Tumor Heterogeneity in Uveal Melanomas

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Tumor heterogeniteit in uveamelanomen

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Chapter 1

Genetics of Uveal Melanoma

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INTRODUCTION

Uveal Melanoma

Uveal melanoma (UM) is the most common primary intraocular malignancy in adults with an incidence of 7-10/ million and has a predilection for hematogenous dissemination to the liver.[1] Despite improvements in diagnosis and treatment of this intraocular tumor, there has not been a change in survival in the past decades. There is no effective treatment for liver metastases resulting in tumor-related death in about 45% of UM patients within 15 years after the initial diagnosis.[2, 3]

Uveal melanoma usually arises 'de novo' from melanocytes derived from neural crest cells that have migrated to the epidermis and uveal tract during embryogenesis. Intraocular melanoma may arise in the choroid (70-80%), ciliary body (10-20%) and iris (5-10%). Iris melanomas are the least common and tend to present at a smaller size, probably because pigmented lesions of the iris are usually visible to the patient. Iris melanomas can cause drainage angle blockage and secondary elevation of the intraocular pressure.[4] Melanomas located in the ciliary body are associated with a high metastatic potential.[5] Choroidal melanomas compromise the majority and grow subretinal in a discoid, dome-shaped or mushroom-shaped pattern.

The diagnosis is based on the clinical appearance of the tumor on ophthalmic examination. Ancillary tests include (Doppler-) ultrasonography, transillumination, fluorescein angiography and optical coherence tomography. For the diagnosis of melanoma, biopsy is reserved for tumors of uncertain origin. Conversely, tumor biopsy gains territory in vision-sparing therapies for cytogenetic analysis providing risk assessment of metastatic disease.

Upon diagnosis of the primary tumor, patients are screened for metastases by liver enzyme tests and liver ultrasound. At time of diagnosis less than 2% of the patients have detectable metastases.[6]

The diagnosis is usually confirmed by histopathological examination if tissue is available from biopsy or enucleation. (Figure 1) Hematoxylin and eosin (H&E) staining is used to differentiate between cell types. Melanomas consist of spindle, epithelioid cells or a combination of both cell types. Periodic-acid Schiff (PAS) staining helps to identify microvascular patterns (three closed loops located back to back).[7] Additionally, immunohistochemistry is performed: S-100 is expressed by cells of neuroectodermal origin, and HMB-45 binds to gp100, an antigen expressed by melanocytes.

Predisposing factors

Certain phenotypes that predispose to uveal melanoma have been described. Caucasian race is the most important one, as uveal melanoma is approximately 150 times more

common in Caucasians than in Africans.[8, 9] Furthermore, blue or gray eyes as well as fair skin type have been suggested to predispose for uveal melanoma.[10-12]
The role of UV-radiation in uveal melanoma is inconclusive. In cutaneous melanoma sun light exposure is a commonly know risk factor. However, in uveal melanoma the data are contradictory.[13-17]
Ocular and oculodermal melanocytosis (Nevus of Ota),[18, 19] neurofibromatosis type I,[20] dysplastic nevus syndrome[21] are all associated with an increased incidence of uveal melanoma. Familial uveal melanoma is rare, only 0,6% of all uveal melanoma cases.[22, 23] Uveal melanoma have been reported to occur in several familial cancer syndromes: xeroderma pigmentosa, Li-Fraumeni syndrome and familial breast and ovarian cancer.[24, 25]

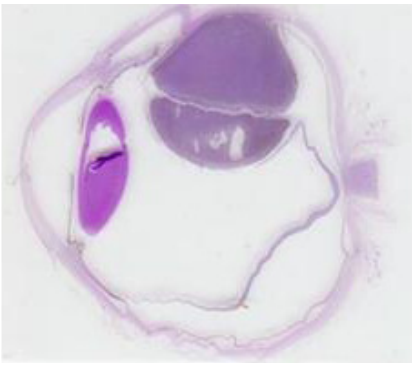

Figure 1. Hematoxylin and eosin staining of a choroidal tumor, Erasmus Ocular Melanoma (EOM) 488, composed of spindle and epithelioid cells with a diameter of 14 mm and prominence of 8 mm

Molecular Tumor Research

Transformation of a healthy cell into a neoplastic cell is caused by alterations of the genome leading to changes in gene expression, activity and localization. Mutations in cellular proto-oncogenes lead to activation of oncogenes that disrupt cell growth and differentiation. Mutations can also lead to loss of function of tumor suppressor genes (for example p53), thereby causing uncontrolled growth. Cancer development is often associated with genomic instability and acquisition of genomic heterogeneity,[26] generating both clonal and non-clonal tumor cell populations.[27, 28] Gene mutations, such as p53, disrupting the cell cycle can lead to aneuploidy. Besides, chromosomes are vulnerable to changes during mitosis, spindle checkpoint and chromosome attachment, leading to single point mutations or even gross chromosomal rearrangements.[29, 30] There is a possible benefit from the accumulation of genetic and epigenetic alterations, even so cells become genetically unstable.[31] Polyploidy is also well known in cancer and it tends to occur in tumors with a more aggressive phenotype,[32] probably because of resistance to p53-dependent apoptosis.[33]
Research is focusing on finding pathways involved in carcinogenesis, thereby trying to understand tumor onset and early development. Furthermore, a search for candidate

chromosome regions and candidate genes in relation to metastatic disease is continuing. Highly invasive tumors are compared with poorly invasive ones, primary tumors with its metastases, and therapy-resistant tumors with responsive ones in order to search for differentially expressed genes and differences in chromosomal aberrations.
Nowadays, human cancers are being classified based on their gene expression signature. Gene expression may also be modulated outside changes to DNA sequence. Epigenetics involves the orchestration of DNA methylation and histone modifications to control germ-line and tissue-specific gene expression.[34] A more recently emerged participant in this field are small regulatory RNAs called microRNAs (miRNAs) with an inhibiting effect on the translation of mRNA into proteins. Human cancers are thought to undergo an overall loss of DNA methylation, but also acquire hypermethylation at specific promoters with consequential inactivation of the associated genes.[35] Global demethylation may activate imprinted genes and affect chromosomal stability. The underlying mechanisms that cause these changes are unknown. A summary of the most widely used (cyto-) genetic and genomic research techniques in UM is provided.

Cytogenetics

A wide variety of cytogenetic techniques are available and in development. All these techniques are aimed at the detection of chromosomal changes in a sample. UMs are very suitable for cytogenetic analysis because of their relatively simple karyotype. The kind of tumor tissue needed for a cytogenetic assay differs per technique. For karyotyping and SKY metaphase spreads are required and in UM, cell culture is usually necessary to obtain metaphases. FISH can be applied to fresh or frozen tissues, cell lines, and archival formalin-fixed paraffin-embedded samples.

Karyotype Analysis (Figure 2)
Conventional karyotyping is able to detect large chromosomal gains, deletions and translocations, but for higher resolution delineation newer molecular cytogenetic techniques are indispensable.

Fluorescence in situ Hybridisation (FISH) (Figure 3a)
Specific translocations and gains and losses of chromosomal regions can be detected using FISH. FISH uses fluorescent probes that bind to only those parts of the chromosome with which they show a high degree of sequence similarity. In dual color FISH two probes, for example bacterial artificial chromosomes (BACs), are labeled with digoxygenin and biotin. The technique is rapid and has high resolution and specificity. More over, it is possible to detect clonal gains and losses that are present in a low percentage of the tumor cells.[36, 37]

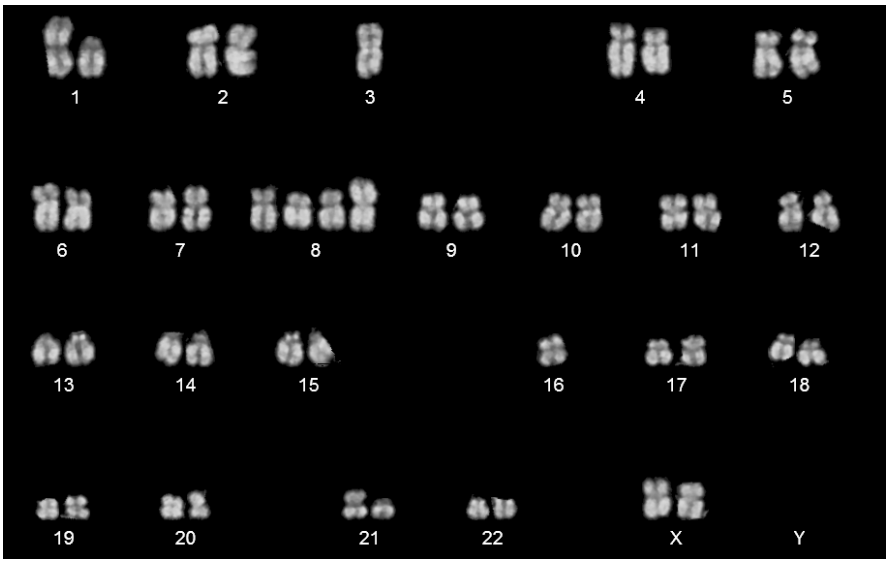

Figure 2. Karyotype of EOM 328 with the for UM typical anomalies: loss of chromosome 1p and 3; gain of chromosome 8 (+8 and isochromosome 8q).

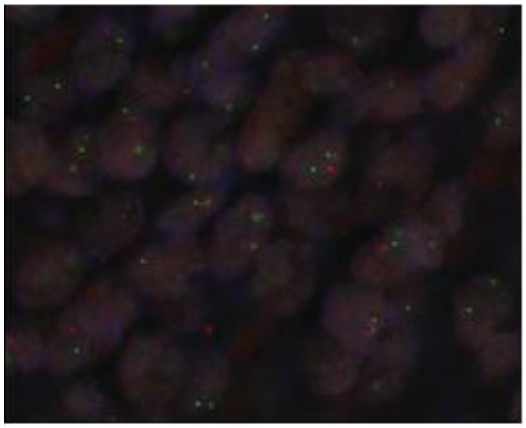

Figure 3a. FISH of a paraffin-embedded tissue section of EOM 149 shows 2 copies of the bio-labeled 8q probe and dig-labeled centromere 8 probe.

A drawback of FISH is that it focuses on a very limited number of loci analyzed in a single experiment. Single cell suspensions are easier to analyze than paraffin sections, because of possible truncation artifacts and signal reduction due to pigmentation.

Spectral Karyotyping (SKY) and Multiplex Fluorescence In Situ Hybridization (M-FISH)
SKY and M-FISH permit the simultaneous visualization of all chromosomes in different colors, thus facilitating karyotype analysis.[38, 39] The origin of a translocated chromosomal

region can easily be identified, because it has the same color as the chromosome it derives from. This technique is especially useful for characterization of complex rearrangements and chromosomal breakpoints.

Molecular cytogenetics

The techniques described below have a higher resolution than conventional cytogenetic assays. They identify changes on the DNA level.

Comparative Genomic Hybridization (CGH)

CGH allows a complete copy number analysis of the entire genome. It involves the competitive hybridization of differentially labeled genomic sample and reference DNA to immobilized normal human metaphase spreads. Deletion and amplifications can be detected by differences in fluorescence signals. However, balanced rearrangements and abnormalities that are only present in small subclones of a tumor cannot be detected with CGH.[40, 41] Moreover, confirmation of chromosomal aberrations with higher resolution techniques seems appropriate.

Loss of heterozygosity (LOH)

Microsatellites are tandem repeats of simple polymorphic sequences randomly distributed in non-coding regions of DNA. Microsatellite instability (MSI), characterized by length changes at tandem repeats, was first described in sporadic colon cancer and in hereditary nonpolyposis colorectal cancer (HNPCC-Lynch) syndrome.[42-44] MSI analysis can be used in loss of heterozygosity (LOH) studies to detect the presence or absence of two different alleles. If an allele has been lost in a tumor, one band is absent. Such deletions are termed LOH and indicate a probable site of tumor suppressor genes. A drawback of this technique is that only a limited number of markers can be analyzed in one single experiment.

Polymerase Chain Reaction (PCR)

PCR is used to amplify specific regions of a DNA strand (the target DNA). This can be a single gene, a part of a gene, or a non-coding sequence.

Methylation-specific PCR (MSP) was developed to detect methylation of CpG islands in genomic DNA.[45] DNA is first treated with sodium bisulfite, which converts unmethylated cytosine bases to uracil, which is recognized by PCR primers as thymine. This allows the PCR primers to discriminate between methylated and unmethylated DNA.

Multiplex ligation-dependent probe amplification (MLPA) (Figure 3b)

MLPA is a PCR-based-amplification method. Simultaneous hybridization of multiple (up to 50) probes, each of unique length, to tumor DNA is followed by amplifying the probes

ligated using polymerase chain reaction and separating the resulting products by capillary electrophoresis. The amount of amplified probes depends on the quantity of target sequences in the sample DNA, therefore a relative quantification (RQ) can be used to detect copy number changes.[46]

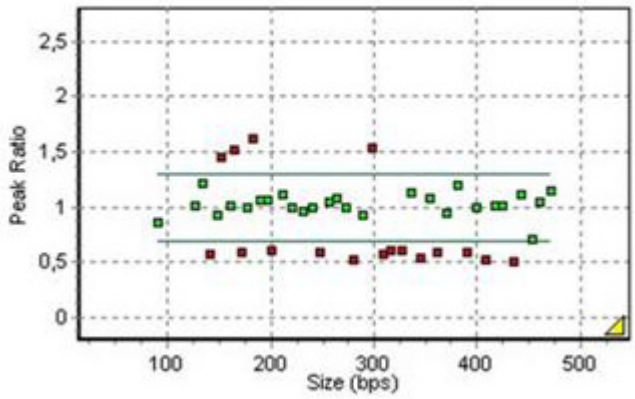

Figure 3b. MLPA of EOM 461 shows loss of chromosome 3 and gain of 8q. The peak ratio on the Y-axis represents the relative quantification (RQ). Deletion of DNA-sequence was defined as a RQ < 0.7, and amplification was defined as RQ > 1.3. The X-axis represents the PCR product size. All products above the 1.3 line represent probes on chromosome 8q and all products below the 0.7 line represent probes on chromosome 3.

