. Proximal 6q, a region showing allele loss in primary breast cancer. *Br J Cancer* 1995; **71**: 290-293.

13 Sheng ZM, Marchetti A, Buttitta F *et al*. Multiple regions of chromosome 6q affected by loss of heterozygosity in primary human breast carcinomas. *Br J Cancer* 1996; **73**: 144-147.

14 Healy E, Belgaid C, Takata M *et al*. Prognostic significance of allelic losses in primary melanoma. *Oncogene* 1998; **16**: 2213-2218.

15 Mancini M, Vegna ML, Castoldi GL *et al*. Partial deletions of long arm of chromosome 6: biologic and clinical implications in adult acute lymphoblastic leukemia. *Leukemia* 2002; **16**: 2055-2061.

16 White VA, Chambers JD, Courtright PD *et al*. Correlation of cytogenetic abnormalities with the outcome of patients with uveal melanoma. *Cancer* 1998; **83**: 354-359.

17 Aalto Y, Eriksson L, Seregard S *et al*. Concomitant loss of chromosome 3 and whole arm losses and gains of chromosome 1, 6, or 8 in metastasizing primary uveal melanoma. *Invest Ophthalmol Vis Sci* 2001; **42**: 313-317.

18 Prescher G, Bornfeld N, Becher R. Nonrandom chromosomal abnormalities in primary uveal melanoma. *J Natl Cancer Inst* 1990; **82**: 1765-1769.

19 Naus NC, Zuidervaart W, Rayman N *et al*. Mutation analysis of the PTEN gene in uveal melanoma cell lines. In: *Int J Cancer*, Vol. 87, 2000: 151-153.

20 Shaffer LG, Tommerup N. *ISCN (2005): An International System for Human Cytogenetic Nomenclature*. S. Basel: Karger, 2005.

21 Kilic E, Naus NC, van Gils W *et al*. Concurrent loss of chromosome arm 1p and chromosome 3 predicts a decreased disease-free survival in uveal melanoma patients. *Invest Ophthalmol Vis Sci* 2005; **46**: 2253-2257.

22 Lapierre JM, Tachdjian G. Detection of chromosomal abnormalities by comparative genomic hybridization. In: *Curr Opin Obstet Gynecol*, Vol. 17, 2005: 171-177.

23 Mooy CM, Luyten GP, de Jong PT *et al*. Immunohistochemical and prognostic analysis of apoptosis and proliferation in uveal melanoma. *Am J Pathol* 1995; **147**: 1097-1104.

24 Coleman K, Baak JP, Van Diest P *et al*. Prognostic factors following enucleation of 111 uveal melanomas. *Br J Ophthalmol* 1993; **77**: 688-692.

25 Folberg R. Tumor progression in ocular melanomas. *J Invest Dermatol* 1993; **100**: 326S-331S.

26 Singh AD, Rennie IG, Kivela T *et al*. The Zimmerman-McLean-Foster hypothesis: 25 years later. *Br J Ophthalmol* 2004; **88**: 962-967.

27 Tschentscher F, Husing J, Holter T *et al*. Tumor classification based on gene expression profiling shows that uveal melanomas with and without monosomy 3 represent two distinct entities. *Cancer Res* 2003; **63**: 2578-2584.

28 Onken MD, Worley LA, Ehlers JP, Harbour JW. Gene expression profiling in uveal melanoma reveals two molecular classes and predicts metastatic death. *Cancer Res* 2004; **64**: 7205-7209.

29 Naus NC, Verhoeven AC, van Drunen E *et al*. Detection of genetic prognostic markers in uveal melanoma biopsies using fluorescence in situ hybridization. *Clin Cancer Res* 2002; **8**: 534-539.

30 Cross NA, Rennie IG, Murray AK, Sisley K. The identification of chromosome abnormalities associated with the invasive phenotype of uveal melanoma in vitro. *Clin Exp Metastasis* 2005; **22**: 107-113.

31 Horsman DE, White VA. Cytogenetic analysis of uveal melanoma. Consistent occurrence of monosomy 3 and trisomy 8q. *Cancer* 1993; **71**: 811-819.

32 Hughes S, Damato BE, Giddings I, Hiscott PS, Humphreys J, Houlston RS. Microarray comparative genomic hybridisation analysis of intraocular uveal melanomas identifies distinctive imbalances associated with loss of chromosome 3. *Br J Cancer* 2005, **93**: 1191-1196.

33 Ericsson C, Seregard S, Bartolazzi A *et al*. Association of HLA class I and class II antigen expression and mortality in uveal melanoma. *Invest Ophthalmol Vis Sci* 2001; **42**: 2153-2156.

34 Blom DJ, Mooy CM, Luyten GP *et al*. Inverse correlation between expression of HLA-B and c-myc in uveal melanoma. *J Pathol* 1997; **181**: 75-79.

35 Stokke T, DeAngelis P, Smedshammer L *et al*. Loss of chromosome 11q21-23.1 and 17p and gain of chromosome 6p are independent prognostic indicators in B-cell non-Hodgkin's lymphoma. *Br J Cancer* 2001; **85**: 1900-1913.

36 Verhagen PC, Hermans KG, Brok MO *et al*. Deletion of chromosomal region 6q14-16 in prostate cancer. *Int J Cancer* 2002; **102**: 142-147.

37 Saito S, Saito H, Koi S *et al*. Fine-scale deletion mapping of the distal long arm of chromosome 6 in 70 human ovarian cancers. *Cancer Res* 1992; **52**: 5815-5817.

38 Wang VW, Bell DA, Berkowitz RS *et al*. Whole genome amplification and high-throughput allelotyping identified five distinct deletion regions on chromosomes 5 and 6 in microdissected early-stage ovarian tumors. *Cancer Res* 2001; **61**: 4169-4174.

39 Henderson LJ, Okamoto I, Lestou VS *et al*. Delineation of a minimal region of deletion at 6q16.3 in follicular lymphoma and construction of a bacterial artificial chromosome contig spanning a 6-megabase region of 6q16-q21. *Genes Chromosomes Cancer* 2004; **40**: 60-65.

40 Merup M, Moreno TC, Heyman M *et al*. 6q deletions in acute lymphoblastic leukemia and non-Hodgkin's lymphomas. *Blood* 1998; **91**: 3397-3400.

41 Bosga-Bouwer AG, Kok K, Booman M *et al*. Array comparative genomic hybridization reveals a very high frequency of deletions of the long arm of chromosome 6 in testicular lymphoma. *Genes Chromosomes Cancer* 2006; **45**: 976-981.

42 Wang J, Kataoka H, Suzuki M *et al*. Downregulation of EphA7 by hypermethylation in colorectal cancer. *Oncogene* 2005; **24**: 5637-5647.

43 Depaepe V, Suarez-Gonzalez N, Dufour A *et al*. Ephrin signalling controls brain size by regulating apoptosis of neural progenitors. *Nature* 2005; **435**: 1244-1250.

44 Ray ME, Wistow G, Su YA *et al*. AIM1, a novel non-lens member of the betagamma-crystallin superfamily, is associated with the control of tumorigenicity in human malignant melanoma. *Proc Natl Acad Sci U S A* 1997; **94**: 3229-3234.

45 Li H, Lahti JM, Valentine M *et al*. Molecular cloning and chromosomal localization of the human cyclin C (CCNC) and cyclin E (CCNE) genes: deletion of the CCNC gene in human tumors. *Genomics* 1996; **32**: 253-259.

46 Lapouge G, Millon R, Muller D *et al*. Cisplatin-induced genes as potential markers for thyroid cancer. *Cell Mol Life Sci* 2005; **62**: 53-64.

47 Cross NA, Ganesh A, Parpia M, Murray AK, Rennie IG, Sisley K. Multiple locations on chromosome 3 are the targets of specific deletions in uveal melanoma. *Eye* 2006; **20**: 476-481.

Part V

General Discussion and Summary

Chapter 9

General discussion and future prospects

Chapter 9

General discussion and future prospects

Objectives of this work

In uveal melanoma a number of clinical and histopathological prognostic parameters have been described. [1, 2] Furthermore, certain chromosomal aberrations were found to have a prognostic value. [3-5] However, none of these factors were discriminative enough and the described prognostic chromosomal regions were relatively large, containing numerous genes. The aim of this thesis is to tackle both issues by developing a more reliable prognostic set of parameters based on a further delineation of prognostic relevant chromosomal regions complemented with gene expression analysis.