Multiplex ligation-dependent probe amplification has several advantages over FISH in that it (1) simultaneously tests multiple genomic sequences (on different chromosomes) in one experiment; (2) has a higher resolution; (3) requires a smaller sample.[47]
A variation is methylation-specific MLPA (MS-MLPA), which can be used for both copy number quantification and methylation profiling with the use of a methylation-sensitive restriction enzyme.[48]

Micro-array technology

The completion of the human genome project led to the development of DNA array technology. Micro-array technology is available for many different genomic approaches using cDNA or oligonucleotides, which represent specific gene coding regions, spotted on a chip. The sample is fluorescently labeled and hybridized to the slide. Raw data are obtained by scanning laser or autoradiographic imaging, and subsequently entered into a database and analyzed by statistical methods. (Figure 4)
Micro-array based CGH, SNP analysis and gene expression analysis are the most frequently applied techniques. A drawback of array-based approaches is that the analyzed signal represents the average value of all cells in the tumor sample, requiring a high

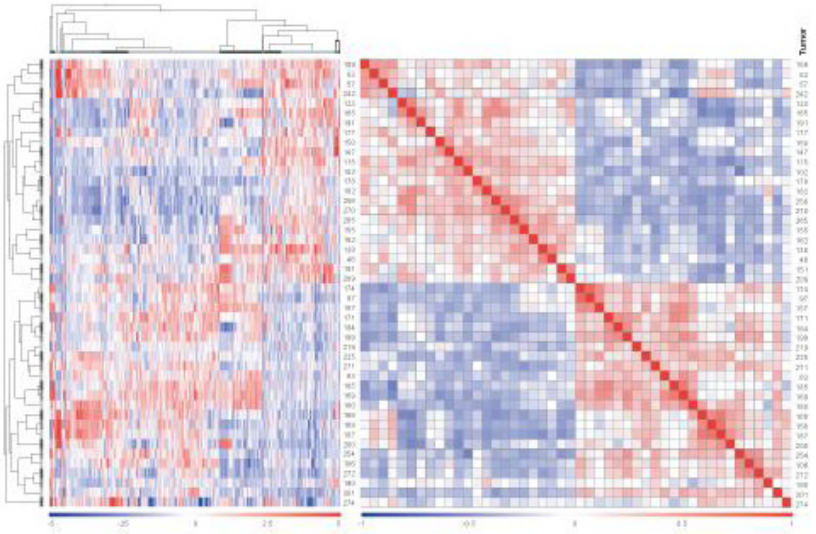

Figure 4. Gene expression analysis of 46 UM involving 528 probes results in 2 clusters of UM that correlate with disomy 3 and monosomy 3. On the left, unsupervised hierarchical clustering of probes and tumors with a K-means algorithm. On the right, the results of heat mapping cluster analysis with Pearson's correlation algorithm (own research data, adjusted from Van Gils et al, IOVS 2008 Oct;49(10):4254-62).

signal-to-noise ratio to quantitatively and reliably detect low-level DNA copy changes on individual array elements. [49] As a result, balanced anomalies and genomic abnormalities appearing in frequencies below 50% of the analyzed nuclei will not be detected. For these low volume abnormalities FISH approach is a better option.
The great advantage is that information on thousands of locations or genes can be obtained in a single experiment.

Array-CGH offers the potential for CGH analysis with a higher resolution. Additionally, there are CGH micro-arrays that cover specific chromosomal regions with a high resolution of approximately 1 kb.[50]
Gene expression micro-array enables the measurement of the expression of thousands of genes in a mRNA sample.
The discovery of single-nucleotide polymorphisms (SNPs) led to the construction of increasingly dense SNP maps. This offers a mapping tool designed to identify regions of the genome linked to or associated with a particular trait or phenotype. SNP analysis is useful for the determination of allele frequencies in various populations and for mapping regions with loss of heterozygosity in oncogenesis. SNP arrays can simultaneously be used to define copy number changes in tumors from signal intensities reflecting the amount of hybridized DNA.[51] (Figure 5)
Table 1 gives an overview of the resolution of the different cytogenetic approaches.

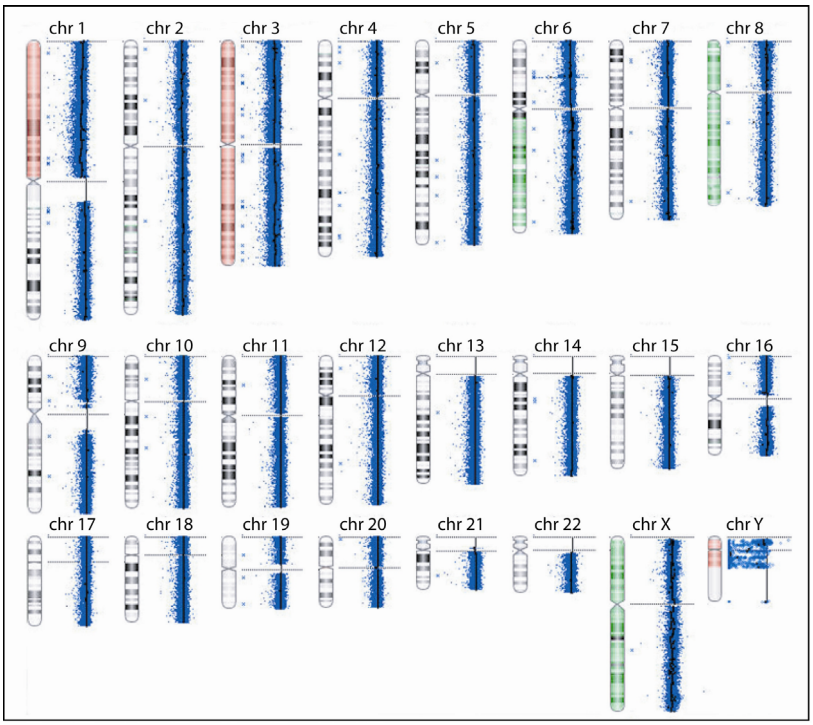

Figure 5. Summary view of the SNP array of EOM 488 revealing loss of chromosome 1p and 3, and gain of 6q and chromosome 8 (and a female patient). Chromosome colours: red = loss, green = gain, white = normal. The SNP array copy number results are projected on chromosomes using Nexus Copy Number software (Biodiscovery).

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

Method	Resolution	Detection balanced anomalies?	Detection unbalanced anomalies?
Karyotype G-banding	~ 5 – 10 Mb	Yes	Yes
FISH	~ 100 kb	Yes	Yes
SKY	~ 1 - 2 Mb	Yes	Yes
MSI	<1 kb	No	Yes
CGH	~ 5 – 20 Mb	No	Yes
PCR	~10-40 kb	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.*[108-110] ** Depending on type and number of probes (BAC, oligonucleotides or SNP) and/or their distribution.

Prognostic factors in UM

Prognostic factors to identify patients at risk for metastasizing disease include clinical (tumor location, tumor size), histological (cell type, tumor-infiltrating lymphocytes, vascular loops) and genetic (chromosomal aberrations, expression profiling) parameters.[2, 52-55] Tumors located in the ciliary body[5] and the size of the tumor correlate with progressive disease.[56, 57] The same holds true for the presence of epithelioid cells[58] and closed vascular patterns,[7] as well as scleral invasion.[59] Genetic parameters will be discussed in greater detail below.

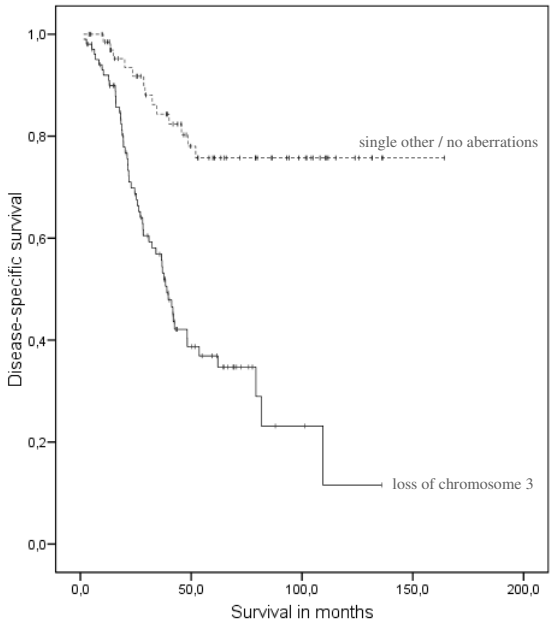

Figure 6. Kaplan-Meier Survival Curve of 172 UM patients showing decreased survival for tumors with loss of chromosome 3.

Chromosomal aberrations

Monosomy of chromosome 3 is the most frequent found non-random chromosomal aberration in UM and is predominantly found in metastasizing tumors.[60] (Figure 6) It is considered to be a primary event, because it is seen in combination with all other chromosomal aberrations in UMs such as loss of chromosome 1p, gain of 6p and gain of 8q. [61] In the majority of tumors with chromosome 3 loss there is complete monosomy, although occasionally isodisomy of this chromosome is acquired.[62-64] Only rarely, melanomas with partial aberrations on chromosome 3 or translocations have been described, making it difficult to map putative tumor suppressor genes. Loss of heterozygosity studies demonstrate common regions of allelic loss located at 3p25 and on the long arm spanning from 3q24 to 3q26.[65, 66]

Gain of chromosome 8, or acquisition of an isochromosome 8q, is suggested to be a secondary event in UM, because variable copynumbers of 8q can be present in one tumor. [67, 68] It occurs frequently in tumors that have lost one copy of chromosome 3 and it is an independent prognostic factor of progressive disease.[64, 69, 70] The shortest region of overlapping gain spans from 8q24.1 to 8qter.[53, 71]

Loss of the chromosomal region 1p36 is often found in UM with loss of chromosome 3.[62, 71] Concomitant loss of chromosomes 1p and 3 has a stronger correlation with metastasizing disease than either one of them.[72] The common deleted regions on chromosome 1 ranges from 1p34.3 to 36.2.[62, 71, 73]

At first, chromosome 6 abnormalities were thought to be late occurring events in UM.[74] Deletion of 6q is present in many neoplasms such as breast and prostate carcinomas and cutaneous melanomas,[75-77] which suggest it to be a possible region of tumorsuppressor genes. More recently, tumors with gain of chromosome 6p have been proposed to represent a separate group of UMs with an alternative genetic pathway in carcinogenesis, because gain of 6p is found frequently in tumors with disomy 3.[69, 78, 79] Other studies could not confirm these results, because of gain of 6p concomitant with loss of chromosome 3.[80] Whereas gain of 6p indicates a favorable patient outcome, loss of 6q implies the contrary.[62] Gain of 6p and loss of 6q can occur in one tumor, but do not necessarily mean there is an isochromosome 6p. The shortest region of overlap on the short arm has been narrowed down to 6p22.3 to the telomeric region by array-CGH.[71] The region of common deletion on the long arm ranges from 6p16.1 to 22.3.

Abnormalities of other chromosomes have also been detected in UM. However, they often lead to contradictory results regarding the prognostic impact. Chromosome 18q22 has been suggested to play a prognostic role,[81] but this could not be confirmed by other groups.[82] Chromosome 9p21[63, 83] and chromosome 16q[84] have been described to be important in UM as well.

Gene expression profiling

Gene expression profiling has identified two distinct classes of UMs with a class II expression profile that predicts metastatic disease. Additionally, the tumors with a class II expression signature correlate with monosomy 3 strongly and gene expression profiling outperforms clinical and chromosomal prognostic markers in relation to prognosis.[52, 55, 85-87] These expression studies yielded sets of classifier genes and although it is encouraging to see that these different sets of genes result in a similar classification of the UM,[87] these classifier genes are merely markers of tumor progression and metastatic potential whereas genes involved in the underlying cause still have to be discovered. However,

in case of genes encoding cell surface markers, they could be a target for cell therapy aimed at an immunological response to eliminate tumor cells.

Methylation

The limited studies on promotor hypermethylation in primary UM have not yet elucidated the role of genes commonly methylated in cutaneous melanoma -such as APC, RASEF, RARB, TIMP3 and CDKN2A (p16)- or genes located on chromosome 3, such as RASSF1 and FHIT.

In UM, methylation of CDKN2A is present in 4 to 32%,[83, 88] RASSF1 in 13 to 70%,[89] RARB up to 7% and TIMP3 in 9%. RASEF is targeted by LOH in combination with methylation in primary UM; there is only low percentage methylation.[90] hTERT, an important gene in carcinogenesis, is methylated in up to 52% of UM, whereas FHIT and APC are never hypermethylated.[91-93] In none of these studies hypermethylation of a gene correlated with metastatic disease.

Recent developments

Even fewer studies are available on the role microRNAs. In 24 primary UM, miRNA expression corresponds to gene expression profiling and is also able to divide tumors into two groups with a low and high risk potential for metastatic disease. The most important differentially expressed miRNAs are let-7b and miR-199a, not located on the chromosomes involved in UM.[94]

Recently, several groups reported on oncogenic G protein alpha subunit q (GNAQ) mutations in UM. These mutations occur at a high frequency of approximately 50% in uveal melanoma, although they do not correlate with metastatic disease. Therefore, it has been suggested that GNAQ mutation is an early event in UM and justifies further research.[95-97]

Box 1. Prognostic parameters associated with metastatic risk in uveal melanomas

- Large tumor size
- Ciliary body involvement
- Scleral invasion
- Epithelioid cells
- Closed vascular patterns
- Loss of chromosome 3 (and 1p)
- Gain of chromosome 8q
- Class II expression signature

Conclusion

Studies on primary UM are valuable in understanding tumor progression, because the biological information of the primary tumor encodes the metastatic risk. Although expression arrays are the best in predicting progressive disease, they are currently too expensive for routine use and moreover, modification of the tumor sample can be

difficult. In expression profiling, the average pattern of poor prognosis primary tumors and good prognosis are similar.[98] Only few genes are differentially expressed between aggressive and low-risk tumors.

There are also differences in chromosome copy numbers measurements in various studies, which can be related to the techniques used. Probe locations are not always completely analogous and shorter sequences provide higher resolution. Other reasons comprise that centromeric and subtelomeric regions contain ALU-repeats, which are subjective to artifacts. In addition, the differences in sensitivity and specificity have already been described.

In this review cell-line studies were not included, because cell culturing can lead to clonal advantages and artifacts.

Difficulties that arise with predicting individual patient outcomes are that the study of prognostic factors must be objective and reproducible. Caveats in studying prognostic factors are flaws in method design, subjective bias and statistical inaccuracy, leading to inaccurate and conflicting data. The reasons for this are the use of biased and semiquantitative methods, associated with small sample sizes, and inappropriate cut-off points when allocating variables to different categories.[99]

Routine identification of high-risk patients based on (cyto-) genetic markers is being installed in the ocular oncology clinic. This has implications for the management of the patient, i.e. the frequency of follow-up visits, and low-risk patients can be reassured of their small chance of metastatic disease. Most small and medium-sized UMs are currently managed by eye-saving treatments and, consequently, the available tissue for assessment of prognosis is limited. Depending on tumor location, biopsies can be obtained via a transscleral or transvitreal route. It is clear that intratumor heterogeneity is present in uveal melanoma, however it does not seem to influence the chromosome status of the tumor.[80] Some reports do mention that monosomy 3 is found more frequently in the tumor base than apex.[100] The best way to acquire representative material of the tumor is to take biopsies from different locations within the tumor.

Studies reveal that fine-needle aspiration biopsies provide sufficient material for FISH analysis and is a reliable method.[100-102] Recently, however, it was found that a 'dry tap'- insufficient harvest of tumor tissue through FNAB- is a positive prognostic factor. (J. Augsburger, personal communication)

Five-year view

In order to imply prognostic risk assessment in standard clinical practice and uveal melanoma care, the genetic approaches and tests need to be standardized. This will also allow easier comparison between different ocular oncology centers and larger study populations.

We think that in the very near future chromosome 3 analyses will be imposed as a standard diagnostic tool in UM. In addition, results on expression profiling are exciting and groups from the USA and Europe have comparable outcomes. The challenge is to make expression arrays a fast cost-effective technique, with for example a small set of genes to identify class II expression signature UM.

Still there is little hope for curative treatment of metastatic UM. At present, small liver metastases in one liver lobe alone are suitable for resection. Isolated liver perfusion is another option only available for early detected metastases with partial responses merely and reasonable comorbidity. Although several agents such as cisplatin, dacarbazine (DTIC), fotemustine, IFNγ, and IL-2 have shown objective remissions, currently no regimen has unequivocally demonstrated a significant impact on survival.[103-107] Therefore, no standard treatment is available for metastasized uveal melanoma patients.

We think adjuvant therapies will become available for high-risk UM. Numerous immunotherapies, that are also suitable as adjuvant systemic therapies, are in development. Immunotherapy aims to activate the immune system to eradicate the tumor and to induce specific and long lasting immunity to protect against recurrent disease. Dendritic cells are antigen-presenting cells with the unique capacity to induce naïve antigen-presenting T cells and by this means are very suitable to induce an immunological response. Dendritic cell based immunotherapy looks promising in skin melanoma patients, and we think such a vaccine should be subjected to clinical trials in high-risk UM patients. At last, much can be learnt from experience with treating and investigating other cancers such as skin melanomas and solid tumors arising in immune privileged sites.

Aims and scope of this thesis

Uveal melanomas can be classified into high-risk and low-risk tumors. High-risk melanomas are aggressive tumors that disseminate to the liver within 5 years, whereas low-risk melanomas have very low metastatic potential with good patient survival consequently. The past decades survival of uveal melanoma patients has not changed significantly.

Genetic analysis of uveal melanomas helps to get a better understanding of tumor biology. Identifying critical chromosomal regions and differentially expressed genes is essential to the understanding of disease progression and aids to isolate targets for future therapies. High-risk uveal melanoma patients probably need different management than low-risk patients, with more frequent follow-up visits to be able to detect metastasis at an early stage. Molecular diagnostic assays lead to some obstacles as well. This thesis aims to address those obstacles and provide information and tools for reliable and reproducible molecular testing. It further aims to gain more insight into various markers of poor survival in uveal melanoma patients and into the differences between melanomas growing in different parts of the eye. Finally, this thesis aims to elucidate the role of escape mechanisms used by progressive growing tumors.

In **Chapter 2,** a newly identified prognostic marker in choroidal melanomas, hyperdiploidy, that correlates with a poor prognosis is discussed. **Chapter 3a** describes genetic heterogeneity within choroidal melanomas, which is important when using biopsies for diagnostic purposes. In **chapter 3b**, chromosomal aberrations in iris melanomas are compared with those in choroidal and skin melanomas. In **chapter 4a**, MLPA, a relatively new cytogenetic technique, is used to analyze the chromosomal regions 18q and 21q as prognostic factors in uveal melanomas. **Chapter 4b** addresses the role of the tumor suppressor gene APITD1, well known from other neural-crest derived tumors. **Chapter 5** addresses the role of immune privilege in the eye and tumor escape mechanisms in a mouse model. Finally, in **chapter 6**, all results and conclusions are summarized and discussed.

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Chapter 2

Hyperdiploidy Is an Indicator of Poor Prognosis in Uveal Melanoma

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ABSTRACT

Background. Uveal melanoma is a highly malignant disease with a mortality of roughly 50%. Previous studies have shown that certain chromosomal changes, such as monosomy 3, are associated with a decreased patient survival. Surprisingly, metastatic disease also occurs in about 10-20% of patients having melanomas with 2 or more copies of chromosome 3. In the current study, tumors with hyperdiploidy and the impact of hyperdiploidy on survival are evaluated.

Methods. 144 uveal melanomas were characterized for numerical chromosomal changes with molecular cytogenetic techniques. In 14 tumors we detected signs of hyperdiploidy and those were analyzed in detail with multiplex ligation-dependent probe amplification (MLPA) and with single nucleotide polymorphism (SNP) arrays. The influence of this type of chromosomal aberration on patient survival was compared with tumor cell type, size and vascular pattern in a multivariate Cox proportional hazard analysis.

Results. In our prospective study of cytogenetic changes in uveal melanoma we encountered 14 cases with a hyperdiploid character with gains on many chromosomes (high hyperdiploid to near-tetraploid). These hyperdiploid tumors had a median largest tumor diameter of 17 mm (range 10-20 mm) and eleven tumors were composed of epithelioid or mixed cells. Seven hyperdiploid melanomas had relative loss of chromosome 3, and, interestingly, loss of heterozygosity (LOH) of chromosome 3 was present in 8/8 tumors. Hyperdiploidy is an independent negative prognostic factors for metastasis free survival ($p<0.001$).

Conclusions. Hyperdiploidy correlates with metastatic disease in uveal melanoma. Surprisingly, these hyperdiploid tumors revealed LOH of chromosome 3. Efforts should be undertaken to consider isodisomy 3 in hyperdiploid tumors, and in other metastasizing melanomas without loss of chromosome 3.

INTRODUCTION

Uveal melanoma (UM) is the most common primary intraocular malignancy in adults with an incidence of approximately 7-10 / million and has a predilection for hematogenous dissemination to the liver.[1] Prognostic factors to identify patients at risk for metastasizing disease include clinical (tumor location, tumor size), histological (cell type, vascular loops) and genetic (chromosomal aberrations, expression profiling) parameters.[2, 3] UMs are very suitable for cytogenetic analysis because of their relative simple karyotype. Usually the karyotype is near-diploid with only few chromosomal changes. Aberrations such as loss of chromosome 1p, monosomy 3 and gain of chromosome 8q are classic parameters of poor prognosis.[4, 5]

Besides these near-diploid populations, 10-20% of the uveal melanomas display a chromosomal content in the high diploid to tetraploid range.[6, 7] Hyperdiploid patterns were found to correlate with worse clinical outcome,[8] but since most other studies concentrated on the near-diploid population, no association between high DNA index and survival was observed.[9-11]

During the last decade, research into chromosomal aberrations in UM has predominantly focused on chromosome 3 and although the association of monosomy 3 with poor prognosis is highly significant, over 15% of the disomic 3 UM patients die of metastatic disease.[12] We encountered signs of hyperdiploidy in 10% of the uveal melanomas by using karyotyping and FISH, indicating that polyploidy is not as common as monosomy 3.

Polyploidy is well known in cancer and it tends to occur in tumors with a more aggressive phenotype,[13] probably because of resistance to p53-dependent apoptosis.[14] There is a possible benefit from the accumulation of genetic and epigenetic alterations enabling the escape of immune elimination.[15] This study attempts to gain insight in hyperdiploidy in UMs.

MATERIALS AND METHODS

Patients and tumor samples

We collected tumor material from 186 uveal melanoma patients who underwent enucleation. 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 processed for karyotyping, FISH and/or molecular genetic analysis and was successful in 144 cases. Conventional histopathologic examination was performed on all tumors and confirmed the origin of the tumor. Follow-up data from time of diagnosis till the end of the study in January 2009 were obtained by reviewing each patient's charts and contacting their general physician. Only patients who died of liver metastases qualified to tumor-related death. 3/144 patients were lost to follow-up.

Cytogenetic and molecular analysis

Chromosome abnormalities were described following the recommendations for cytogenetic nomenclature.[16]

Cytogenetic analysis

Chromosome preparations were made following standard procedures and stained with acridine orange or atebrine to obtain R or Q banding.

FISH analysis

Fresh tumor material from enucleated eyes containing uveal melanoma was routinely cultured for karyotyping and/or directly used for FISH (chromosome 1p, 3, 6 and 8).[17] Dual color FISH was performed with the following probes: RP11-48E9 (1p36), Pα3.5 (centromere 3), RP11-64F6 (3q25), RP11-356B3 (6p22), RP11-787I22 (6q21), RP11-24P4 (8p21), D8Z2 (centromere 8) and RP11-88J22 (8q22). The concentration for centromeric probes was 5 ng per slide and for BAC probes 50 to 75 ng per slide. As a control for aneuploidy a probe on chromosome 5 was used: RP11-1059N10 (5q12). After hybridization and washing, slides were counterstained with 4', 6-diamidino-2-phenylindole and mounted in anti-fade solution (Dabco-Vectashield 1:1). 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]

DNA isolation

A 5µm section was made from fresh frozen tumor material for H&E staining, and depending on the size of the tumor 10 to 15 sections of 20µm were used for DNA isolation with QIAamp DNA mini-kit (Qiagen) according to the manufacturers' instructions. DNA quantity was measured using the NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware USA).

Comparative genomic hybridization (CGH)

CGH was performed as has been described previously.[19] In brief, tumor DNA and reference DNA were labeled, denatured, and hybridized to normal male metaphase chromosomes. Loss of DNA sequences was defined as chromosomal regions with a mean green : red (test : reference DNA) ratio < 0.8; gain was defined by a ratio > 1.2. These threshold values were based on a series of normal controls in our laboratory.

Multiplex ligation-dependent probe amplification (MLPA)

MLPA to detect loss or gain of specific chromosomes was performed with the salsa MLPA P095 aneuploidy kits containing probes for chromosomes 13, 18, 21 and sex chromosomes (MRC-Holland, Amsterdam, The Netherlands). Deletion of DNA-sequence was defined as a RQ < 0.7 and amplification was defined as RQ > 1.3 as recommended by the manufacturer.

High-density SNP-based oligonucleotide array analysis

For additional whole genome analysis we used the Illumina Infinium 610 quad Beadchip (Illumina San Diego, CA). Genomic DNA (200 ng) from the tumor sample was processed using reagents and protocols as described by the supplier. The data were analyzed us-

ing Beadstudio software (Illumina, San Diego, CA), which allows for the visualization of several different variables relevant to the detection of copy number alterations (CNA), including the B-allele frequency (BAF) and log R ratio (LRR).[20] The normalized intensity ratio for each SNP in the tumor sample was compared with a 120 HapMap reference set. The BAF and LRR output data were also analyzed for CNA's by using the software program Nexus Copy Number (Nexus BioDiscovery, El Segundo, CA). Results were compared with an in-house database of known copy number variations, and a public copy number variations dataset containing ~3500 healthy controls (DGV Dataset of Genomic Variants).

Data classification

We subdivided the copy number variation in chromosomes 1p, 3, 6p, 6q and 8q using FISH analysis into 3 categories: loss of a chromosome, normal chromosome numbers (two copies) and gain of one or more chromosomes. The hyperdiploid melanomas were defined as having gain on chromosome 1p and the 5q control FISH probe (as part of the diagnostic FISH analysis) and at least one other chromosomes (total number of chromosomes 47-57, ranging from hyperdiploid to tetraploid). Some tumors showed clones with high hyperdiploidy (51-70 chromosomes). When different subclones were identified, only FISH findings of the largest clone were included.

Statistical analysis

The primary end point for disease-free survival was the development of metastatic disease. The importance of prognostic factors on metastases-free survival was assessed using a log rank test (for categorical variables) or Cox proportional hazard analysis (for continuous variables). The significance of associations 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 metastases-free survival we used a multivariate Cox proportional hazard analysis and the likelihood ratio test. The following factors were evaluated: age, gender, cell type (spindle, mixed or epithelioid cells), vascular pattern (closed loops or not), largest tumor diameter (LTD), chromosomal aberration (loss of chromosome 3, single other or no chromosomal aberrations, or hyperdiploidy). The statistical analyses were performed with SPSS 15.0 software.

RESULTS

To asses whether the unusual findings, gain on chromosome 1p and hyperdiploidy, were related to specific tumor characteristics and patient survival, the chromosomal aberrations of 144 UMs were compared. As previously described, these tumors were analyzed

for chromosomal changes using karyotyping and FISH analyses as a standard and in some cases CGH, MLPA, LOH or SNP analyses additionally. The median clinical follow-up of the patients was 37,4 months (range 1-208 months).

In our prospective study of cytogenetic changes in uveal melanoma we encountered eleven cases with a hyperdiploid chromosome content and three tumors with complex karyotypes including a triploid clone (Figure 1). These fourteen UM demonstrated a median largest tumor diameter of 17 mm (range 10-20 mm) and eleven tumors were composed of epithelioid or mixed cells (Table 1). The gain of the chromosome 1p signal was not limited to a specific area and these tumors turned out to display a hyperdiploid character with gains on many chromosomes. In spite of that, seven tumors (EOM 174,193,211,262,296,355 and 358) showed relative loss of chromosome 3 (compared to other chromosomes). Additional SNP-array analysis revealed LOH of chromosome 3 in 8/8 melanomas (Figure 2). This implies that the two (ore more) signals for chromosome 3 counted in FISH analysis are due to the presence of more copies of the same allele. Unfortunately, we only had paraffin-embedded tumor tissue of the five other melanomas, which is not suitable for SNP-arrays.

Tumor proliferation was not higher in the hyperdiploid tumors as assessed by mitotic rate. Only three tumors, EOM 253, 283 and 329, had high proliferative activity (Table 1).

Table 1. Clinical and histological characteristics of 14 hyperdiploid uveal melanomas.
EOM 174,193,211,262,296,355 and 358 are hyperdiploid with relative loss of chromosome 3.

EOM	Age	Gender	LTD	Cell type	Vascular pattern	Proliferation	Surival
	yrs		mm	epithelioid	closed	mitoses / HPF	months
174	59	F	19	-	-	1 / 15 HPF	82*
193	44	M	16	+	+	4 / 10 HPF	42*
211	66	F	14	-	-	2 / 15 HPF	20*
253	74	M	19	+	+	20 / 10 HPF	6*
262	64	F	13	+	+	4 / 15 HPF	19*
283	44	F	12	+	-	11 / 15 HPF	40
296	66	M	19	+	+	5 / 8 HPF	13*
316	71	F	18	+	+	3 / 15 HPF	26*
329	71	M	16	+	not available	9 / 15 HPF	1*
353	65	f	10	+	-	2 / 15 HPF	10*
354†	81	m	17	+	+	2 / 15 HPF	13*
355	72	f	20	+	+	4 / 15 HPF	19*
358	57	m	12	-	+	3 / 15 HPF	47*
464	73	f	17	+	-	0 / 15 HPF	5

HPF: High power field

* metastases or death of metastases

† ciliary body melanoma

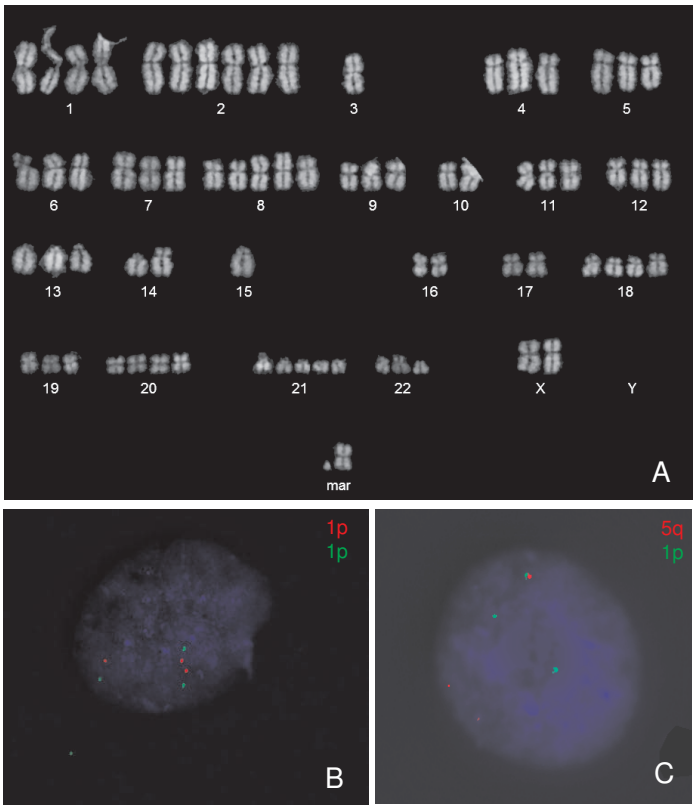

Figure 1. EOM 329: The karyogram shows high hyperdiploidy and loss of chromosome 3 (A). FISH analysis demonstrates 3 signals for both bio and dig probes on chromosome 1p (B) and 3 signals for the bio probe on chromosome 5q and the dig probe on chromosome 1p (C).