Prognostic signature of Uveal Melanoma based on gene-expression profiling data

Micro array-based gene expression analysis is an important tool in current cancer research that can be used to predict prognosis and for improved understanding of the genetic pathways leading to tumor development and progression. We and others have used this approach to develop classifiers to discriminate tumors with for example a high risk of systemic tumor spread.[6, 7] These studies, including our own analysis described in chapter 2, indicate the existence of two distinct molecular classes in primary uveal melanomas and we developed gene expression classifiers for disease-free survival and for unsupervised tumor class. The tumor class signature correlates with known prognostic markers and has strong prognostic value, outperforming all known prognostic indicators for uveal melanomas (HR 7.7, $p<0.001$). Despite the fact that in the comparable studies different gene-sets were identified, we were able to show that with our gene-set of 32 genes we could identify the same two separate tumor categories Class 1 correlates with a better prognosis and class 2 with a poor prognosis; (Class 1: sensitivity 1 and specificity 0,94; class 2: sensitivity 0,93 and specificity 1, respectively). The genes that are most differentially expressed in the micro-array classifier, predictive of tumor class, can be used in rapid diagnostic screening with Real-Time PCR. With this method, a small number of genes can be analyzed in high-throughput screening.[8] This will allow the application of recently collected micro-array knowledge in a diagnostic setting. Since it has been shown that tumor class can be predicted from fine-needle aspirate biopsies (FNAB)s, a more accurate and possibly eye-sparing prognostic screening has now become possible.[9]

Pathway analysis of Uveal Melanoma based on gene-expression profiling data

To unravel the molecular basis of uveal melanoma development and progression the differential expression between sub-groups was estimated in Significance Analysis of Micro arrays (SAM) implemented in Omniviz according to the principles of Tusher. [10] All comparisons were made using our extensive patient database containing follow-up, histo-pathological, clinical and

cytogenetic data. The data were explored using Ingenuity Pathways Analysis, a Web-based application that enables the discovery, visualization, and exploration of interaction networks with significantly changed gene expression in micro array data sets. When looking at the significance of functions and diseases, we did not find any significant functions in the SAM dataset based on tumor micro array class. The groups that were analyzed originated from unsupervised hierarchical clustering, resulting in two groups that have the most opposing gene expression profile, Therefore, there are many different genes in many different pathways and functions that discriminate between the two groups, irrespective of biological functions and pathways. Ingenuity function analysis compares the percentage of associated genes in each specific function group with the percentage of associated genes in total, which makes it unlikely that one specific function will reach significance.
However, when we looked at the most significant functions in the data of SAM analysis on disease-free survival with 4 years as the cut-off value, the most significant deviant biological functions are cell cycle, cellular assembly and organization, embryonic development, DNA replication, recombination and repair, lipid metabolism and small molecule biochemistry, all with a –log(significance) value of 0.0015.

This indicates that tumors in the poor DFS group in general have higher mitotic and metabolic rates, an up-regulated DNA-repair mechanism and upregulation of a number of apoptosis mediating genes and also have a number of unregulated genes that are involved in the development of metastases, like *Col18A1*, *HTATP2*, *MAPK1*, *PTPRA* and *STAT6*. This is in line with what could be expected from tumors in a poor prognosis group, and it partly explains why these tumors are more prone to develop metastatic disease than tumors in the better prognosis group. The data are indicative of faster tumor growth, a higher incidence of chromosomal aberrations and up regulation of genes involved in migration and the development of metastases in the poor prognosis group, all typical for a more aggressive phenotype. The initiation of this aggressive signature, however, remains concealed with this approach.

Prognostic parameters

In this thesis, we have re-assessed a number of clinical and histopathological known predictors of UM survival in a prognostic analysis that also included chromosomal changes. The single prognostic factors were analyzed in a univariate and a multivariate setting. Loss of chromosome 3, largest tumor diameter, gain of chromosome 8q and a mixed/epitheloid cell type were the only significant predictors of poor survival in univariate analysis. The significance of monosomy of chromosome 3, largest tumor diameter and cell type did not alter when correcting for possible confounders. Of this set of prognostic markers, loss of chromosome 3 is widely accepted as the most reliable prognostic parameter. One of the findings described in this thesis is that when this loss is accompanied by loss of chromosome 1p, disease-free survival chances are further decreased, with a HR of 7.8 ($p<0.001$) of developing metastatic disease. Micro array classification has a HR of 7.7 ($p<0.001$). The major difference between the parameters micro array class and concurrent loss of chromosomes 1p and 3 is that with micro array classification the poor prognosis group encompasses 50% of all patients, instead of only one fifth of all patients with the chromosomal predictor. In other words, micro array classification has a strong predictive value

for a highly increased risk in a larger number of patients. The combination of concurrent loss of chromosome 3, epitheloid cell type and micro array class 2, the three most significant individual predictors for poor prognosis, results in an almost ten times higher risk of developing metastatic disease for 37% of all patients in the analyzed cohort. When additional parameters are added to the analysis, like loss of chromosome 1p, the relatively small size of the total cohort of 46 patients that were analyzed on micro-arrays results in rather small sub-groups of patients that lack statistical power. It would therefore be of great interest to use the expression based classifier gene set to determine array class in an additional series of patients, preferably by micro-array analysis of more UMs, which will also lead to more data on affected pathways, or by analysis with the relatively cheap and fast technique of Quantitative Real-Time PCR. This will lead to a larger patient cohort, in which more prognostic parameters can be combined, resulting in a very strong prognostic prediction for groups of patients. In that context, the finding that we could classify all patients in our study to the correct class with only the top four predicting genes opens great perspectives for a rapid prognostic class prediction using real-time PCR.

Chromosomal regions

Earlier studies have revealed prognostic significant chromosomal regions of gain and loss in uveal melanoma. The most frequently affected chromosomes are chromosomes 1, 3, 6 and 8. We and other research groups have published data on the smallest recurrently affected chromosomal regions, (summarized in fig. 1-4 of the introduction) and together with the experimental research, described in chapters 2-8 of this thesis, a few remarkable observations can be made.

Improvement of diagnostic FISH probe set

Loss of chromosome 3 is the most frequent adverse event in uveal melanoma. Combined delineation studies revealed smaller regions of deletion on both arms. The combined results of different studies, including our studies described in chapters 6 and 7 of this thesis, show different demarcated regions. However, a region ranging from 3q25.1 to 3q26.1 is involved in most studies and our routinely used diagnostic FISH probe RP11-64F6 maps to this region. Two regions on the p-arm of chromosome 3 showed decreased expression in UMs with short survival, 3p12-3p14.1 and 3p23-3p25.3 (chapter 2). These regions overlap with the regions described by Cross *et al*. and Parrella *et al*.[11, 12], resulting in common regions of 3p12-3p14.1 and 3p25.1-3p25.2. Because of the strong relation of gene expression on those regions with survival and the finding that these regions of significantly decreased gene expression overlap with SROs of frequently deleted genomic regions in UM, it is important to include these 3p regions in the routine diagnostic FISH-panel in our laboratory, which currently only includes the q-arm and the centromere of chromosome 3.

The FISH probe that we currently use as a diagnostic probe on chromosome-arm 6q, RP11-787I22, is located in the middle of the smallest region of overlap (SRO) ranging from 6q16.3 –6q23 that results from different studies on uveal melanoma, together with the region described in chapter 8. The diagnostic probe on chromosome-arm 6p, RP11-356B3, is located slightly outside the smallest overlapping region of amplification described in chapter 8 of this

thesis (6p22.3 –p25.3). Since amplifications on chromosome-arm 6p in almost all cases include the tip of the chromosome, extension of the diagnostic FISH set with a more telomeric probe on chromosome-arm 6p is urgently needed.

The findings described above have lead to a proposal for a renewed set of diagnostic FISH probes, including probes on chromosome-arm 3p and a new 6p probe, mapping to the subtelomeric region of chromosome-arm 6p. The locations of the current and suggested probes for FISH for routine diagnostic screening of UMs are listed in table 1. This improved FISH panel will increase the sensitivity since it includes the analysis of additional important regions.

Table 1. **Current and suggested locations for the diagnostic FISH probes**

Chromosomal location	Current diagnostic probe set	New diagnostic probe set
Chromosome 1	1p36	1p36
Chromosome 3	 #3 3q24	**3p25.1** **3p13** #3 3q24
Chromosome 6	6p22 6q21	**6pter** 6q21
Chromosome 8	8p11 #8 8q24	8p11 #8 8q24

Role of chromosome 8 alterations

Our expression studies showed that genes on the whole long arm of chromosome 8 were expressed at higher levels and genes on 8p were expressed at lower levels in tumors with a short survival time. In our genomic studies we observed that both loss of 8p and gain of 8q are strongly related to loss of chromosome 3, whereas gain of 8p is not related with loss of chromosome 3 (chapter 3). Apparently, iso-chromosome formation of the long arm of chromosome 8, and not gain of an entire chromosome 8 copy contributes to a poorer prognosis of UM patients. As a validation of this hypothesis, Kaplan Meier survival analysis of patients in our database showed a significant poorer survival for patients with either iso-8q formation, or, when karyotyping was not possible, concurrent loss of chromosome-arm 8p and gain of 8q, when compared with gain of an entire chromosome 8. The log-rank significance of this difference was 0.029 (fig.1). This suggests that both loss of chromosome-arm 8p and gain of chromosome-arm 8q are important in tumor progression.

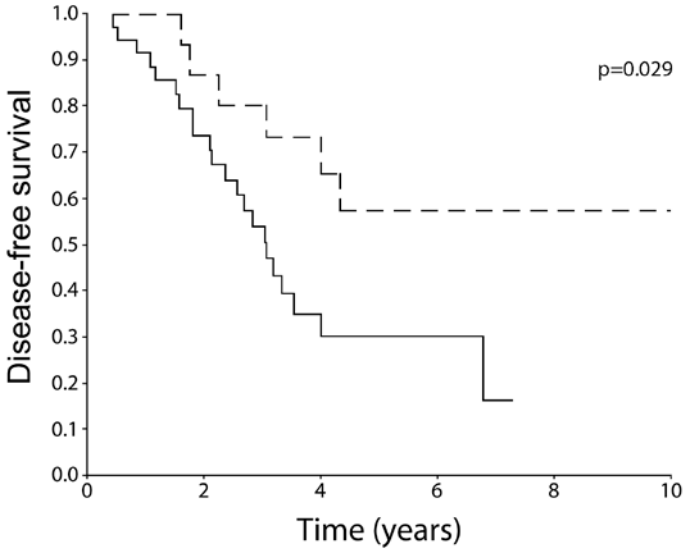


Figure 1. **Kaplan-Meier survival analysis of UM patients with tumors presenting with gain of chromosome 8 or isochromosome formation of chromosome-arm 8q.** Dashed line: tumors with gain of an entire chromosome 8; solid line: tumors with presence of 8q iso-chromosomes.