Hyperdiploidy was identified as a significant prognostic factor for tumor-related death compared to monosomy 3 or single other/no aberrations ($p<0.001$) as identified by log-rank analysis of Kaplan-Meier curves (Figure 3). Univariate analysis of the other single prognostic risk factors showed a significant decreased survival for patients having UMs consisting of epithelioid cells ($p=0.001$), closed vascular loops ($p<0.001$) or large tumor diameter ($p=0.023$). Patient age ($p=0.096$) and gender ($p=0.880$) did not influence metastasis-free survival significantly.

To exclude confounding variables a Cox proportional hazard analysis was performed including all prognostic factors tested (Table 2). The prognostic factors tumor diameter and closed vascular loops lost significance. The presence of epithelioid cells in the tumor did nearly reach significance. Monosomy 3 and hyperdiploidy were independent negative prognostic factors for metastasis-free survival.

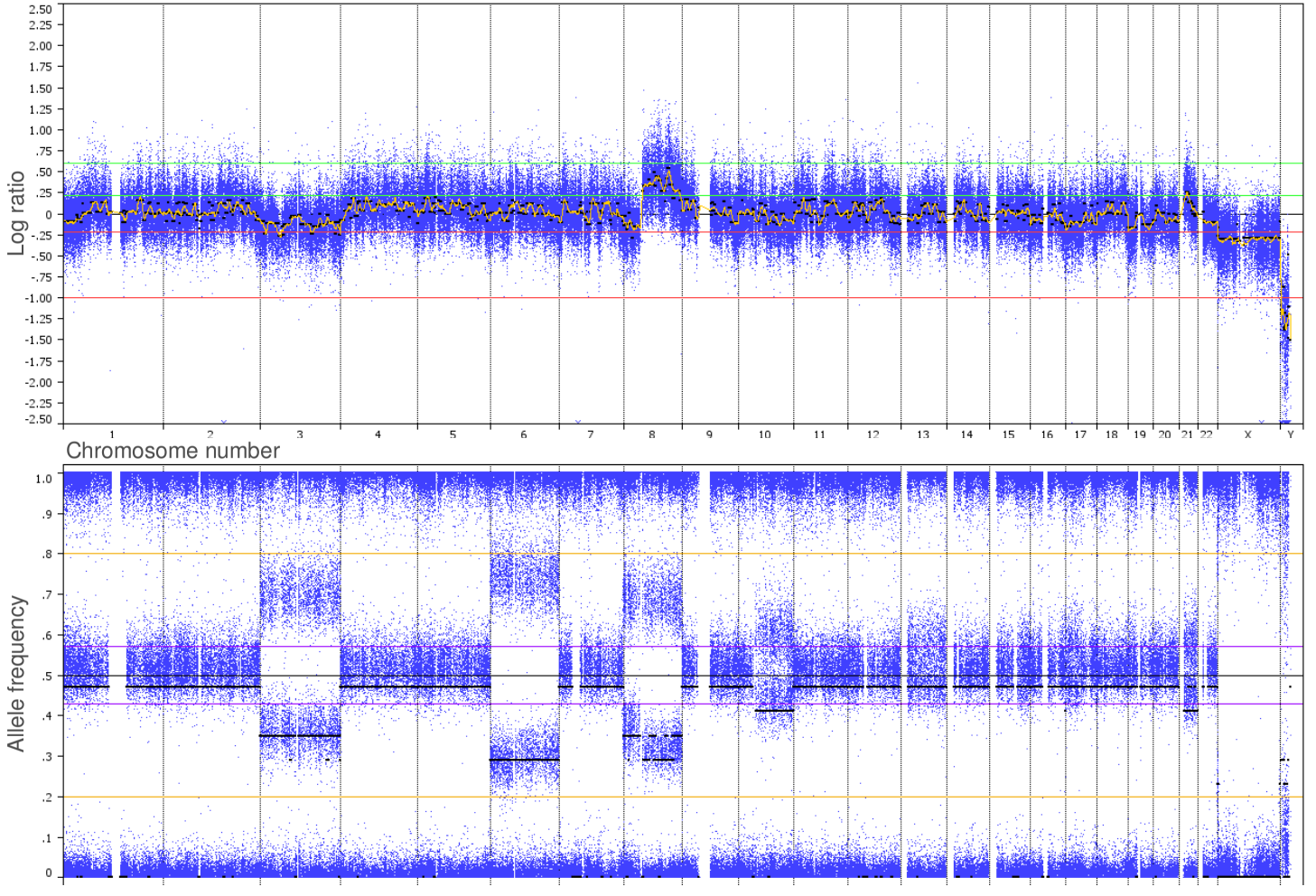

Figure 2. Whole genome SNP-array analysis of EOM 296. The top panel shows the log ratio to detect copy number alterations. Deletions of chromosome 3, 8p and X are visible, as well as amplification of chromosomal region 8q (chromosome 8 could be a mosaic). The lower panel shows the allele frequency. There is about 65% of one allele and 33% of the other allele of chromosome 3 indicating a region of LOH. Chromosome 6 and 8 both demonstrate similar percentages of LOH as chromosome 3, whereas the X chromosome shows only one type of allele (0 and 100%).

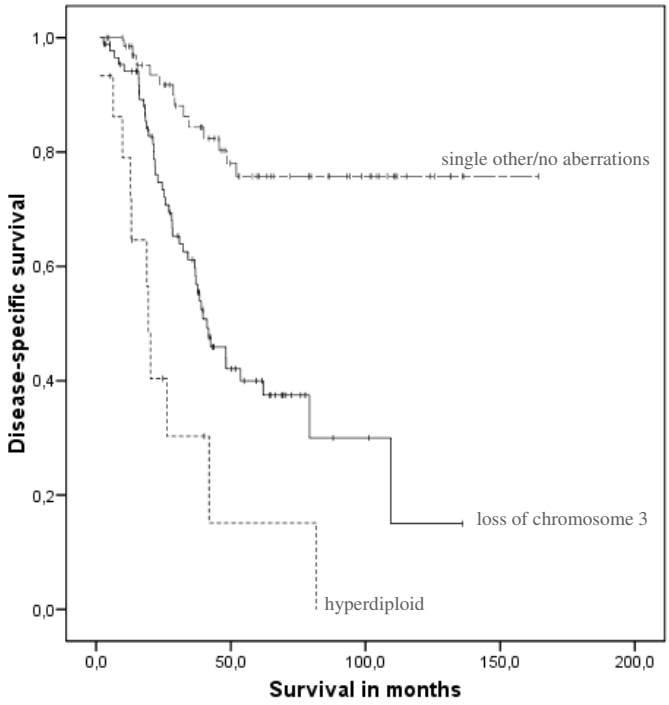

Figure 3. Kaplan-Meier survival curve based on aneuploidy in 172 UM.

Table 2. Cox regression analysis

Variable	n	Hazard Ratio (95% C.I.)	P-value
No aberrations / other than chrom 3	56	1	
Hyperdiploid	13	10,388 (3,787-28,495)	0,000
Monosomy 3	75	4,091 (1,941-8,622)	0,000
Cell type spindle vs epithelioid/mixed	144	1,802 (0,994-3,265)	0,052
Vascular loops open vs closed	144	1,676 (0,945-2,972)	0,077
Age	144	1,019 (0.997-1,042)	0,096
Gender female vs male	144	0,892 (0,513-1,552)	0,685
Largest Tumor Diameter	144	0,996 (0,919-1,084)	0,965

DISCUSSION

Monosomy 3 is an excellent prognostic marker and is commonly used to predict metastatic disease and to select patients at risk.[4] However, 10-20% of the UM patients without loss of chromosome 3 still develop metastases.[12] In a few of these patients isodisomy of chromosome 3 could be demonstrated, implicating a rescue mechanism to compensate for the net loss of chromosome 3. On the other hand, duplication of the diploid genome with selective loss of specific chromosomes as chromosome 3, commonly seen in other tumors, could also be an explanation.

In the present study, we demonstrate that hyperdiploidy is an independent prognostic risk factor for metastasizing disease in UM patients. Chromosome 1p gain, indicative of hyperdiploidy, was used to select possible hyperdiploid tumors and we confirmed in 11 patients the presence of hyperdiploid clones in the tumor specimen. Additionally, we identified two tumors with a triploid clone and one tumor with a tetraploid clone by using classic chromosome banding techniques. In total, 14 tumors with hyperdiploid chromosome content were collected. Yet, hyperdiploidy might be underreported in UM, because it is easily missed by molecular cytogenetic techniques as FISH, MLPA, CGH and micro-arrays.[21, 22] These latter DNA based techniques only recognize relative DNA changes thus 'pure' polyploidy will not be noticed. LOH can also be missed by some of these techniques, but with the introduction of SNP-arrays LOH is easily detected.

Nine of the hyperdiploid tumors demonstrated loss of chromosome 3, either a relative loss or loss of heterozygosity. This suggests a common etiology with tumors that have monosomy 3, and would fit with the assumption that loss of chromosome 3 is considered to be an early event, and is seen in combination with all other chromosomal aberrations in UMs.[24, 25] Because hyperdiploidy is only seen in a small subset of tumors this implies that hyperdiploidy is a late event during tumor progression in tumors that have already lost a copy of chromosome 3.

SNP-array analysis provided important information on the presence of isodisomy 3 in our hyperdiploid tumor population. The results suggest that LOH of chromosome 3, in other words loss of one of the alleles of the chromosome, represents the loss of important genetic information. Only one of the hyperdiploid tumors has been studied by expression profiling. This tumor, EOM 174 (a hyperdiploid tumor with LOH of chromosome 3) classified to molecular array class 2 and thus correlated with monosomy 3 and poor disease-free survival.[26] It would be interesting to know the expression array profiles of all the hyperdiploid tumors and whether tumors without a relative loss of 3 display the same pattern or are clustered in a separate class.

Nevertheless, the hyperdiploid tumors demonstrate LOH or relative loss of chromosome 3. This probably explains the association with metastatic disease and puts forward the question if hyperdiploidy should be considered as a separate group of UMs. A bias in

our study might be that the Erasmus Ocular Melanoma (EOM) database includes only enucleated melanomas, and thus the majority are medium to large size melanomas. The use of tumor biopsies in conservative treatment regimens gains territory, because patients often wish to be informed about their prognosis even when knowing metastatic disease is untreatable.[12]

The majority of chromosomal aberrations in our EOM database are loss of chromosome 3, which is easily detectable by genomic arrays because of it's presence in high volume. Our current results draw the attention to an unusual finding in UMs, hyperdiploidy, and indicate that effort should be undertaken to analyze chromosome 3 for LOH. In assessing fine needle aspiration biopsies, hyperdiploidy and LOH of chromosome 3 should be considered besides monosomy of chromosome 3 for prognostication.

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Chapter 3

Cytogenetic Abnormalities in Uveal Melanoma

Chapter 3a

Chromosome 3 Intratumor Heterogeneity in Uveal Melanoma

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ABSTRACT

Purpose. To investigate the presence of focal or diffuse heterogeneity of monosomy 3 in uveal melanoma, by using fluorescence in situ hybridization (FISH).

Methods. Direct interphase FISH in a series of 151 uveal melanomas revealed 82 tumors with loss of chromosome 3. Tumors with monosomy 3 were suspected to be heterogeneous if there were low percentages of monosomy 3, triploid clones, inconsistencies between FISH on centromere 3 and the long arm of chromosome 3, or discrepancies between fine needle-aspiration biopsies (FNABs) and the main tumor. These tumors (n=16), all choroidal melanomas, were selected and analyzed for intratumor heterogeneity by using FISH on paraffin-embedded tissue sections.

Results. Different sections of each tumor were evaluated with FISH: 6 tumors showed monosomy 3 in the same percentage throughout the tumor, and 10 showed multiple clones with different percentages of monosomy 3. However, these tumors did not show focal heterogeneity with respect to the chromosome 3 status and difference in monosomy 3 distribution between the base and apex of the tumor could not be identified.

Conclusions. Although a small number of uveal melanomas show heterogeneity for chromosome 3, it does not affect survival. In the presence of triploid clones, the loss of chromosome 3 is more difficult to interpret. In general, tumor biopsies in uveal melanoma provide an accurate prediction of the patient's prognosis.

INTRODUCTION

Uveal melanoma (UM) is the most common primary intraocular malignant tumor in adults and has a predilection for hematogenous dissemination to the liver. There is no effective treatment for liver metastasis resulting in tumor-related death in about 45% of patients with UM.[1] Prognostic factors to identify patients at risk for metastasizing disease include clinical (tumor location, largest tumor diameter), histological (cell type, vascular mimicry) and genetic (chromosomal aberrations, gene expression profiling) parameters.[2-4] Gene expression profiling identifies two distinct classes of UMs with a class II expression profile that predicts metastatic disease.[5] Loss of chromosome 3 (monosomy 3) and a class II expression profile are considered to be most accurate in selecting high-risk patients.[6] A correlation between gene expression profile and monosomy of chromosome 3 has also been established.[7]

Identification of high-risk patients has implications for follow-up and would allow the use of adjuvant therapy in the future. Most small and medium-sized UMs are currently managed by eye-saving treatments, and consequently, the available tissue for assessment of prognosis is limited. Depending on tumor location, biopsies can be obtained via a transscleral or transvitreal route. Recent studies reveal that fine needle-aspiration biopsy (FNAB) provide sufficient material for FISH analysis.[8-10]

In our laboratory, all UMs treated with enucleation are screened for the classic genetic parameters loss of the short arm of chromosome 1, monosomy 3, chromosome 6 and chromosome 8 abnormalities by fluorescence in situ hybridization (FISH) and karyotyping. However, we could not demonstrate loss of chromosome 3 in all cases of UM with metastatic disease (12/51 metastatic tumors had disomy 3). It is possible that some tumors evolved in a different manner, but it could also be an inability to detect, for example, partial loss of chromosome 3 (often screening is done by FISH analysis of the centromere), isodisomy 3 (duplication of one copy of a chromosome) or intratumor heterogeneity. Intratumor heterogeneity is considered a consequence of cancer pathogenesis. Cancer development is often associated with genomic instability and acquisition of genomic heterogeneity[11], generating both clonal and non-clonal tumor cell populations.[12] Morphologic heterogeneity is well recognized in UM showing variable proportions of epithelioid and spindle cells. Epithelioid cells lose chromosome 3 more frequently than do spindle cells.[13, 14] White et al. [15] and Sandinha et al. [13] reported cytogenetic heterogeneity in UM. However, both these studies describe morphologic heterogeneity corresponding with cytogenetic heterogeneity. In case of FNAB, intratumor heterogeneity would interfere with a correct prediction of the patient's prognosis. In this study we investigate intratumor heterogeneity of chromosome 3 in different areas of a tumor (posterior, anterior, base, and apex) in UM. Furthermore, we wanted to know whether a transscleral or a transvitreous approach for FNAB is preferable.