Candidate genes in common deleted and or amplified regions

In this thesis the strong negative effect on prognosis of concurrent loss of chromosome region 1p36 and chromosome 3 is described (chapter 3). In the search for a possible candidate gene responsible for the adverse effect of chromosome 1p loss in uveal melanoma, we have studied expression and performed copy number analysis of one particular gene in this region, *APITD1*. Although expression of this gene, that contains an apoptosis-inducing domain, is affected in neuroblastoma, its expression is not related with survival or DNA copy number in uveal melanoma. Therefore, another gene must be responsible for the adverse effect of 1p loss. The gene *TP73*, also located on chromosome 1p36, was studied in a differential expression study and revealed increased expression of the dominant negative p73Δex2 transcript in tumors with loss of chromosome 1p. This suggests a role of this gene in uveal melanoma (Kilic *et al.*, submitted). [13] Other interesting candidates could be *UBE4B*, *IF1B*, *PGD*, *DFFA* and *PEX14*, which are down-regulated in neuroblastomas with poor prognosis and belong to the same cluster of genes to which *APITD1* belongs. [14]

The affected regions on chromosome 3, described in chapter 6 of this thesis, harbor potential tumor suppressor genes. Candidates on the 3p region are *THRB* and *RARβ2*. The human *THRB* locus acts as a transcriptional activator and silencer. Aberrant expression and/or mutations in *THR* genes could be associated with carcinogenesis. Decreased levels of the receptor *RARβ2* are associated with malignancies, like breast tumors, lung cancer and squamous cell cancer of the head and neck.[15] As addressed in chapter 2, also *MITF* is a promising candidate gene since it is located in one of the regions on chromosome-arm 3p with lower expression in UMs of patients with a short survival time.

In addition, the gene expression classifiers described by Onken *et al*. and Tschentscher *et al*. harbor two genes mapping to the 3p region, *NR1D2* and *RPL15,* and three genes mapping to the 3q region*, PIK3R4*, *EIF2A* and *KIAA0678*. [6, 7] In particular, these 3p genes are excellent candidates for future analysis.

The deleted region on chromosome-arm 6q described in chapter 8 of this thesis contains many genes, with among them the candidate tumor suppressor genes *EphA7*, *AIM1* and *CCNC.* In addition, three genes of the gene expression classifiers described by Onken *et al*. and Tschentscher *et al*. map to the amplified 6p region described in chapter 8, namely *HCGIV.8*, *ALDH5A1* and *TFAP2A*. [6, 7]

Abnormal expression of genes in poor prognosis uveal melanomas could, besides chromosomal aberrations, also be caused by epigenetic mechanisms like methylation of gene promoter regions. Blocks of genes with lower expression in particular chromosomal regions without deletions could indicate epigenetic mechanisms of regulation of gene expression in uveal melanoma. Previously, promoter methylation has been identified as the mechanism responsible for lower expression of the classifier gene *TIMP3* in uveal melanoma. [16] In addition, methylation of *RASSF1A* has been shown to be a sensitive prognostic marker for prognosis in uveal melanoma. [17] Therefore it is interesting to look at the methylation status of genes in prognostic relevant regions of uveal melanoma in tumors of patients with a poor survival and without chromosomal aberrations of those regions. It could well be that the promoter regions of genes in those regions have abnormal methylation patterns in these tumors. Epigenetic studies would, therefore, lead to a better understanding of the genetic and epigenetic mechanisms underlying decreased and increased expression of genes associated with poor prognosis in uveal melanoma.

Future developments and new directions

Global screening of the tumors with high resolution techniques will provide more knowledge on the involved chromosomal areas in uveal melanoma. In particular the development of Single Nucleotide Polymorphism (SNP) micro arrays is very promising, since it allows a rapid high resolution screening of both copy number and the number of different alleles in one single run. In a recent SNP analysis study from Onken *et al.* it was shown that loss of heterozygosity screening provides a stronger prognostic marker for uveal melanoma than monosomy of this chromosome. [18] The combination of screening for the presence of different allelic variants and screening for copy number using SNP micro arrays offers the opportunity to further delineate the critical chromosomal regions in UM. This approach has already proven its advantages in a large scale study on childhood acute lymphoblastic leukemias, in which the frequent mutation of genes encoding transcription factors important for B cell development, was detected by SNP micro array analysis.[19] Also, the findings in a large breast cancer study, in which SNPs in five novel independent loci that exhibited strong and consistent evidence of association with breast cancer were found, underline the value of large-scale SNP analysis in tumors. [20] Although for this kind of studies large numbers of case and control samples have to be included to identify true candidate SNPs, these findings open wide perspectives for high resolution SNP analysis in other types of tumors.

About 5 years ago another new high-throughput method has been developed for genetic screening. This method, called Multiplex ligation-dependent probe amplification or MLPA, allows determination of copy number presence of up to 60 sequences in one reaction, thereby enabling a rapid screening of a series of DNA samples.[21] The technique is very promising for UM, now that the prognostic relevant regions become more and more specified. There are also possibilities for MLPA to be applied in tumor gene expression screening and tumor DNA methylation. [22, 23] Methylation of cytosines in CpG dinucleotides forms the main epigenetic modification in humans.[24] In cancer the amount of methylated CpG dinucleotides is often aberrant compared to normal tissues, resulting in uncontrolled gene expression levels.[25, 26] As mentioned above, in uveal melanoma hypermethylation plays a role in abnormal expression of the p16 gene, as well as for *RASSF1* and *TIMP3*. [16, 17, 27] It would be interesting to look at the methylation status of genes in uveal melanomas of patients with a poor survival and without chromosomal aberrations of the prognostic relevant regions.

However, with regard to these high-throughput methods one has to keep in mind that an average signal of the specimen is measured. It is known that there are interesting UM cases that display a heterogenic pattern of morphological and chromosomal abnormalities. With respect to chromosomal abnormalities, in our tumor collection, there are UM cases found that display monosomy of chromosome 3 in one region, whereas retention of both alleles is found in other parts of the same tumor (unpublished data, personal communication H.W. Mensink). It would be interesting to further investigate to what extent intratumoral heterogeneity plays a role in UM and to study the prognostic value of smaller sub clones with loss of chromosome 3. If smaller sub clones are indeed important for patient survival chances, the new micro array-based techniques and multiplex analysis, in spite of their impressive high-throughput capacities, cannot replace the established karyotyping and FISH techniques, which allow analysis of single nuclei and thereby also smaller tumor subclones. However, a retrospective study has indicated that UMs can be classified correctly from the gene expression profile obtained from FNABs. [9] Although these findings have to be confirmed in a prospective study, this suggests that gene expression profile is relatively constant throughout the tumor. This opens doors for diagnostic and prognostic screening on FNABs.

In the gene-expression profiling study described in chapter 2, LAP analysis was successfully used to identify chromosomal regions with differential expression. Next to the regions on chromosomes 3 and 8, a number of smaller regions were identified on chromosomes less frequently involved in UM. These regions might provide interesting starting points for future investigations. LAP analysis can be applied relatively easy to existing micro-array datasets to pinpoint chromosomal regions that are critical in malignant processes, both for tumor initiation and tumor progression. Therefore, LAP analysis could also be very useful in studies on other types of tumors.
Complementary to the screening at the DNA and RNA level, gene expression profiling has offered a number of candidates for immunohistochemistral approaches in UM, both for diagnostic/ prognostic and for therapeutic purposes. In our tumor-class prediction signature, there are 9 genes of which the protein products are located at the plasma membrane. This makes it possible to screen for expression of those proteins and to assay their possible use as therapeutical targets