MATERIAL AND METHODS

Patients, tumor selection and follow-up

Between March 1992 and May 2006, tumor tissue was collected from 151 patients with UM who underwent enucleation. Informed consent was given prior to enucleation, and the study was performed according to the tenets of the Declaration of Helsinki. A conventional histopathologic examination was performed on all tumors and confirmed the origin of the tumor, as well as the tumor size, cell-type, and vascular pattern. Follow-up data regarding metastases and tumor-related death were obtained by reviewing each patient's charts and contacting the general physician. Three patients were lost to follow-up. The primary clinical endpoint was metastatic disease measured from the time of enucleation.

Fluorescence in situ Hybridisation (FISH)

Fresh tumor tissue from enucleated eyes containing UM were routinely cultured for karyotyping or directly used for FISH (chromosome 1p, 3, 6 and 8) as previously described.[9] Cutoff limits for deletion was >15% of the nuclei with one signal and for amplification was >10% of the nuclei with three or more signals.[16] Chromosome abnormalities were described following the recommendations for human cytogenetic nomenclature (ISCN, 2005).[17]

Tumors were considered of possible intratumor heterogeneity if FISH showed a low percentage of monosomy 3 (n=8) or inconsistencies between centromere 3 and the long arm of chromosome 3 (n=2). Furthermore, when triploid clones were present (n=4) or if FISH results differed between ex vivo FNABs and the main tumor (n=3). The latter were selected from a group of 40 UM, in which FISH analysis on chromosomes 1, 3, 6, and 8 was compared between ex vivo FNAB material and single cell suspension from fresh tumor material. In three tumors (6/249 hybridizations) the results were incongruent.[9]

From 16 tumors, with possible heterogeneity, 5-µm paraffin-embedded tissue sections were pretreated by dewaxing with xylene, permeabilizing with sodium thyocyanate, proteolysis and denaturation. Dual color FISH was performed with the following probes: Pα3.5 (centromere 3) and RP11-105N10 (5q12.1). Chromosome 5 is rarely involved in genetic changes in UM and was used as a control for aneuploidy and truncation or cutting artifacts. In some tumors, additional FISH was performed for 1p36 (probe RP11-48E9), 6p22 (probe RP11-356B3), 6q21 (probe RP11-787I22), and 8q21 (probe RP11-88J22). The concentration of the centromeric probe was 5 ng per slide, whereas for the BAC-clones, 15 to 25 ng per slide was used. After hybridization and washing, slides were counterstained with 4′, 6-diamidino-2-phenylindole and mounted in anti-fade solution (1:1; Dabco-Vectashield; Vector Laboratories, Burlingame, CA). Tumor sections were screened

for difference in signals between tumor base and apex, and anterior and posterior part of the tumor. 200 nuclei per sample were counted.

Statistical analysis

The influence of single prognostic factors on metastasis-free survival was assessed by using log rank analysis (for categorical variables) or Cox proportional hazard analysis (for continuous variables). The statistical analyses were performed with commercial software (SPSS 14.0 software; SPSS, Chicago, IL).

RESULTS

Interphase FISH on direct fresh tumor material was performed routinely in 151 UMs, to screen for chromosomal aberrations. The variation in the status of chromosome 1p, 3, 6p, 6q, and 8q, assessed using FISH analysis, was subdivided into three categories: loss of one chromosome, two copies of a chromosome (disomy), and gain of one or more chromosomes. Univariate analysis of the single prognostic risk factors showed a significant decreased survival for patients with UM consisting of epithelioid cells, closed vascular loops, monosomy 3, and gain of 8q (*Table 1)*. Tumor diameter and patient age were significant prognostic risk factors as well. The other chromosomal changes loss of 1p, gain of 6p, and loss of 6q did not reach significance. In addition, tumor thickness, tumor location and patient gender did not influence metastasis-free survival significantly. Eighty two choroidal melanomas showed monosomy 3. As commonly observed in UM patient populations, monosomy 3 was identified as a significant prognostic factor for metastatic disease by log-rank analysis of Kaplan-Meier curves ($p<0.001$). Whereas 15% is the detection border for chromosome loss, in the majority of UMs monosomy 3 was detected in ~90% of the counted nuclei. Sixteen tumors were selected for further analysis of intratumor heterogeneity. Six UMs showed monosomy 3 in the same percentage throughout the tumor (EOM 193, 206, 207, 208, 237 and 305). The other melanomas showed clones with various percentages of monosomy 3 (*Table 2)*. No structural difference in monosomy 3 distribution between the base and apex of the tumor could be identified. *Figure 1* shows a paraffin-embedded tissue section of a choroidal melanoma with low mitotic activity and a more pigmented area of the tumor composed of spindle and epithelioid cells, whereas the other part of the tumor was composed of epithelioid cells only. FISH showed a higher percentage of monosomy 3 in the pigmented area, but also the other part had loss of chromosome 3 (*Figure 2)*. When comparing the UMs with a low percentage of monosomy 3 with UMs with monosomy 3 in most nuclei, no significant difference in metastasis-free survival was observed ($p= 0.573$).

Table 1. Univariate analysis of prognostic markers on disease-free survival in 151 uveal melanomas

Variable	**Median**	**p-value***
Age at time of diagnosis (yrs)	62	0.004
Tumor thickness (mm)	8	0.337
Largest tumor diameter (mm)	13	0.002
Variable	**No. of patients (%)**	**p-value†**
Male gender	81 (54)	0.601
Involvement ciliary body	22 (15)	0.544
Mixed / epithelioid cell type	80 (54)	0.018
Closed vascular loops	67(49)	<0.001
loss of 1p	45/143 (31)	0.103
loss of chromosome 3	82/148 (55)	<0.001
gain of chromosome 6p	51/126 (40)	0.280
loss of chromosome 6q	39/119 (33)	0.313
gain of chromosome 8q	87/129 (67)	<0.001

* Cox-regression analysis
† Log-rank test

Table 2. 16 UMs with percentage chromosome 3 loss by FISH

EOM	**paraffin-embedded tissue section**			
	anterior	posterior	base	apex
139 *	28	14	24	-
144	74	81	79	53
193	62	58	50	52
203	74	50	50	76
206 †	57	57	57	53
207 †	94	76	76	80
208 †	82	84	80	90
225	62	56	52	46
237	62	70	-	-
240	58	52	34	50
253 ‡	16	33	32	33
262 ‡	72	84	88	96
303	32	60	-	-
305	60 §	66 §	56	56
321 *	28	34	44	24
341 ‡	28 §	63 §		

* discrepancy between centromere 3 and 3q
† discrepancy between FNAB and main tumor
‡ triploid clones present
§ which part is lying anterior and posterior could not be identified

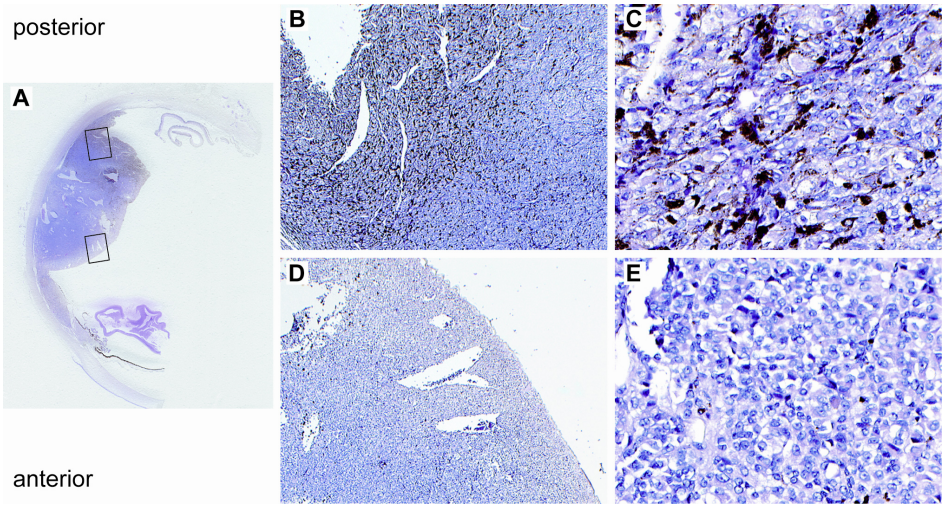

Figure 1. Heterogeneity of uveal melanoma EOM 303:section stained with hematoxylin and eosin staining. A) Choroidal melanoma located at the equator extending to the pars plana (magnification x 10), B) and C) present epithelioid and spindle cells with melanine-containing macrophages in between, D) and E) less pigmented part of the tumor with predominantly epithelioid cells. B+D magnification x 25, C+E magnification x 200.

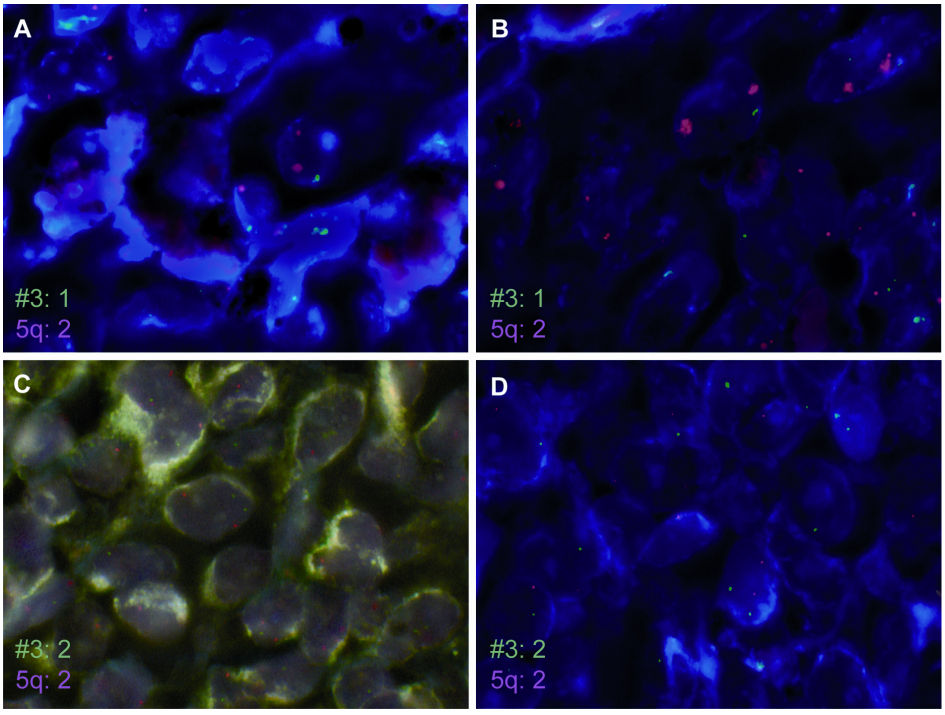

Figure 2. EOM 303 Paraffin-embedded tissue section FISH: the posterior part (A+B) shows 60% monosomy 3 and the anterior part (C+D) shows 19% monosomy 3. (green signals: centromere 3, red signals: 5q)

In all sixteen tumors, monosomy 3 was found in every part of the tumor. In four UMs, we observed a difference in chromosome 3 status between interphase FISH on single-cell suspension from fresh tumor tissue and FISH on paraffin-embedded tissue. In the tissue sections, we observed a 14% to 96% loss of chromosome 3, whereas interphase FISH revealed only a relative loss of chromosome 3. These four UMs contained triploid subpopulations of tumor cells with only two copies (instead of three) of chromosome 3.

DISCUSSION

In the study presented in this article, we attempted to gain insight to intratumor heterogeneity. Subclones with different percentage of monosomy 3 could be identified within the tumor in 10 cases. None of the analyzed choroidal melanomas required adjustment of risk calculation for metastasizing disease based on chromosome 3 status throughout the tumor. Intratumor heterogeneity should be considered in tumor biopsies, although it does not seem to influence survival. UMs with a low percentage of chromosome 3 loss also result in reduced survival; therefore, it would be interesting to examine the liver metastases to investigate which clone or clones have led to tumor progression. Identifying high-risk patients based on examining tumor biopsies appears reliable, because UM do not demonstrate abundant chromosomal focal heterogeneity.

Another group of interesting UMs are the ones with disomy 3 that do metastasize. Of 51 metastasized primary tumors, 12 showed disomy 3. Additional analysis such as karyotyping and comparative genomic hybridization (CGH) did not suggest loss of chromosome 3 or heterogeneic loss of chromosome 3. Currently, we are investigating these tumors by high-density array CGH.

Sandinha et al.[13] have examined morphologic and cytogenetic heterogeneity in UM. They found monosomy 3 in 12 of 22 mixed cell–type UMs. In seven of these tumors, monosomy 3 was found only in the epithelioid cells and not in the spindle cells. In the present study, we were more interested in cytogenetic differences between tumor areas, because that might influence the route (transscleral or transvitreal) to choose for FNAB. We found monosomy 3 in nine spindle cell–type UMs, four epithelioid cell–type UMs, and three mixed cell–type UMs.

Maat et al.[18] have pointed out that heterogeneity of monosomy 3 is a frequent event in uveal melanoma. In their study, 7 of 50 UMs were found to be heterogeneous, but FISH was unsuccessful in 17 UMs and difficult to interpret in 8. Furthermore, FISH was only performed for chromosome 3, without a control probe for aneuploidy and/or truncation artifacts, and no cut off values for loss or gain of a chromosome were reported. Possible caveats include truncation artifacts in paraffin-embedded sections leading to cutting of nuclei and as a result to the deletion of chromatin material from a section, sampling

errors in FISH (e.g. looking at macrophages instead of tumor cells), and signal reduction due to pigmentation.[19] However, truncation artifacts can be controlled by assessing a chromosome not involved in UM for aneuploidy. Few stroma are present in UMs and melanoma nuclei are larger than nuclei of macrophages and fibroblasts, making identification of tumor cell nuclei easy and probe signals distinguishable.

A more important drawback in interphase FISH of paraffin-embedded tissue sections and other techniques, as well, is that the tumor is observed in one dimension, which limits insight into the entire lesion, possibly missing intratumor heterogeneity. Previous studies by our group demonstrate that FISH analysis of FNAB biopsy specimens gives reliable results of cytogenetic changes within the tumor. Chromosome status in the biopsy specimens was consistent with the results in the main tumor tissue. In 6 of 249 hybridizations FNAB showed normal copy numbers while a direct single-cell suspension of the main tumor showed gain or loss of chromosomes in small subclones.

In our present study, the incongruent chromosome 3 status of four tumors in different experiments might be the result of relative loss in triploid clones or genetic imbalance. Two of these tumors metastasized within 19 months of diagnosis. All four tumors have triploid clones, and tissue was taken from different parts of the tumors. Yet, the results were obtained from different FISH techniques, and consequently, conclusions about focal heterogeneity may be indefinite. Different available FISH techniques need further attention and standardization in ocular oncology research.