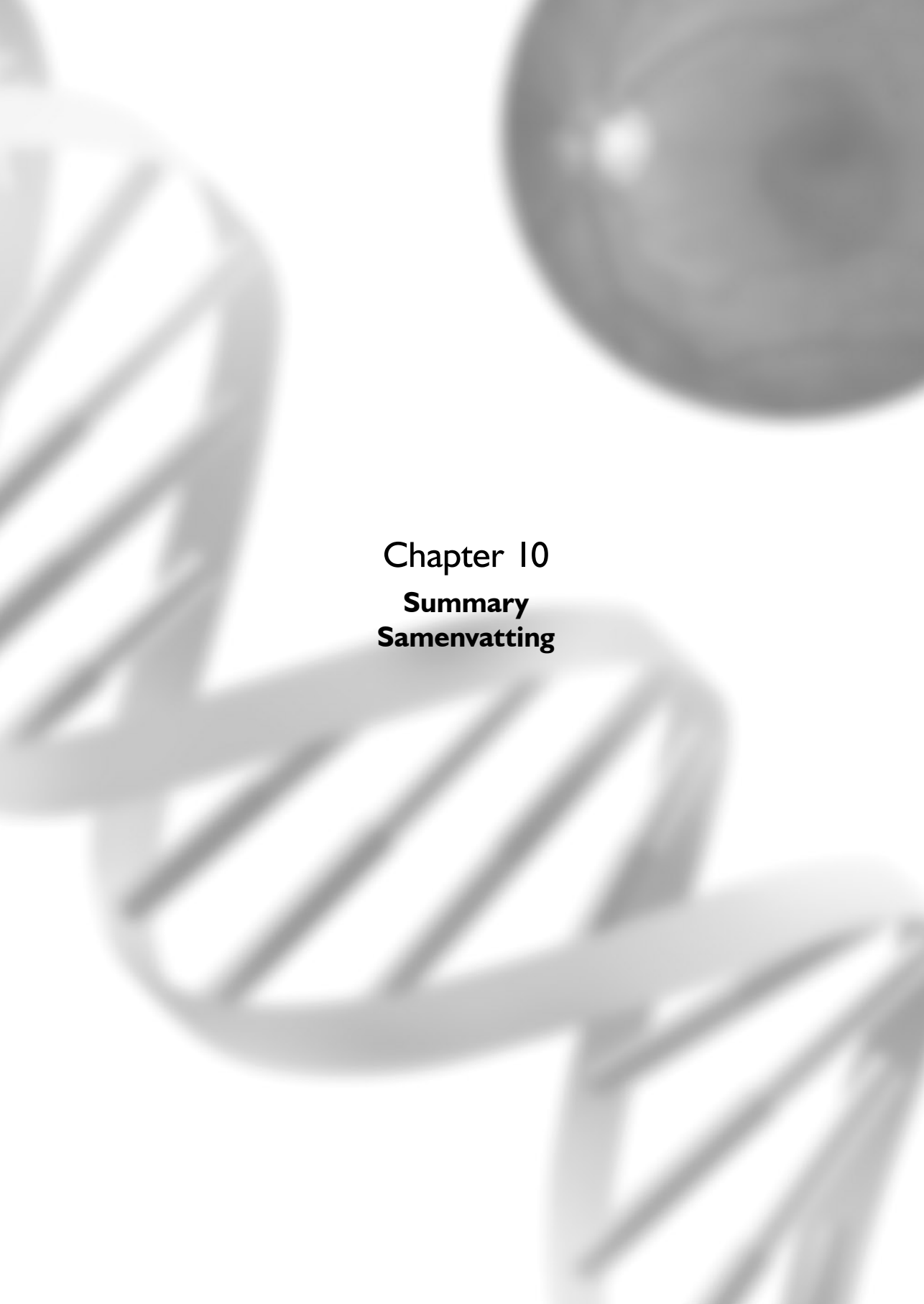
when their protein expression is found to be tumor specific and high enough for detection. A very promising pathway to study therapeutical purposes is the interaction of MSH with the RAS pathway. MSH, that also induces expression of the *MITF1* gene described in chapter 2 of this thesis,[28] binds to the MC1 receptor on melanocytes. This leads to accumulation of cAMP and PKA dependent activation of BRAF. Eventually, Ras/raf signaling results in ERK activation. Subsequently, ERK is able to activate transcription factors that in turn regulate various functions under which cell cycle control and proliferation and pigment synthesis. Since cutaneous melanoma is characterized by loss of the p16 encoding gene (CDKN2A) and activation of the RAS pathway,[29, 30] it could well be that in uveal melanoma this pathway is critical in tumor development and thereby becomes a potential target for tumor treatment. In the pathway analysis of our gene expression data, Erk expression was found to be upregulated in both tumors classes, however only in class 2 tumors also Braf expression is increased. Perhaps activation of BRAF, plays a critical role in the poor survival of patients suffering from class 2 tumors specific.

The importance of the work described in this thesis is not only the development of a new prognostic parameter and the delineation of already known prognostic relevant regions, but it also provides new opportunities for rapid prognostic screening as well as possible targets for anti-cancer therapy. Furthermore, the data generated by gene expression profiling provides new insights in the molecular biological behavior of uveal melanoma, which will ultimately lead to a better treatment of this disease.

References

1. Mooy CM, De Jong PT. Prognostic parameters in uveal melanoma: a review. *Surv Ophthalmol*. 1996;41:215-228
2. Singh AD, Shields CL, Shields JA. Prognostic factors in uveal melanoma. *Melanoma Res*. 2001;11:255-263.
3. Prescher G, Bornfeld N, Friedrichs W, Seeber S, Becher R. Cytogenetics of twelve cases of uveal melanoma and patterns of nonrandom anomalies and isochromosome formation. *Cancer Genet Cytogenet*. 1995;80:40-46
4. Sisley K, Rennie IG, Parsons MA *et al.* Abnormalities of chromosomes 3 and 8 in posterior uveal melanoma correlate with prognosis. *Genes Chromosomes Cancer*. 1997;19:22-28.
5. Kilic E, Naus NC, van Gils W *et al.* Concurrent loss of chromosome arm 1p and chromosome 3 predicts a decreased disease-free survival in uveal melanoma patients. *Invest Ophthalmol Vis Sci*. 2005;46:2253-2257
6. Onken MD, Worley LA, Ehlers JP, Harbour JW. Gene expression profiling in uveal melanoma reveals two molecular classes and predicts metastatic death. *Cancer Res*. 2004;64:7205-7209
7. Tschentscher F, Husing J, Holter T *et al.* Tumor classification based on gene expression profiling shows that uveal melanomas with and without monosomy 3 represent two distinct entities. *Cancer Res*. 2003;63:2578-2584
8. Bustin SA, Mueller R. Real-time reverse transcription PCR (qRT-PCR) and its potential use in clinical diagnosis. *Clin Sci (Lond)*. 2005;109:365-379
9. Onken MD, Worley LA, Davila RM, Char DH, Harbour JW. Prognostic testing in uveal melanoma by transcriptomic profiling of fine needle biopsy specimens. *J Mol Diagn*. 2006;8:567-573
10. Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci U S A*. 2001;98:5116-5121
11. Parrella P, Fazio VM, Gallo AP, Sidransky D, Merbs SL. Fine mapping of chromosome 3 in uveal melanoma: identification of a minimal region of deletion on chromosomal arm 3p25.1-p25.2. *Cancer Res*. 2003;63:8507-8510
12. Cross NA, Ganesh A, Parpia M *et al.* Multiple locations on chromosome 3 are the targets of specific deletions in uveal melanoma. *Eye*. 2006;20:476-481
13. Emine Kilic, Hennie T. Brüggenwirth, Marit Meier, Nicole C. Naus, H. Berna Beverloo, Jules P. Meijerink, Gre P. Luyten, Annelies de Klein. Differential expression of TP63 and TP73 in Uveal Melanoma reveals an increased expression of p73Dex2 transcript in tumours with loss of chromosome 1p. *submitted*

14. Caren H, Ejeskar K, Fransson S *et al.* A cluster of genes located in 1p36 are down-regulated in neuroblastomas with poor prognosis, but not due to CpG island methylation. *Mol Cancer*. 2005;4:10
15. Yang Q, Yoshimura G, Mori I, Sakurai T, Kakudo K. Chromosome 3p and breast cancer. *J Hum Genet*. 2002;47:453-459
16. van der Velden PA, Zuidervaart W, Hurks MH *et al.* Expression profiling reveals that methylation of TIMP3 is involved in uveal melanoma development. *Int J Cancer*. 2003;106:472-479
17. Maat W, van der Velden PA, Out-Luiting C *et al.* Epigenetic inactivation of RASSF1a in uveal melanoma. *Invest Ophthalmol Vis Sci*. 2007;48:486-490
18. Onken MD, Worley LA, Person E *et al.* Loss of heterozygosity of chromosome 3 detected with single nucleotide polymorphisms is superior to monosomy 3 for predicting metastasis in uveal melanoma. *Clin Cancer Res*. 2007;13:2923-2927
19. Mullighan CG, Goorha S, Radtke I *et al.* Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. *Nature*. 2007;446:758-764
20. Easton DF, Pooley KA, Dunning AM *et al.* Genome-wide association study identifies novel breast cancer susceptibility loci. *Nature*. 2007;447:1087-1093
21. Schouten JP, McElgunn CJ, Waaijer R *et al.* Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. *Nucleic Acids Res*. 2002;30:e57
22. Eldering E, Spek CA, Aberson HL *et al.* Expression profiling via novel multiplex assay allows rapid assessment of gene regulation in defined signalling pathways. *Nucleic Acids Res*. 2003;31:e153
23. Nygren AO, Ameziane N, Duarte HM *et al.* Methylation-specific MLPA (MS-MLPA): simultaneous detection of CpG methylation and copy number changes of up to 40 sequences. *Nucleic Acids Res*. 2005;33:e128
24. Nguyen CT, Gonzales FA, Jones PA. Altered chromatin structure associated with methylation-induced gene silencing in cancer cells: correlation of accessibility, methylation, MeCP2 binding and acetylation. *Nucleic Acids Res*. 2001;29:4598-4606
25. Feinberg AP, Vogelstein B. Hypomethylation distinguishes genes of some human cancers from their normal counterparts. *Nature*. 1983;301:89-92
26. Agrawal A, Murphy RF, Agrawal DK. DNA methylation in breast and colorectal cancers. *Mod Pathol*. 2007;20:711-721
27. van der Velden PA, Metzelaar-Blok JA, Bergman W *et al.* Promoter hypermethylation: a common cause of reduced p16(INK4a) expression in uveal melanoma. *Cancer Res*. 2001;61:5303-5306
28. Aberdam E, Bertolotto C, Sviderskaya EV *et al.* Involvement of microphthalmia in the inhibition of melanocyte lineage differentiation and of melanogenesis by agouti signal protein. *J Biol Chem*. 1998;273:19560-19565
29. Haluska FG, Tsao H, Wu H *et al.* Genetic alterations in signaling pathways in melanoma. *Clin Cancer Res*. 2006;12:2301s-2307s
30. Smalley KS. A pivotal role for ERK in the oncogenic behaviour of malignant melanoma? *Int J Cancer*. 2003;104:527-532



Chapter 10

Summary
Samenvatting

Chapter 10

Summary

Uveal melanoma (UM) is the most common primary tumor in the eye with an incidence of approximately 7 per million every year in the Western World. The clinical outcome of patients with uveal melanoma depends on the development of metastases. A number of clinical and histopathological parameters have been described to predict for outcome of the disease. Also cytogenetic prognostic factors are reported. Previous studies from our group and others have shown that the most frequent aberrations, seen in UM, are loss of 1p36, loss of chromosome 3, abnormalities of chromosome 6 and gain of chromosome 8 or 8q. The use of only clinical and histopathological parameters is not conclusive enough; even in combination those parameters cannot provide a sound estimation of prognosis. Therefore it is of great importance that also cytogenetic prognostic factors are included in the establishment of a patients' prognosis. Several studies have focused on the delineation of commonly affected regions on chromosomes 1, 3, 6 and 8 in UM. This thesis describes different genomic and cytogenetic approaches to make advances in prognostic screening of UM and to provide new insights in the search for candidate genes, responsible for tumor initiation and development, and possible targets for therapy.

In the first part of this thesis (**chapter 2**), a gene expression study of uveal melanoma performed with micro array technology is described. The existence of two distinct molecular classes in primary uveal melanomas is confirmed and the development of two gene-expression classifiers that predict for disease-free survival and for micro-array class is described. The results indicate that micro-array classification outperforms known prognostic indicators for uveal melanomas, such as cytogenetic abnormalities. In addition, two regions on chromosome-arm 3p were found to have decreased gene expression in tumors with shorter disease-free survival. Those regions likely harbor genes responsible for the poor prognosis of UM patients with chromosome 3 loss.

In the second part, described in **chapters 3** and **4**, studies on different clinical and cytogenetic analyses of uveal melanoma and findings on correlations of a range of parameters with prognosis are described.

Chapter 3 describes a study in which the cytogenetic changes were correlated with clinical and histopathological parameters. The karyotypes of 74 primary uveal melanomas were analyzed, resulting in identification of the most frequent chromosomal abnormalities involved in uveal melanoma: chromosome 8 (53%); loss of chromosome 3, p-arm (41%) and q-arm (42%); partial loss of chromosome 1, p-arm (24%); and abnormalities in chromosome 6 that resulted in gain of 6p (18%) and/or loss of 6q (28%). In univariate analysis, monosomy 3 and largest tumor diameter were the most significant in determining survival of patients with uveal melanoma in this study, in which gene expression-based uveal melanoma class was not included.

Chapter 4 describes the investigation of independent numerical changes in chromosomes 1, 3, 6, and 8 on disease-free survival (DFS) in tumors of 120 uveal melanoma patients. Univariate analysis, loss of chromosome 3, gain of 8q, largest tumor diameter and the presence of epitheloid cells were associated with a decreased DFS. Multivariate analysis showed that the effect of

monosomy 3 on survival was strongly affected by changes in 1p36. Of all chromosomal changes, only the concurrent loss of the short arm of chromosome 1 and all of chromosome 3 was an independent prognostic parameter for disease-free survival ($P < 0.001$). Therefore, the conclusion of this part is that in uveal melanoma, concurrent loss of the short arm of chromosome 1 and all of chromosome 3 is an independent and strong predictor of decreased DFS.

The third part of this thesis focuses on studies of critical regions and genes in uveal melanoma, aiming on further delineation of the critical regions in uveal melanoma, which will lead to the identification of genes that are critical in uveal melanoma.

Chapter 5 focuses on a candidate tumor suppressor gene called *APITD1* that is located on the critical region on chromosome-arm 1p. It was hypothesized that lower expression levels of this gene could lead to decreased survival in patients with concurrent loss of a region on chromosome-arm1p and chromosome 3. We have found that expression of APITD1 mRNA was not related to DNA copy number ($p=0.956$) or chromosome 3 status ($p=0.958$). Kaplan-Meier survival analysis showed very similar survival curves for tumors with high and low APITD1 expression with a log-rank significance value of $p=0.9682$. These results indicate that APITD1 is not the tumor suppressor gene on 1p36 responsible for the negative prognostic effect in uveal melanoma with concurrent loss of chromosomes 1p36 and 3.

Delineation studies of chromosome 3 in uveal melanoma are described in **chapters 6** and **7**.

Chapter 6 outlines the demarcation of partial losses of chromosome 3 in a uveal melanoma cell line, Mel270, which is derived from a primary tumor. LOH and FISH studies revealed a chromosome 3q deletion ranging from 3q21.2-3q24 in Mel270 and the cell line OMM2.3 that was derived from one of the metastases of the same primary tumor. In addition, a region of possible allelic loss, mapping to 3p24, was found in these cell lines. Since allelic loss could not be confirmed by FISH, this could point to a hemizygous region. These results contribute to a further demarcation of candidate regions for tumor suppressor genes on chromosome 3 in uveal melanoma.

Chapter 7 considers a delineation study of chromosome 3 by investigating tumors with partial chromosome 3 losses using FISH and CGH. This study led to demarcation of a region that spans from the centromere to approximately 3q26.2. Interestingly, all patients in our cohort with UMs, presenting with partial deletions of chromosome 3, involving the q-arm only, did not die from the disease. Apparently, poor prognosis in UM patients is not caused by loss of the 3q region only.

Chapter 8 describes a delineation study on chromosome 6. In an interesting UM case, karyotyping and FISH analysis revealed a deleted region on chromosome 6q that was further delineated to a region ranging from 6q16.1 to 6q22 using CGH and FISH analysis. Furthermore, a region of gain spanning from 6pter to 6p21.2 was also demarcated with CGH and FISH. No other deletions or amplifications on recurrently involved chromosomes were found in this patient. This study indicates the presence of one or more tumor suppressor genes on chromosomal region 6q16.1-6q22 and the presence of one or more oncogenes on chromosomal region 6pter-6p21.2, which are likely to be important in uveal melanoma and probably also in other tumors.

In **chapter 9** the findings of this thesis and future directions of uveal melanoma research are discussed. The work described in this thesis has led to a further delineation of chromosomal

regions that are of importance in uveal melanoma development. In addition, it is shown that molecular class based on gene expression profile and concurrent loss of chromosome-arm 1p and chromosome 3 are the most significant predictors for poor prognosis in UM patients.

Samenvatting

Oogmelanomen zijn de meest voorkomende primaire tumoren in het oog met een incidentie van ongeveer 7 per miljoen per jaar in de Westerse wereld. De klinische afloop van de ziekte hangt af van de ontwikkeling van metastasen. Een aantal klinische en histopathologische parameters zijn beschreven waarmee de prognose voor een oogmelanoom patiënt bepaald kan worden. Ook van enkele cytogenetische parameters is een prognostische waarde beschreven. Eerder onderzoek door onze en andere onderzoeksgroepen heeft aangetoond dat de meest voorkomende chromosomale afwijkingen in oogmelanomen bestaan uit het verlies van chromosoom-arm 1p36, verlies van chromosoom 3, afwijkingen op chromosoom 6 en winst van chromosoom-arm 8q. Het gebruik van enkel klinische en histopathologische parameters is niet conclusief genoeg, aangezien met deze parameters zelfs in combinatie niet met voldoende zekerheid de overlevingskansen voor een oogmelanoom patiënt kunnen worden voorspeld. Het is daarom van belang dat ook cytogenetische factoren worden meegenomen in de bepaling van de prognose voor een patiënt. Verscheidene eerdere studies hebben zich gericht op de afbakening van gemeenschappelijk betrokken chromosomale regio's op de chromosomen 1, 3, 6 en 8. Dit proefschrift beschrijft verschillende genomische en cytogenetische benaderingen om vooruitgang te boeken in de prognostische screening van oogmelanomen en het verwerven van nieuwe inzichten in de zoektocht naar kandidaat-genen, welke verantwoordelijk zijn voor de initiatie van oogmelanomen en de ontwikkeling ervan, en naar mogelijke targets voor behandeling.

In het eerste gedeelte van de thesis (**hoofdstuk 2**), wordt een gen expressie studie op oogmelanomen beschreven, waarbij gebruik is gemaakt van micro array technologie. Het bestaan van twee verschillende moleculaire klassen bij oogmelanomen wordt bevestigd en de ontwikkeling van twee gen expressie classifiers indicatief voor ziekte vrije overleving en moleculaire micro array klasse wordt beschreven. De resultaten geven aan dat micro array classificatie een betere prognostische marker vormt dan andere bekende prognostische markers bij oogmelanomen, zoals cytogenetische parameters. Verder zijn twee regio's op chromosoom-arm 3p gevonden waarvan de genexpressie significant verlaagd is in tumoren met een kortere overleving. Deze regio's bevatten waarschijnlijk genen die verantwoordelijk zijn voor de slechte prognose van oogmelanoom patiënten met verlies van chromosoom 3.