Monosomy of chromosome 3 is the most frequent found nonrandom chromosomal aberration in UM and is predominantly found in metastasizing tumors.[20, 21] In the majority of tumors with chromosome 3 loss there is complete monosomy, although occasionally isodisomy of this chromosome is acquired. Rarely, melanomas with partial aberrations on chromosome 3 or translocations have been described, making it difficult to map putative tumor suppressor genes. Loss-of-heterozygosity studies demonstrate common regions of allelic loss located at 3p25 and on the long arm spanning from 3q24 to 3q26.[22, 23]

Monosomy 3 is considered to be a primary event, because it is seen in combination with all other chromosomal aberrations in UMs such as loss of 1p, gain of 6p, and gain of 8q.[24] Tumors with gain of chromosome 6p have been proposed to represent a separate group of UMs with an alternative genetic pathway in carcinogenesis.[25, 26]

Reviewing our Erasmus Ocular Melanoma (EOM) database of 151 UM, we found that 61 tumors with loss of chromosome 3 gained 8q concurrently, whereas no tumors with disomy 3 gained 8q. Tumors with monosomy 3 had gained 6p concurrently in 17 (21%) cases and had lost 6p in one case. Gain of 6p was also identified in 32 tumors with disomy 3. In a recent study by Ehlers et al.[26], integrative genomic analysis of 49 UMs also revealed concurrent gain of 6p and loss of chromosome 3 in 4% of the tumors. Gain of 6p does not reach significance as a single prognostic factor in our study population.

Furthermore, gain of 6p occurs more often in tumors with monosomy 3, suggesting that it is unlikely to represent an alternative pathway of UM pathogenesis. We do agree that gaining 6p is probably an early event in carcinogenesis.

This study is the first to systematically evaluate genetic heterogeneity, and it confirmed that monosomy 3 remains an important prognostic factor in UM that can be determined with relative easy cytogenetic techniques. Overall, our findings support the use of tumor biopsies for diagnosis, prognosis and therapeutic decision-making. A transscleral or transvitreal approach can be chosen, depending on tumor location. To reduce the possible impact of heterogeneity on identification of high-risk patients, FNABs should be obtained from several areas of the tumor.

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Chapter 3b

Chromosomal Aberrations in Iris Melanomas

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ABSTRACT

Background. Uveal melanomas can develop in the choroid, ciliary body and iris. In choroidal and ciliary body melanomas specific chromosomal changes correlate with metastatic disease. Iris melanomas have a better prognosis than choroidal melanomas and it would be interesting to know if they share chromosomal changes. In addition, in iris melanomas UV-induced mutations of tumour suppressor genes, such as PTEN and CDKN2A can be expected.

Methods. Twenty patients with iris melanomas were analyzed for chromosome 1p, 3, 6, 8, 9p and 10q abnormalities using fluorescence in situ hybridization. These results were correlated to clinical follow-up data using statistical analyses.

Results. Loss of chromosome 3 was observed in 9 iris melanomas and gain of 8q was present in 7 tumours. Loss of chromosome 9p was demonstrated in 7 tumours, but no deletions of the PTEN region of chromosome 10 were found. Three patients died of metastatic disease. However, no significant correlations between histopathological parameters or chromosomal changes were found.

Conclusion. CDKN2A mutations might play a role in iris melanoma and monosomy 3 is not an indicator of poor prognosis.

INTRODUCTION

Iris melanomas are the least common of uveal melanomas (< 6%) and tend to present at a smaller size than posterior melanomas.[1] They can cause drainage blockage of the anterior chamber angle and secondary elevation of the intraocular pressure. They carry a better prognosis compared to other uveal melanomas with a metastatic rate of 0.5% at 3 and 7% at 10 years, respectively.[2] Melanocytic lesions of the iris are usually managed by a 'wait and see policy' with regular follow-ups to detect possible growth. Biomicroscopy, gonioscopy and slit lamp measurements are standard tests. Ancillary tests such as fluorescein angiography and ultrasound biomicroscopy are sometimes needed to assess growth more accurately. Metastases are more likely to develop in those patients who are older or with tumour features as iris root location, ciliary body invasion, elevated intraocular pressure, or extraocular extension.[3] The differential diagnosis of iris melanoma includes Cogan Rees syndrome, nevus, melanocytoma, metastasis, adenoma, adenocarcinoma, cysts and pigment dispersion syndrome.[4]

The favourable outcome of iris melanomas compared to choroidal and ciliary body melanomas is poorly understood. To determine the prognosis on only histological classification proved to be difficult.[5] Therefore more accurate tests are needed. The different anatomical locations of the intraocular melanomas result in different microenvironments. Aqueous humour and vitreous contain various soluble factors that might influence cell growth.[6] Another differential factor is UV-radiation. Its role in uveal melanoma is inconclusive, but arguably the iris is more exposed to the sun.[7-13] While this might induce changes in the genome, intrinsic genetic differences may also play a role.[14]

To further elucidate the genetic differences between choroidal and iris melanomas, the following study was performed. Twenty iris melanomas were analyzed for chromosomal aberrations well known in choroidal melanomas, such as loss of chromosome 3 and gain of chromosome 8q,[15] in addition to common changes in skin melanomas, such as loss of 9p21 and 10q23 (regions that harbour the tumour suppressor genes CDKN2A and PTEN, respectively).[16]

MATERIALS AND METHODS

The research methodology followed the tenets of the Declaration of Helsinki. Informed consent was obtained for the use of human tissue. The research was approved by the local research ethics committee. All cases of iris melanomas were collected from the files from the departments of pathology in Rotterdam (1993-2009) and Leiden (1980 to 2009) and reviewed retrospectively. Cases that originated from the ciliary body were excluded as well as patients who had no available follow-up information. This effected in

22 iris melanomas available for our study. FISH (Fluorescence in situ hybridisation) was unsuccessful in 2 cases.

The enucleation and biopsy specimens were fixed in standard 10%, buffered formalin for 24 h. They were embedded in conventional, paraffin wax and sections were cut at 5 µm. Sections were stained with Haematoxylin and Eosin (H&E) in order to obtain a histopathological diagnosis.
Paraffin-embedded iris melanoma sections were used for FISH (chromosome 1p, 3, 6, 8, 9p and 10q). The pathologist located the tumour and dual colour FISH was performed with the following probes: RP11-48E9 (1p36), RP11-522N9 (3p13), Pα3.5 (centromere 3), RP11-64F6 (3q25), RP11-356B3 (6p22), RP11-787I22 (6q21), RP11-24P4 (8p21), D8Z2 (centromere 8), RP11-88J22 (8q22), P1.063 (9p21) and PAC190D6 (10q23).[17] The concentration for centromeric probes was 5 ng per slide and for BAC probes 50 to 75 ng per slide. As a control for aneuploidy a probe on chromosome 5 was used: RP11-1059N10 (5q12). After hybridization and washing, slides were counterstained with 4', 6-diamidino-2-phenylindole and mounted in anti-fade solution (Dabco-Vectashield 1:1). Cut-off limits for deletion (>25% of the nuclei with one signal) or amplification (>10% of the nuclei with 3 or more signals) were adapted from own experiments and the available literature.[18]

The primary endpoint for disease free survival (DFS) was the time to development of metastatic disease, whereby death due to other causes was being censored. Statistical analyses were performed with SPSS software, release 15.0. Actuarial probabilities of disease free survival (with an event defined as development of metastatic disease or metastasis-related death) 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. The following factors were evaluated: age, cell type (spindle, mixed or epithelioid cell), Jacobiec classification, involvement of ciliary body, tumour invasion of the chamber angle, chromosomal aberrations (chromosomes 1, 3, 6p, 6q, 8q, 9p and 10q).

RESULTS

Tumour characteristics

Twenty iris melanomas were included in our study. The specimens were reviewed and classified according to the Jacobiec histopathologic classification of iris melanocytic tumours:[5] 3 borderline spindle cell nevi, 4 spindle cell melanomas, 6 mixed spindle and epithelioid cell melanomas and 7 epithelioid cell melanomas (Figure 1).

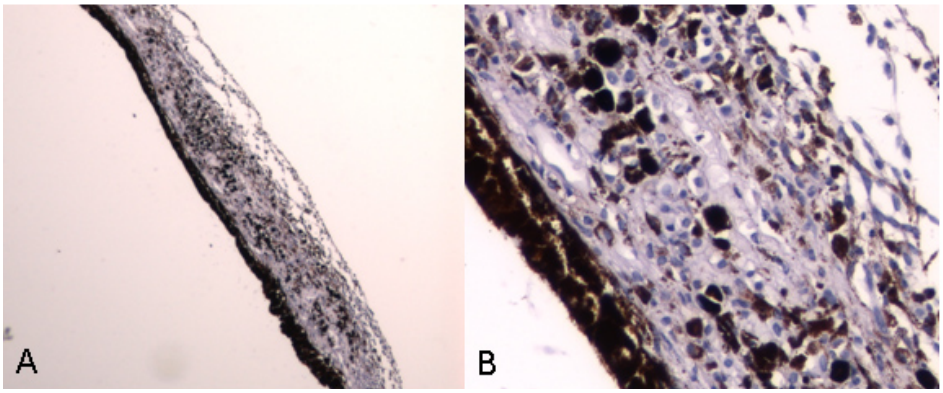

Figure 1. Histology of no.2, EOM 186,(H&E stain) shows an epithelioid cell iris melanoma. A) original magnification ×100 and B) original magnification ×500.

Fifteen melanomas grew in the chamber angle resulting in secondary glaucoma in 5 cases caused by angle closure. Nine iris melanomas that spread into the angle also spread into the ciliary body, but primarily originated from the iris and in all of them epithelioid cells were present (Table 1).

Table 1. Tumour characteristics

Iris melanoma no#	Jacobiec	Age years	Ciliary body involved	Cell type	Angle invasion	Survival in months	Tumour-related death
1	9	53	yes	epithelioid	yes	48	yes
2	9	63	no	epithelioid	yes	112	no
3	9	41	yes	epithelioid	yes	20	no
4	9	55	yes	epithelioid	yes	16	no
5	6	77	no	spindle	yes	62	other cause
6	6	76	no	spindle	no	10	no
7	6	71	no	spindle	no	2	no
8	9	42	no	epithelioid	yes	144	no
9	8	52	yes	mixed	yes	37	yes
10	8	59	yes	mixed	yes	13	no
11	8	71	no	mixed	yes	23	no
12	7	46	no	spindle	no	96	no
13	8	49	no	mixed	no	157	no
14	7	62	no	spindle	yes	62	no
15	9	69	no	epithelioid	yes	5	no
16	8	66	yes	mixed	yes	35	yes
17	8	56	yes	mixed	yes	86	no
18	7	69	no	spindle	no	24	no
19	9	83	yes	epithelioid	yes	157	other cause
20	7	67	no	spindle	no	204	no

Clinical data

The median age of the patients at the time of diagnosis was 62 years. Three patients had a trabeculectomy for glaucoma in their medical history. Surgery or Ruthenium plaque irradiation was chosen to treat the iris melanoma.
The median follow-up time was 42 months (range 2-204 months). Three patients died of metastases. One patient had been treated with a trabeculectomy 12 years earlier (histopathological diagnosis at that time: Cogan Reese syndrome) and died of metastatic disease. The second patient presented with metastases, located in the bones and submandibular lymph nodes, 3 years after a trabeculectomy (histopathological diagnosis at that time: iris nevus without signs of malignancy). The third patient received Ruthenium plaque irradiation and did not have intraocular surgery.

Cytogenetic results

All twenty iris melanoma tissue slides could be hybridized with the chromosome 3p and centromere 3 probe. For the other FISH probes it was not always possible to obtain data, because often the material was to sparse to give a definite answer. See table 2 for the results. FISH Analysis of paraffin sections demonstrated monosomy 3 in nine tumours. Seven melanomas with monosomy 3 had concomitantly gained chromosome 8q (see Figure 2). Loss of the chromosomal region 9p21 was present in seven tumours, and six of those had concomitant loss of chromosome 3 and 9p. Five tumours had all three chromosome aberrations (loss of chromosome 3, 9p and gain of 8q) mentioned above. Chromosome 10q was normal in all cases.

The tumours of the patients who died of metastases revealed loss of chromosome 3. Two of them also lost 9p21, and two melanomas had gained 8q. Two of the progressively growing melanomas were composed of mixed spindle and epithelioid cells and one was composed of epithelioid cells only. All three spread into the angle of the chamber and into the ciliary body. Three melanomas in our group were spindle cell nevi with borderline malignancy (Jacobiec group 6). Interestingly, two of them did not have any chromosomal aberrations, but the third one did lose one copy of chromosome 3q and 9p simultaneously with gain of chromosome 8q.

Statistical analysis

Univariate analysis was performed for all parameters, showing a lower disease free survival for patients with iris melanomas having monosomy 3 ($p=0,055$) or invading the ciliary body ($p=0,02$). None of the chromosomal aberrations showed significant correlations. In a multivariate Cox proportional hazard analysis including all histopathological parameters and chromosomal variations, the association of ciliary body involvement with disease free survival was no longer significant ($p=0,656$).

Table 2. FISH results

Iris melanoma	Copy number per chromosomal region										
no#	1p36	3p13	cen3	3q25	6p22	6q21	8p21	cen8	8q22	9p21	10q23
1	1	1	1	1	2	2	2	3	3	1	2
2	2	1	1	1	2	1	3	3	3	1	2
3	2	2	2	2	2	2	2	2	2	2	2
4	2	2	2	2	2	2	2	3	2	1	2
5	2	2	2	1	2	2	2	3	3	1	2
6	2	2	2	2	2	2	2	2	2	2	2
7	2	2	2	2	2	2	2	2	2	2	2
8	1	2	2	2	2	2	2	2	2	2	2
9	2	1	1	1	2	2	2	3	2	1	2
10	2	1	1	1	2	2	1	3	3	2	2
11	2	1	1	1	2	2	4	3	3	1	2
12	2	2	2	2	2	2	2	2	2	2	2
13	n/a	2	2	n/a	n/a	n/a	n/a	3	n/a	n/a	n/a
14	1	1	1	n/a	n/a	n/a	n/a	n/a	3	1	2
15	1	1	1	1	2	2	1	3	3	2	2
16	1	1	1	n/a	2	1	n/a	n/a	3	2	2
17	n/a	2	2	n/a	n/a	n/a	n/a	n/a	n/a	2	2
18	2	2	2	2	2	2	2	2	2	2	2
19	1	1	1	n/a	n/a	n/a	n/a	n/a	3	2	2
20	2	2	2	n/a	n/a	n/a	n/a	n/a	2	2	2
Total aberrations	6/18	9/20	9/20	6/14	0/15	2/15	4/14	9/15	9/18	7/19	0/19

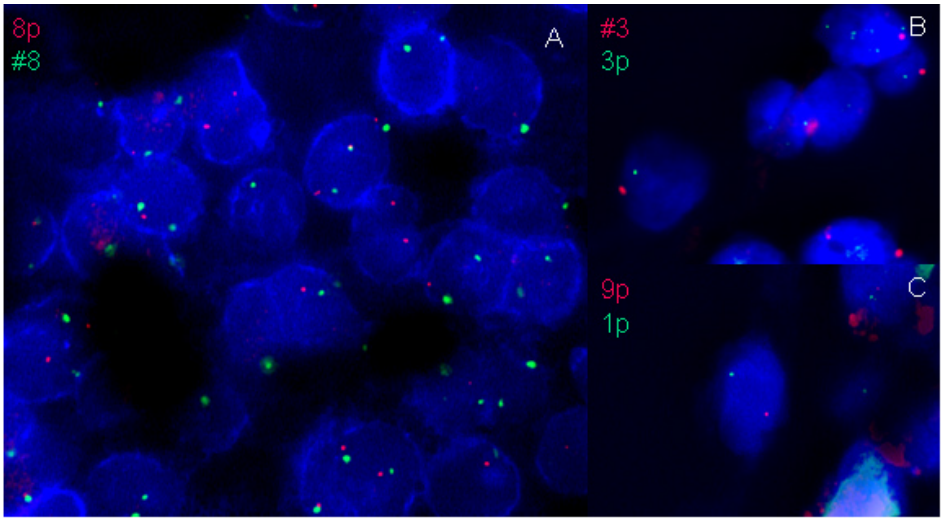

Figure 2. FISH analysis of iris melanoma no.2, EOM 186, demonstrates 3 signals for both bio and dig probes on chromosome 8 (A), 1 signal for both bio and dig probes on chromosome 3 (B), and 1 signal for the bio probe on chromosome 9p and 1 signal for the dig probe on chromosome 1p (C).