Het tweede gedeelte van dit proefschrift, beschreven in **hoofdstukken 3 en 4**, beslaat studies naar verschillende klinische en cytogenetische factoren in oogmelanomen en de bevindingen over correlaties van een reeks parameters met de prognose.
Hoofdstuk 3 beslaat een studie waarin cytogenetische veranderingen worden gekoppeld aan klinische en histopathologische parameters. De karyotypes van 74 primaire tumoren zijn vergeleken, wat heeft geresulteerd in de identificatie van de meest voorkomende chromosomale afwijkingen in oogmelanomen, namelijk chromosoom 8 (53%), verlies van chromosoom 3, namelijk de p-arm (41%) en de q-arm (42%), verlies van een deel van chromosoom 1 op de p-arm (24%) en afwijkingen op chromosoom 6 resulterend in winst van 6p (18%) en/of verlies van 6q (28%). In univariate analyse is gevonden dat monosomie van chromosoom 3 en de grootste tumor diameter de meest significante predictors vormen voor overleving van oogmelanoom patiënten in deze studie, waarin tumor classificatie op basis van genexpressie profiel niet is opgenomen.
Hoofdstuk 4 beschrijft een onderzoek naar de invloed van onafhankelijke numerieke veranderingen op de chromosomen 1, 3, 6 en 8 op ziektevrije overleving in de tumoren van 120 oogmelanoom patiënten. In univariate analyse werd gevonden dat verlies van chromosoom 3, winst van 8q, grootste tumor diameter en de aanwezigheid van epitheloid cellen geassocieerd waren met een verminderde ziektevrije overleving. Multivariaat analyse toonde aan dat het effect van monosomie van chromosoom 3 op de overleving versterkt werd door verlies van 1p36. Van alle chromosomale afwijkingen, was gezamenlijk verlies van chromosoom 3 en chromosoomregio 1p36 de enige onafhankelijke prognostische marker voor ziektevrije overleving ($P<0.001$). De conclusie van dit gedeelte is dan ook dat het gecombineerde verlies van chromosoom 3 en de korte arm van chromosoom 1 een onafhankelijke en sterk voorspellende prognostische marker voor oogmelanomen vormt.

Het derde gedeelte van dit proefschrift bestaat uit studies naar kritische regio's en genen in oogmelanomen, met als doel de voor oogmelanomen kritische regio's te verkleinen, wat zal leiden tot de identificatie van genen die kritiek zijn voor oogmelanomen.
Hoofdstuk 5 richt zich op een kandidaat tumor suppressor gen, genaamd APITD1, dat is gelokaliseerd in het kritische gebied op chromosoomregio 1p. De hypothese was dat verlaagde expressie van dit gen zou kunnen leiden tot een verslechterde overleving bij patiënten met gezamenlijk verlies van de kritische regio op 1p en chromosoom 3. We vonden dat expressie van APITD1 mRNA niet gerelateerd is aan het aantal DNA kopieën ($p=0.956$) of chromosoom 3 status ($p=0.958$). Kaplan-Meier overlevingsanalyse liet zien dat tumoren met een hogere en een lagere APITD1 expressie een vergelijkbare overleving hadden, met een log-rank significantie van $p=0.9682$. Deze resultaten geven aan dat APITD1 niet het tumor suppressor gen is op 1p36 dat verantwoordelijk is voor het negatieve effect op de prognose in oogmelanomen met gezamenlijk verlies van chromosoom 3 en chromosoomregio 1p36.
Hoofdstukken 6 en **7** behandelen delineatie studies op chromosoom 3 in oogmelanomen.
In **hoofdstuk 6** wordt een afbakeningsstudie beschreven van partiële deleties op chromosoom 3 in een oogmelanoom cellijn, genaamd Mel270, welke is verkregen uit een primaire tumor. LOH en FISH analyse duidden op een chromosoom 3q deletie van 3q21.2 tot 3q24 in Mel270 en de cellijn OMM2.3, welke is afgeleid van één van de metastasen van dezelfde primaire tumor.

Ook werd een regio van mogelijk allelisch verlies gevonden in beide cellijnen, gelokaliseerd op 3p24. Omdat het allelisch verlies niet bevestigd kon worden met FISH, zou het een hemizygote regio kunnen betreffen. Deze resultaten dragen bij aan een verdere afbakening van kandidaat-regio's voor tumor suppressor genen op chromosoom 3 in oogmelanomen.
Hoofdstuk 7 betreft een delineatie studie van chromosoom 3 waarbij tumoren met partiële verliezen op chromosoom 3 onderzocht zijn met FISH en CGH. De studie heeft geleid tot de afbakening van een regio vanaf het centromeer tot ongeveer 3q26.2. Opvallend is dat alle patiënten in het cohort met oogmelanomen welke een partiële deletie van chromosoom 3 bevatten die enkel de q-arm beslaat, niet overleden zijn ten gevolge van metastasen. Het lijkt erop dat de slechte prognose in oogmelanoom patiënten niet veroorzaakt wordt door verlies van de 3q regio alleen.
Hoofdstuk 8 beschrijft een delineatie studie op chromosoom 6. In een interessante oogmelanoom casus, werd met karyotypering en FISH analyse een gedeleteerde regio aangetoond welke verder is afgebakend tot een gebied van 6q16.1 tot 6q22 met CGH en FISH analyse. Ook werd een regio van winst van 6pter tot 6p21.2 afgebakend met CGH en FISH. Verder werden geen deleties of amplificaties gevonden op vaak in oogmelanomen betrokken chromosomen waargenomen in deze tumor. Deze resultaten duiden op de aanwezigheid van één of meer tumor suppressor genen in het gebied 6q16.1-6q22 en de aanwezigheid van één of meer oncogenen op de chromosomale regio 6pter-6p21.2, welke waarschijnlijk van belang zijn voor oogmelanomen en wellicht ook in andere tumoren.

In **hoofdstuk 9** worden de bevindingen van deze thesis en mogelijke nieuwe onderzoeksrichtingen volgend uit dit proefschrift besproken. Het werk wat wordt beschreven in deze thesis heeft geleid tot een verdere afbakening van chromosomale regio's die van belang zijn in de ontwikkeling van oogmelanomen. Verder is in dit werk aangetoond dat moleculaire klasse gebaseerd op gen expressie profiel en gemeenschappelijk verlies van chromosoomgebied 1p36 en chromosoom 3 de meest significante voorspellers zijn van een slechte prognose voor oogmelanoom patiënten.

List of abbreviations

CGH	Comparative Genomic Hybridization
DFS	Disease-Free Survival
FISH	Fluorescence In Situ Hybridization
FNAB	Fine-Needle Aspiration Biopsy
HR	Hazard Ratio
LAP	Locally Adaptive statistical Procedure
LOH	Loss Of Heterozygosity
LTD	Largest Tumor Diameter
MM	MisMatch
MLPA	Multiplex Ligation-dependant Probe Amplification
PAM	Predictive Analysis of Micro-arrays
PM	Perfect Match
SKY	Spectral KarYotyping
SNP	Single-Nucleotide Polymorphism
SRO	Shortest Region of Overlap
TSG	Tumor Suppressor Gene
UM	Uveal Melanoma
VSN	Variance-Stabilizing Normalization

Curriculum vitae

Walter van Gils was born on the 26th of November in Roosendaal. He passed his secondary school exam (Gymnasium in 1997) at the Norbertus College in Roosendaal and subsequently started his study Biology and Medical Laboratory Investigation at the 'Hogeschool Brabant' in Etten-Leur. After his graduation, he started his Biology study at the Leiden University in 2000. Durig his first internship of 6 months, he studied the expression and localization of the ZDG42II protein in a zebrafish cell line at the Clusius laboratory in Leiden. In the second internship, he investigated the influence of growth factors on the promotor activity of the genes *upa* and *pai-1* at the department of internal oncology of the Erasmus University in Rotterdam. He graduated in Biology October 2002.

After his graduation, he started to work as a PhD-student at the department of Clinical Genetics at the Erasmus MC in Rotterdam on a project financed by the Dutch Cancer Society. Under supervision of his promotors prof. dr. G. van Rij and prof. Dr. G. Luyten and his co-promotors dr. A. de Klein and dr. H. Brüggenwirth, he studied a number of molecular prognostic markers in uveal melanoma, with genomic approaches and a gene expression profiling study. Currently, Walter works in Leiden at BaseClear BV in the functions of Postdoc researcher and product specialist. He expects to defend his thesis 'Molecular prognostic markers in UM: expression profiling and genomic studies' on 30th January 2008.

Dankwoord

Zoals elke promovendus heb ook ik het promotietraject niet in m'n eentje bewandeld. Vele mensen heb een steentje, klein of groot, bijgedragen aan het tot stand komen van dit proefschrift. Hierbij wil ik graag de volgende mensen bedanken.

Mijn promotoren. Eerst één en later twee, prof.dr. G. Van Rij en prof.dr. G. Luyten. Professor Van Rij, dank voor de betrokkenheid bij mijn werk en, samen met uw echtgenote, het beoordelen van een aantal van de stukken.
Gré, bedankt voor de klinische invalshoek in mijn onderzoek. Veel succes met je carrière in de mij niet onbekende stad Leiden.

De overige leden van de kleine commissie. Prof.dr. Van Meurs, hartelijk bedankt voor het beoordelen van het manuscript. Prof.dr. Oostra, dank voor het beoordelen van dit werk en de mogelijkheden die ik heb gehad binnen de klinische genetica om dit werk te verrichten. Dr. Berns, Els, bedankt voor de prettige discussie over dit werk, waarvan de resultaten zeker terug te zien zijn in dit proefschrift. Het is altijd leuk om weer terug te komen bij de interne oncologie.

John Martens, jou moet ik zeker niet vergeten als ik het heb over de interne oncologie. Ik heb zeer goede herinneringen aan mijn stageperiode met jou als begeleider, en sindsdien hebben we altijd een prettig contact gehad. Bedankt voor de gesprekken over het wel en wee van het AIO-schap, veelal tijdens, voor en na het hardlopen.