DISCUSSION

Our study reports on chromosomal abnormalities in the largest group of iris melanomas described thus far. We demonstrated that in iris melanomas monosomy 3 tends to occur often, in 9/20 tumours (45%), but does not correlate with progressive disease, whereas loss of chromosome 3 is present in over 50% of the choroidal melanomas and identifies patients at high risk of metastatic disease.[19] This observation supports the theory that possible environmental influences of aqueous and vitreous humours contribute to the metastatic potential of a melanoma.[6]

Similar to common changes in skin melanomas, such as loss of 9p21 and 10q23, 7/ 20 iris melanomas (35%) revealed loss of chromosome 9p. However, we could not demonstrate aberrations of chromosome 10q in iris melanomas. Previously, it has been reported that the tumour suppressor gene PTEN (located on chromosome 10q) is not of significance in uveal melanomas, whereas mutations of PTEN are present in up to 40% of cutaneous melanomas.[20-22]

UV-radiation induces mutations in the CDKN2A gene that encodes for the p16 protein that acts as a negative regulator of the cell cycle and is located on chromosome 9p21.[23] Alterations of chromosome 9 have been found to occur frequently in cutaneous melanomas and relate to progressive disease.[24-26] Conversely, CDKN2A mutations are rarely observed in UM.[20, 21] The same holds true for N-ras mutations. Mutations of the N-ras gene, located on chromosome 1p13, correlate with sunlight exposure and occur frequently in cutaneous melanoma arising in sun exposed areas.[7] Nevertheless, N-ras mutations are usually absent in uveal melanomas, further questioning the role of sunlight in the pathogenesis of uveal melanoma.[8, 15]

Little is known about somatic mutations and chromosomal aberrations in iris melanomas. To date only 8 iris melanomas have been cytogenetically analyzed and described worldwide. The first karyogram of an iris melanoma revealed extra copies of chromosomes 2, 7 and 18, and loss of chromosome 15. Additionally, rearrangements on chromosomes 8q, 4q, 12p and marker chromosomes were observed.[27] Sisley et al. karyotyped three iris melanomas and did not find specific chromosomal changes common to all tumours. Clonal abnormalities of chromosomes 3, 5, 6, 7, 8, 9, 12, 15, 17, 18, 19, and Y were found, and in one case a large number of marker chromosomes were observed.[14] They concluded that iris melanomas experience relatively high levels of chromosomal alterations compared to choroidal melanomas, which is perhaps reminiscent of cutaneous melanoma. The third study included four iris among other uveal melanomas that were analyzed by comparative genetic hybridization.[28] Gains of chromosome 6p and 8q were identified twice and gains of chromosome 8p and 13q were identified once. One melanoma was completely diploid.

Surprisingly, in the twenty iris melanomas described above chromosome 9p was involved in seven tumours and monosomy 3 in nine melanomas. These results suggest that the tumour suppressor gene CDKN2A, located on chromosome 9p, might play a role in iris melanomas. The role of monosomy 3 in iris melanomas needs further investigation. The prognostic significance seems different from choroidal melanomas. This observation suggests that the possible environmental influences of aqueous and vitreous humours contribute to the metastatic potential of a melanoma, which may have important consequences in developing alternative therapies for uveal melanomas.

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Chapter 4

Candidate Regions and Genes Associated with Choroidal Melanoma Progression

Chapter 4a

Molecular Cytogenetic Analysis of Archival Uveal Melanoma with Known Clinical Outcome

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ABSTRACT

Uveal melanoma (UM) is the most common primary intraocular tumor in the Western world. Cytogenetically this tumor is characterized by typical chromosomal aberrations such as loss of 1p, 3 and 6q, and gain of 6p and 8q. Routinely, karyotyping and fluorescent in situ hybridisation (FISH) on fresh tumor-biopsies are used to identify chromosomal changes. In addition, archival UM samples can be examined using comparative genomic hybridization (CGH). In the presented study, we used CGH on a series of 46 archival uveal melanomas to identify chromosomal changes. In 44 tumors aberrations were present and classic prognostic markers as loss of 1p (12 tumors, 26.1 %), monosomy 3 (26 tumors, 56.5%), loss of 6q (10 tumors, 21.7%), and gain of chromosome 8q (27 tumors, 58.7%) were observed. Gain of chromosomes 18q or 21q was found in 3 UMs. Multiplex ligation-dependent probe amplification (MLPA), a novel technique in UM, was performed to verify this low number of chromosome 18 and 21 abnormalities, but we could not confirm the previously reported gain of chromosome 18q11.2 and chromosome 21q11.2 as poor prognostic factors in UM.

INTRODUCTION

White et al. described gain of chromosome 18q11.2 as a powerful predictor of a poor prognosis in uveal melanoma patients.[1] That group evaluated 100 UMs with an impressive clinical follow-up over 9 years by CGH. These UMs were divided into two survival cohorts of 43 patients having a metastases-free follow-up over 9 years and 39 patients with systemic tumor spread. Six regions of interest differed between these cohorts and serve as prognostic factors in UM: gain of 18q11.2, 6q16, 21q11.2, 9q12, 3q12 and loss of 1p33. The chromosomal regions 18q11.2, 21q11.2 and 9q12 are newly identified prognostic factors in UM.
Prognostic factors to identify patients at risk for metastasizing disease include clinical (tumor location, largest tumor diameter), histological (cell type, vascular mimicry) and genetic (chromosomal aberrations, expression profiling) parameters.[2-4] In our laboratory, uveal melanomas are routinely screened with fluorescence in situ hybridization (FISH) and karyotyping for the following classic genetic parameters: loss of 1p, monosomy 3, and abnormalities of chromosomes 6 and 8. To verify the prognostic markers 18q11.2 and 21q11.2 identified by White et al., we applied multiplex ligation-dependent probe amplification (MLPA) in a series of 46 tumors analyzed by CGH.

METHODS AND RESULTS

CGH was performed as described by White et al.[1] with some modifications. 10μm Formalin-fixed paraffin-embedded tissue sections were used for DNA isolation. In brief, tumor DNA and reference DNA were labeled, denatured and hybridized to normal male metaphase chromosomes. Loss of DNA sequences was defined as chromosomal regions with the mean green: red (test-reference DNA) ratio < 0.8; gain was defined by a ratio > 1.2.
In total, 46 UM were screened for DNA-sequence copy number changes (*Table 1)*. The median follow-up was 43.8 months (range 6-165 months) with tumor-related death in 22 patients and metastatic disease in one patient. The median largest tumor diameter was 14.0 mm (range 7-19 mm).
CGH was completed on 43 choroidal and 3 ciliary body melanomas (*Figure 1)*. The classic prognostic markers loss of 1p, monosomy 3, gain of 6p, loss of 6q and gain of 8q could be identified. Loss of chromosome 18 was found in one choroidal tumor. CGH detected loss of chromosome 18q12 and 18q22 in one melanoma and gain of 18p11.1 in nine others. In one of those nine UMs, karyotyping revealed a small population of chromosome 18 gains. Two UMs showed gain of the long arm of chromosome 21 and fourteen UMs showed gain of the subtelomeric region 21q22. To confirm chromosome 18 en 21 abnormalities, we used MLPA, a PCR-based-amplification method, on 31 UMs: 10 fresh-frozen and 21 paraffin-embedded samples. A Salsa MLPA P095 Aneuploidy kit (MRC-Holland,

Amsterdam, The Netherlands) was used as described by Schouten et al..[5] The amount of amplified probes depends on the quantity of target sequences in the sample DNA, therefore a relative quantification (RQ) can be used to detect copy number changes. Deletion of DNA-sequence was defined as a RQ < 0.7, and amplification was defined as RQ > 1.3.
The target sequences included 8 probes located on chromosome 18 and 8 probes on chromosome 21: 18p11.3, 18p11.2 (2xs), 18q11.2, 18q21, 18q21.1, 18q21.3, 18q23, 21q11, 21q21.1(2xs), 21q21.3, 21q22.1, 21q22.1, 21q22.2 and 21q22.3.
Loss of chromosome 18 could be validated in tumor EOM 174, but gain of 18p11.1 could not be confirmed in any UM. Notably, the region of interested 18q11.2 was amplified in two of the three ciliary body melanomas (EOM 8 and EOM 21), as well as in EOM 257 (one of the 43 choroidal melanomas).
Validation by FISH analysis with the BAC-clones RP11-79F3 (18q11.2) and RP11-1059N10 (5q12.1, as a control for aneuploidy) could be performed in two of these UMs and resulted in normal copy numbers of 18q11.2 (EOM 8:81%, EOM 21:86%). The FISH-probe, however, does not map to the exact same position as the MLPA-probe.
MLPA also identified three UMs with gain of 21q11.2. Those patients all died of metastatic disease. FISH analysis confirmed duplication of 21q11.2.
Log-rank analysis of Kaplan-Meier curves did not show a significant influence of gain of chromosome 18q11.2 or gain of chromosome 21q11.2 on survival. The influence of concurrent loss of chromosome 1p and chromosome 3 did show a trend towards significance (p=0.08) in this cohort. In multivariate Cox regression analysis also, no significant correlates were observed.

DISCUSSION

The 46 uveal melanomas analyzed by CGH are a representative group of our database containing all enucleated uveal melanomas in Rotterdam (from 1992 to date). The follow-up of our patients is relatively short; however, given that the peak incidence of melanoma-related death is 2-3 years after diagnosis[6-8], a 43.8 month median period of follow-up is unlikely to affect any determination of factors of poor prognosis.
Differences in chromosome copy number by CGH, karyotyping, MLPA and FISH could have several causes related to the methodology. The CGH findings on 18p11.1 could be artifacts because the gain is within a centromeric region. Similarly, the subtelomeric gains on 21q22 could be artifacts, because MLPA probes have a shorter sequence than FISH probes and so the locations are not completely analogous. In addition, paraffin gives background noise, which influences the results in CGH and MLPA. Yet another factor is the different detection levels of chromosomal abnormalities in each technique, in which FISH is most sensitive in detecting aberrations present in a low percentage of the cells.

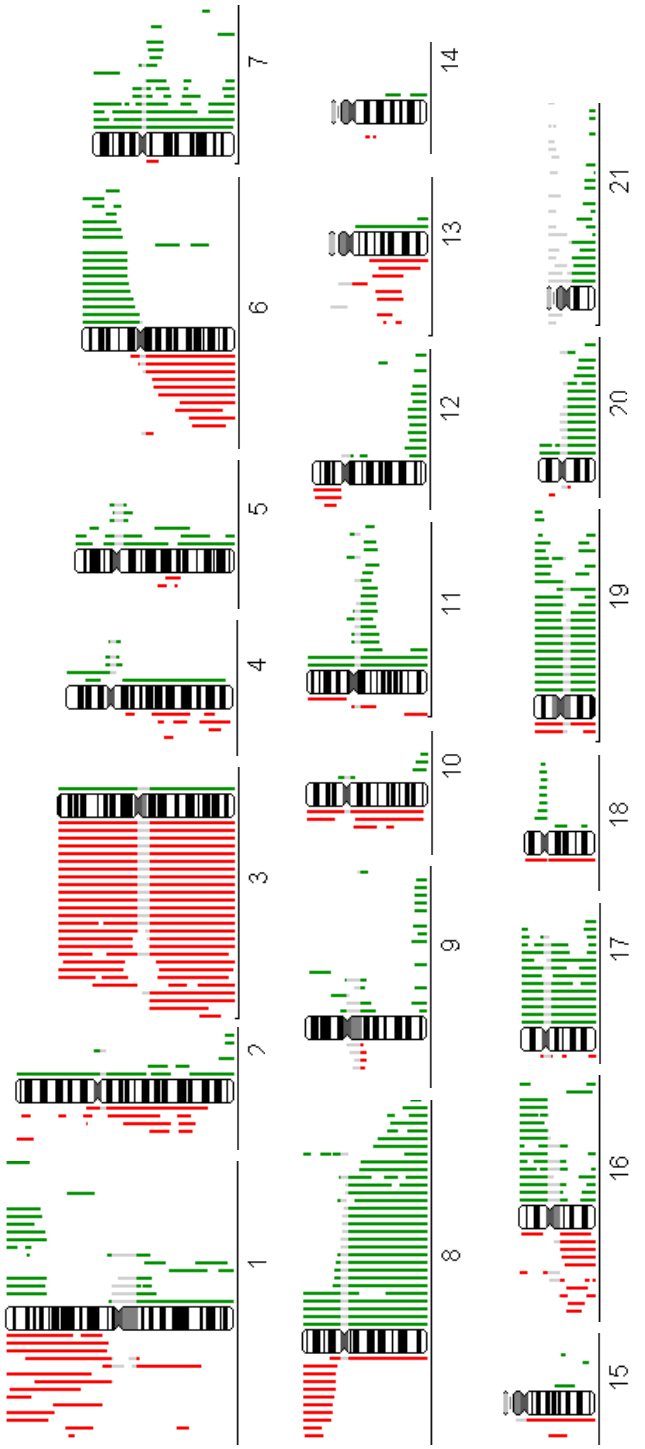

Figure 1. Combined comparative genomic hybridization profile showing DNA copy number changes of 46 archival cases of uveal melanoma. Vertical red bars to the left of the chromosome indicate losses and vertical green bars to the right indicate gains per tumor.