Ik heb deze jaren nauw samengewerkt met de mensen op 16-07 en 16-10, een kleine oogheelkunde-clan. Eerst vooral de AIO's Sjoukje en mijn paranimf Emine, twee harde werkers, maar dan wel afgewisseld met gesprekken over keukens en verhuisklussen onder het genot van een cappuccino en een koffie verkeerd. Sjoukje, ontzettend bedankt voor de morele steun en Emine, bedankt voor het vele constructieve werkoverleg. Ook Hanneke, de nieuwste AIO op de kamer, moet ik bedanken voor de kleurrijke tijd, maar vooral ook voor het doorlezen en bespreken van al het werk. Asma, je was een leuke stagiaire en het is mooi dat we elkaar nog wel eens tegenkomen in de trein. Eline, "mijn" studente, je was ontzettend goed en je hebt veel praktisch werk voor mijn onderzoek verricht. Maar buiten dat was het ook erg leuk om met je te werken. Veel succes in jou promotieonderzoek! Ook Michael heeft ruim 4 jaar op zijn typerende chaotische wijze meegewerkt aan dit onderzoek. Dank hiervoor. Jolanda, mijn andere paranimf, bedankt voor alle arbeid in de laatste periode van het onderzoek, en de gezellige sfeer. Dank ook voor de Brabantse noot op de kamer. Ook Michael hiervoor bedankt, het was wel eens lastig om niet al te onverstaanbaar te worden voor de overige mensen in de kamer. Huib, bedankt dat je zitting wilt nemen in mijn promotiecommissie en bedankt voor het leven in de brouwerij dat je altijd met je meebracht naar de 16e. Verder moet ik wat de 16e betreft natuurlijk niet vergeten Jan-Roelof, Angela, en Elizabeth, sfeerbepalend op de 16e in respectievelijk het begin, einde en de hele periode dat ik gewerkt heb op de 16^{e}. Tot slot ook Chantal, veel succes in de oogmelanomenclub.

Mijn co-promotoren. Hennie, bedankt voor de prettige samenwerking, eerst kort op de 16e, later op iets meer afstand op de 24e. Ik waardeer het zeer dat je elke keer tijd hebt gevonden om mijn werk te bespreken en vooral ook tijd voor een geruststellend woord wanneer dit nodig was. Annelies, bedankt voor de begeleiding. Je no-nonsense aanpak is iets wat ik zeer waardeer en heeft er voor gezorgd dat ik nu zo ver ben. Bedankt voor alle steun en vertrouwen.

Wat betreft het labwerk heb ik veel gehad aan de diverse mensen op de 8e, 9e en 24e verdieping. Marjan en Berna, bedankt voor al het oogmelanoomwerk en het delen van kennis omtrent de tumorcytogenetica. Mijn labgenoten op de 9e, Elly, Rachel, Laura, Laura, Marian, Mark, Leontine, en de vele stagiaires, analisten en AIO's die door de jaren heen met me op het lab hebben gewerkt, bedankt voor de leuke tijd.

De andere collega's op de 9e. Allereerst de AIO's die mij voorgingen, Merel en Sandra. Merel, succes met de opleiding bij de kindergeneeskunde in Maastricht en bedankt voor je les in organisatie. Sandra, bedankt voor de vele zin- en onzinvolle gesprekken. Wat hebben we gelachen met de AIO workshop in Lille! Elisabeth, bedankt voor je talloze uurtjes achter de PC voor het analyseren van mijn data. Ook bedankt voor het opzetten van een zeer effectieve methode voor blinde sample selectie. Guido, bedankt voor de introductie in de wereld van real-time PCR. Daar heb ik nu nog steeds profijt van. Verder wil ik alle andere mensen op de 9e gezamenlijk bedanken voor alle hulp en de gezellige werksfeer, vooral de squash avondjes staan me nog erg bij.

De mensen op de 24e. Met name Bert en Hannie bedankt voor de hulp en het overleg. Alle andere mensen van de cytogenetica, bedankt voor de leuke werksfeer en de hulp met microscopie.

De mensen van de Biomics afdeling. Bedankt voor alle hulp bij de praktische uitvoering van de expressieanalyses en Wilfred bedankt voor het technische overleg.

Bruce Ksander, thank you for reviewing a part of this work. Neeltje, bedankt voor het toepassen en overdragen van je expertise op dit gebied binnen de pathologie.

De medici van de oogheelkunde. Nicole, dank voor het vele werk aan de oogmelanomen. Zonder jouw resultaten had ik dit project nooit uit kunnen voeren. Succes met het vervolgonderzoek op dit gebied. Ruchi, dank voor je enthousiasme en grondigheid bij het beoordelen van één van de stukken. Ook alle anderen bedankt voor de prettige samenwerking en de verbreding van mijn kennis.

De overige leden van de promotiecommissie. Dr. Paridaens, Dion bedankt voor de deelname aan het oogmelanomen onderzoek vanuit het oogziekenhuis, het beoordelen van enkele wetenschappelijke publicaties. Prof.dr. Looijenga. Leendert, bedankt voor je enthousiasme, bij het bespreken van werk, op een congres en bij het hardlopen. Tot slot Prof.dr.Ir. Van der Maarel. Allen bedankt voor het zitting nemen in mijn promotiecommissie.

Tom, bedankt voor het vormgeven van dit proefschrift en daarbij het telkens weer aanpassen van de stukken wanneer er toch nog wijzigingen in aangebracht moesten worden.

Mijn nieuwe werkgever BaseClear. Erna en Bas, bedankt voor de ruimte die jullie me gaven om mijn promotie af te ronden. Het duurt even, maar dan heb je ook inderdaad een Postdoc in huis gehaald!

Ook weet ik me gesterkt door een goede familieband. Ons pa en ma hebben me goed op deze grond gezet en me er vervolgens altijd met beide benen op gehouden. Naast een hoop andere herkenbare trekken heb ik mijn doorzettingsvermogen ook aan jullie te danken.

Mijn schoonouders (en de rest van de familie), bedankt voor jullie interesse en steun bij mijn promotieonderzoek.

Jan-Peter, Jappie en Minie, jullie waren er altijd als dit nodig was.

Tot slot wil ik natuurlijk mijn lieve vouw Judith bedanken. Zij heeft de meeste uitingen van mijn stress meegemaakt en samen met mij naar alle deadlines toegeleefd. Judith, bedankt voor je onvoorwaardelijke steun. Ik beloof dat de computer voorlopig even uit blijft tijdens de weekeinden.

Color figures Chapter 2: see next page

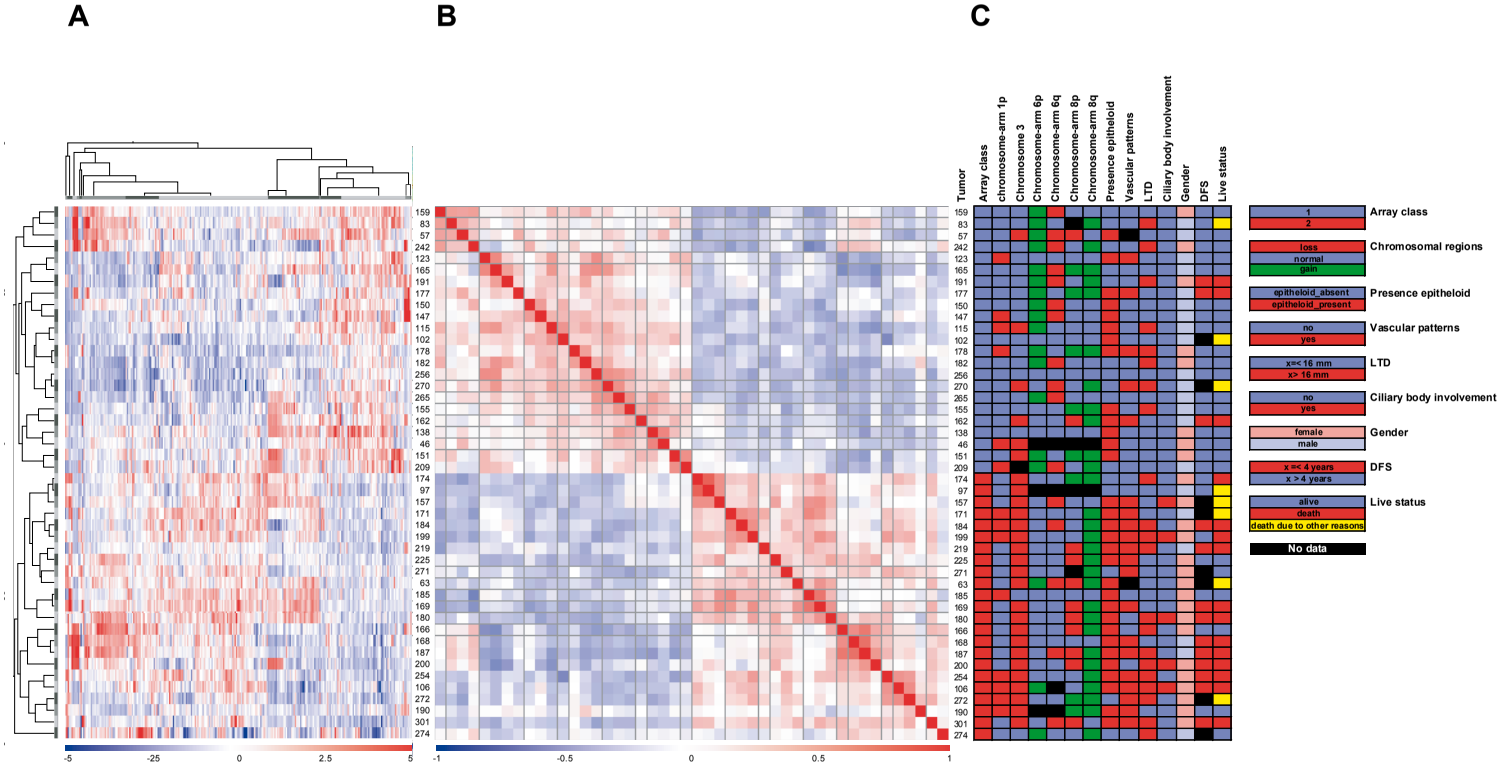


Figure 1, Chapter 2. **Correlation View of Specimens from 46 UM patients involving 528 probes combined with data of follow-up, clinical, pathological and cytogenetic parameters. A)** Results of unsupervised hierarchical clustering of probes and tumors with a K-means algorithm is shown. **B)** The results of heat mapping cluster analysis with Pearson's correlation algorithm. The figure shows 2 clusters identified on the basis of the Correlation View, separated by a black line. **C)** Data of follow-up, clinical, pathological and cytogenetic parameters are shown schematically.

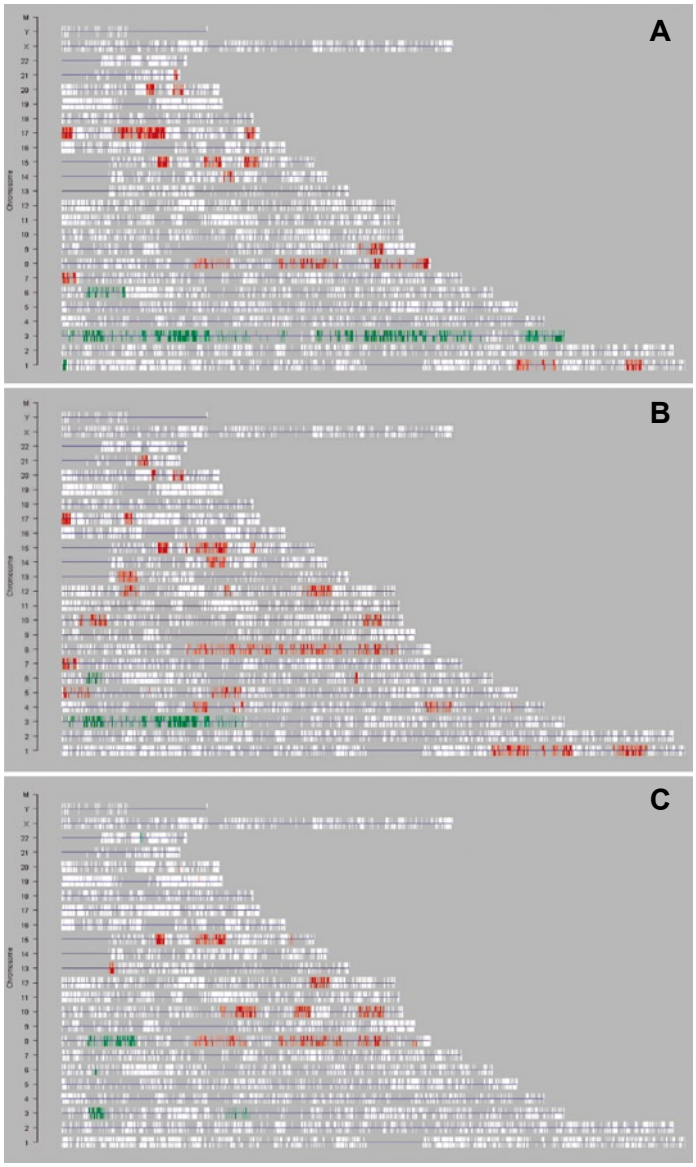


Figure 3, Chapter 2. **Whole genome plot of the differentially expressed genes in LAP analysis at a q-value of <0.05.** The colored perpendicular lines represent the exact chromosomal locations, orientations, and up- (red) or down-regulation (green) states of the differentially expressed genes, while the white bars indicate locations and orientations of all probe sets in the microarray. Positions for both the sense and antisense strands are expressed in numbers of base pairs measured from the p (5' end of the sense strand) to q (3' end of the sense strand) arms; upper and lower bars stand for genes on sense and antisense strands, respectively. **A)** Differential expression of tumors with monosomy of chromosome 3 compared to tumors with normal copy numbers of chromosome 3. **B)** Differential expression of tumors in class 2 compared to tumors in class 1. **C)** Differential expression of tumors from patients with DFS ≤ 4 years compared to tumors from patients with DFS >4 years.

Color figures Chapter 6 and 7: see next page

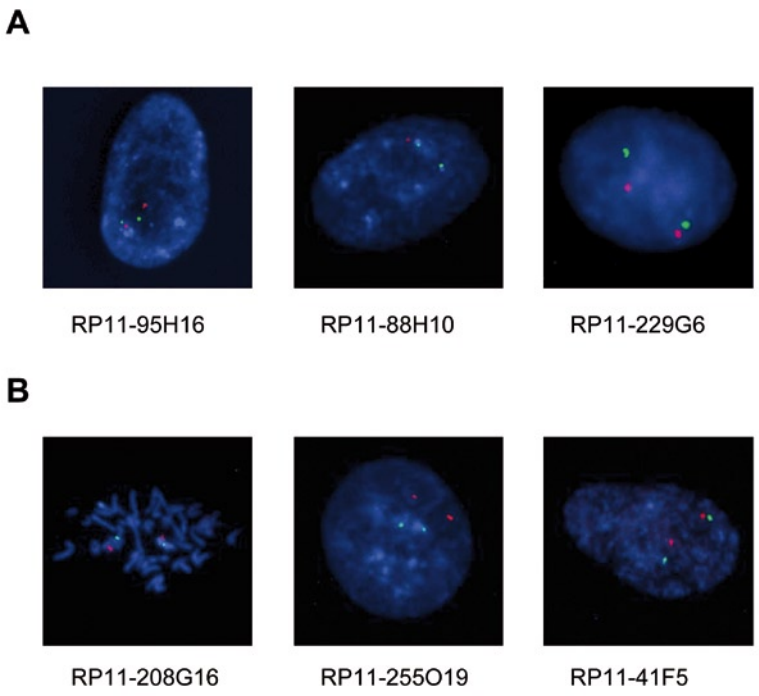


Figure 3, Chapter 6.

FISH analysis of Mel270.
A: representative results of dual color FISH on Mel270 cells, hybridized with a combination of a chromosome 3q probe (red) and a centromere 3 probe (Pα3.5) (#3) (green), except for probe *RP11-95H16* (green) that was combined with a 3pter probe (B47A2) (red). Panel A: 3pter probe and *RP11-95H16*; #3 and *RP11-88H10;* and #3 and *RP11-229G6*.
B: representative results of dual color FISH on Mel270 cells, hybridized with a combination of a chromosome 3p probe (red) and a centromere 3 probe (Pα3.5) (#3) (green). Panel B: #3 and *RP11-208G16*, #3 and *RP11-255O19*, and #3 and *RP11-41F5*.

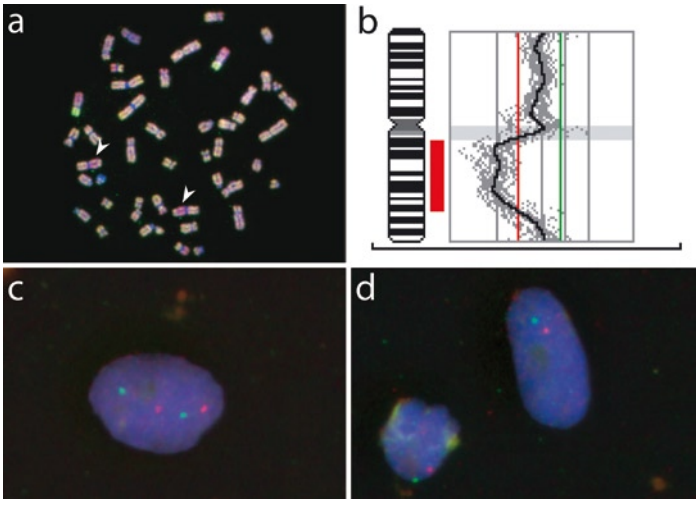


Figure 1, Chapter 7. **CGH and FISH on case 1. A)** Metaphase spread after comparative genomic hybridization with tumor DNA labeled in green, reference DNA labeled in red. The arrowheads point to the red regions on the q-arm of chromosomes 3 indicating a loss. **B)** CGH results on chromosome 3 of ten metaphase spreads processed with Isis version 5 software. The thick line represents the average signal of the ten metaphases. The lower limit was set as green-to-red ratio 0.8, the upper limit as a ratio of 1.2. The partial deletion is indicated with a red bar alongside of the chromosome image. **C)** and **D)** are images of FISH. **C)** FISH on isolated nuclei with probes RP11-816J6 (labeled in red) and RP11-54L9 (labeled in green) showing the presence of two copies of regions 3q26.2 and 3q28, respectively. **D)** Probes RP11-449F7 (red) and RP11-64F6 (green) showed loss of one copy of chromosome 3 in the regions 3q11.2 and 3q25.1 respectively.