Table 1. Clinical characteristics and chromosome 18 and 21 copy number changes in 46 uveal melanoma

EOM	Age/sex	Cell type	Follow-up	Chromosome 18			Chromosome 21		
				CGH	Karyotype	MLPA	CGH	Karyotype	MLPA
7	69 / M	mixed	156	N	-	N	gain 21q22*	-	N
8 +	63 / M	epithelioid	21 †	gain 18p*	-	gain 18q11.2	N	-	N
12	45 / F	mixed	148 †	N	-	-	N	-	-
15 +	80 / F	mixed	17 †	N	-	-	N	-	-
21 +	51 / F	epithelioid	149	gain 18p*	-	gain 18q11.2	N	-	N
28	47 / M	mixed	85 ‡	N	-	-	N	-	-
42	47 / F	mixed	32 †	N	N	loss 18p11.2 and gain 18q23	N	N	N
45	61 / M	epithelioid	29 †	N	N	-	N	N	-
57	74 / M	mixed	132	N	-	N	N	-	N
81	69 / M	mixed	21 †	gain 18p*	-	N	N	-	N
107	76 / M	mixed	63	N	N	N	N	loss 21 (4/6)	N
112	52 / F	mixed	37 †	N	-	-	N	-	-
114	42 / F	spindle	83 †	N	-	-	N	-	-
115	26 / M	spindle	108	N	-	N	N	-	N
138	34 / F	mixed	99	N	-	N	gain 21q22*	-	N
151	48 / F	spindle	92	N	N	loss 18p11.3	gain 21q21 & 22	N	gain 21q11.2
152	76 / F	epithelioid	26 †	# gain 18p* and loss 18q12 & 22	N	N	N	N	N
157	72 / F	mixed	27	N	N	N	gain 21q	N	gain 21q22.3
158	61 / M	spindle	48 †	N	N	N	gain 21q22*	N	gain 21q22
162	54 / M	mixed	25 †	N	-	N	gain 21q22.3*	-	N
173	51 / F	epitheiloid	70	N	-	N	gain 21q22*	-	N
174	59 / F	spindle	91	loss 18	-	loss 18	gain 21q	-	gain 21q

EOM	Age/sex	Cell type	Follow-up	Chromosome 18			Chromosome 21		
				CGH	Karyotype	MLPA	CGH	Karyotype	MLPA
177	58 / M	spindle	34 †	N	N	N	N	N	N
181	47 / M	spindle	86	N	-	-	N	-	-
184	70 / F	mixed	16 †	N	-	N	N	-	gain 21q11
187	65 / M	epithelioid	31 †	N	N	N	gain 21q	N	N
191	46 / F	spindle	46 †	N	N	N	gain 21q22.3*	gain 21p11	N
193	44 / M	epithelioid	42 †	gain 18p*	N	N	gain 21q22.3*	N	N
199	64 / F	spindle	48 †	N	N	-	N	N	-
200	45 / F	epithelioid	40 †	N	N	-	N	N	-
202	65 / M	spindle	71	N	N	-	N	N	-
206	73 / F	mixed	64	gain 18p*	-	N	N	-	N
209	67 / M	spindle	68	N	-	N	N	-	N
218	60 / M	spindle	60	N	N	N	gain 21q22*	loss 21	N
237	77 / M	spindle	32 †	gain 18p*	N	gain 18q23	N	N	N
244	53 / F	spindle	67	N	-	gain 18q23	gain 21q22.3*	-	N
253	74 / M	epithelioid	6 †	gain 18p*	gain 18 (3/13)	n	gain 21q22.3*	N	N
257	87 / M	spindle	12 ?	N	N	gain18q11.2	gain 21q22.3*	N	gain 21q22.3
262	64 / F	epithelioid	19 †	N	N	N	gain 21q22*	N	gain 21q21.1
265	40 / M	spindle	48	N	N	-	N	N	-
270	65 / M	spindle	24 †	N	N	-	N	N	-
272	52 / F	spindle	30	N	N	-	N	N	-
274	42 / M	spindle	44	N	N	-	N	N	-
277	46 / M	mixed	42	gain 18p*	-	gain 18q23	gain 21q22.3*	-	N
279	48 / M	spindle	44	N	-	N	gain 21q22.3*	-	gain 21q22.3
283	44 / F	epithelioid	40	N	-	-	N	-	-

+ ciliary body

† melanoma-related death
‡ metastases present
? cause unknown

*small portion above centromere
labeling switch

*(sub-)telomeric region

White et al. found 18q11.2 to be the most important minimal common region (30% > copy number abnormalities < 70%) with a poor prognosis. Sisley et al. described apparently balanced translocations of chromosome 18 in three UMs.[9] Chromosome 18 is not often involved in malignancies. In colorectal cancer, the DCC-gene on 18q21.2 is associated with a poor prognosis.[10] Chromosomal region 21q11.2 is assumed to harbor tumor-suppressor genes and loss of this region is found in several tumors.[11]

Chromosome 18q11 might be involved in prolonged survival (survival cohort of 9-years or more), but is of less importance in discriminating high-risk patients in early disease, in which monosomy 3 and simultaneous loss of chromosome 1p are of predictive value.[4] Even White et al. found the strongest multivariate prognostic factor to be concurrent loss of chromosome 1p33 and gain of chromosome 18q11.2, which suggests a modest role for chromosome 18q11.2. In the present group of 46 tumors, presumably concurrent loss of chromosome 1p and chromosome 3 was not identified as a significant prognostic factor, because of the small number of patients.

White et al. suggested that CGH did not detect monosomy 3, because of its presence in < 20% of UM cells. In the present study, CGH did identify monosomy 3; in addition, FISH usually identifies monosomy 3 in 80-90% of nuclei (data not shown). In our 46 UMs, we confirmed the following classic parameters with CGH: loss of 1p, monosomy 3, loss of 6q and gain of chromosome 8q. Gain of 18q11.2 was detected in three UMs by MLPA, but could not be confirmed by FISH. Gain of 21q11.2 was confirmed by MLPA and FISH in three UMs. MLPA is a novel technique for UM, one that efficiently identifies chromosomal aberrations in fresh as well as archival tumor material. The prognostic significance of chromosome regions 18q11 and 21q11 in uveal melanoma, as described by White et al, may be reconsidered.

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Chapter 4b

Expression of *APITD1* Is Not Related to Copy Number Changes of Chromosomal Region 1p36 or the Prognosis of Uveal Melanoma

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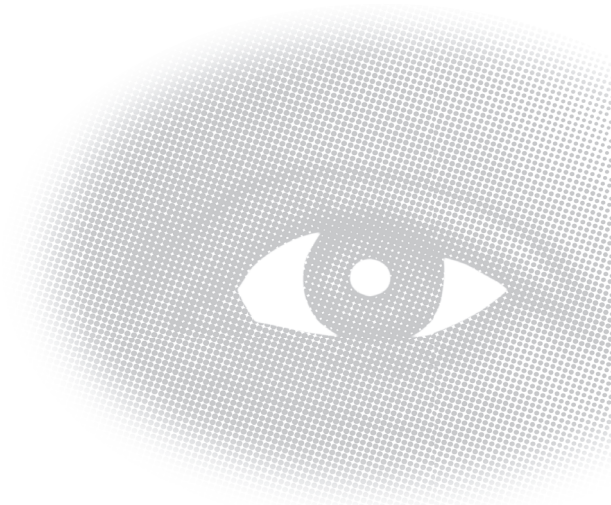

ABSTRACT

Purpose. Concurrent loss of chromosome arm 1p, region 36, and chromosome 3 leads to decreased disease-free survival in patients with uveal melanoma. 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 arm 1p and chromosome 3. Using neuroblastoma cells, which, like uveal melanoma, originate from neural crest cells, a former study showed that *APITD1* has cell growth and/or cell death properties. In this study, *APITD1* expression was analyzed to determine whether it corresponds with the DNA copy number and is related to survival in uveal melanoma.
Methods. To detect whether loss in the copy number of *APITD1* results in lowered mRNA expression of the gene, FISH analysis was combined with real-time PCR. In addition, the effect of *APITD1* expression on survival was studied by 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 chromosome arm 1p, region 36, and chromosome 3.

INTRODUCTION

Uveal melanoma (UM) is the most common primary malignant intraocular tumor in the Western world, with a yearly incidence of six 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 arms 8q and 6p and loss of chromosome 3, the chromosomal arm 6q, and the distal part of chromosome arm 1p are the most frequent.[2,3] Loss of chromosome arm 1p, region 36, 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 1p that were smaller than 1p34-pter, and Hughes et al. identified the 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* (*ap*optosis-*i*nducing, *T*AF9-like *d*omain *1*) positioned at 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 in p53-mediated transcription activation.[8] As p53 is associated with apoptotic cell death and growth arrest, *APITD1* may have a role in tumor suppression. Krona et al.[9] showed that addition of *APITD1* mRNA to neuroblastoma cells results in a reduction of cell growth (up to 90%) compared with nontreated cells, suggesting that *APITD1* indeed has a role in the cell death pathway of neuroblastoma. 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 involved in UM by interfering with the p53 pathway. We have analyzed whether loss of 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 region 1p36 and chromosome 3.

METHODS

Patient samples and cell lines

Fresh tumor tissue was obtained from patients without prior radiation or chemotherapy within 1 hour after primary enucleation. Informed consent was obtained before to enucleation and the study was performed according to the tenets of the Declaration of Helsinki. Tumors were processed for fluorescence in situ hybridization (FISH) and cytogenetic analysis as described previously.[11] Part of the tumor was snap-frozen and stored in liquid nitrogen. In addition, 11 UM-derived cell lines were used. Mel 270, Mel 202, EOM 3, OCM 1 and 92.1 are cell lines derived from primary tumors. OMM 1, -2 and -3 were established from metastases from different patients with UM, and OMM 2.2, -2.3 and -2.6 are all cell lines derived from different metastases of the same patient from whom Mel 270 was also derived.[12-15] Also, two cell lines obtained from normal eye melanocytes , EMC 1 and -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 chromosomes 1 (short arm), 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; 10 with loss of 1p36.33 and 10 with normal copy numbers of this region. All tumors were further analyzed with BAC probe RP11-199O1 mapping to the *APITD1* 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 the University of California at Santa Cruz (http://genome.ucsc.edu/cgi-bin/hgGateway/ provided in the public domain by UCSC Genome Bioinformatics, University of California at Santa Cruz, Santa Cruz, CA) and the National Center for Biotechnology Information (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, 10 metaphases and 100 interphases were analyzed. Abnormal copy numbers were detected in less than 3% 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 three or more signals) were adapted from the available literature.[18]

RNA purification, cDNA synthesis and PCR assay

Five to eight sections with a thickness of 50 µm were made from fresh-frozen tumor tissue, depending on the size of the tumor. RNA was isolated from the sections (RNA-Bee; TelTest Inc, Friendsweed, TX) and cell line RNA was isolated (RNeasy Mini Kit; Qiagen,

Venlo, the Netherlands) according to the manufacturer's protocol. RNA quantity was measured by spectrophotometer (model ND-1000 NanoDrop Technologies, Wilmington, DE) and the quality was assayed (Bioanalyzer 2100; Agilent, Palo Alto, CA). From 1 µg of total RNA cDNA was synthesized using 2 µl of a random hexamer primer (0,5 µg/µl) and 10 U of super-reverse transcriptase (HT Biotechnology, Ltd, Cambridge, UK) according to the manufacturer's instructions. Solutions were diluted to 10 ng/µl for cDNA synthesis. For the PCR, master mix (TaqMan Universal Master Mix; Applied Biosystems, Inc [ABI], Foster City, CA) was used. Gene expression assays were selected for *APITD1* and three reference genes: β-2-microglobulin (*B2M*; 15q21-q22), β-glucuronidase (*GUSB*; 7q21.11) and hypoxanthine phosphoribosyltransferase (*HPRT1*; Xq26.1). Each of these assays consists of two unlabeled primers and a probe labeled with the 5' reporter dye FAM and the 3' quencher TAMRA (ordered from the ABI assays-on-demand platform). Every reaction contained 3.375 µl H_2O, 12.5 µl Master Mix (2x) (without AmpErase UNG; ABI), 2.5 µl of gene expression assay reagent, and 1 µl of cDNA. The reactions were run on a sequence-detection system (Prism 7700; ABI). The solution was subjected to a protocol of 50°C for 2 minutes, 95°C for 10 minutes, and 45 cycles of 95°C for 15 seconds, followed by 60°C for 1 minute.

The efficiency of the PCR 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 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 intratumor 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 10 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 with commercial software (SPSS, ver. 11; SPSS, Chicago, IL).

RESULTS

FISH

Twenty tumors were selected from our UM database on the basis of routine FISH scores on chromosomal arm 1p, region 36. 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 with the expression in the primary tumors ($p<0.001$) (Figure 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 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 probability close to 1 ($p=0.9682$; Figure 2).

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

Tumor	Relative expression of APITD1 (2- DCt 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).

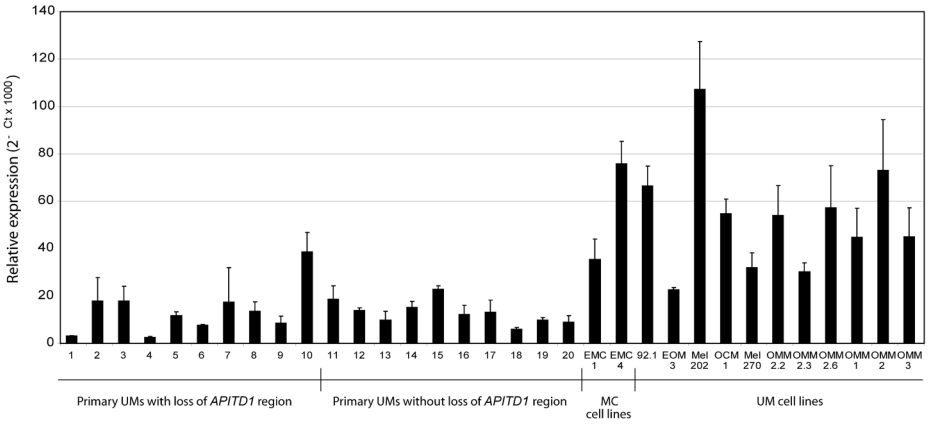

Figure 1. APITD1 expression in UM and MC cell lines and primary UMs. Results of the primary UMs are divided into two groups, based on the number of APITD1 DNA copies. The Y-axis indicates the relative expression of APITD1 compared to the GUSB housekeeping gene, calculated with 2- ΔCt x 1000. The identity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

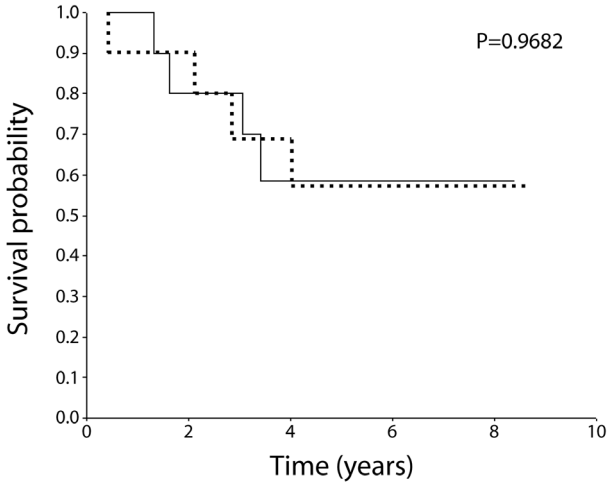

Figure 2. Kaplan-Meier survival analysis of APITD1 expression in 20 uveal melanoma patients. Dashed line: tumors with high APITD1 expression; solid line: tumors with low APITD1 expression. p-value is indicated.

DISCUSSION

A frequent characteristic of UM is deletion of the distal part of chromosome arm 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. Altogether, this evidence suggests a role for these genes at least in high-stage neuroblastoma. [7,9,19-22] In UM, the p53 pathway is not affected through alterations in p53 protein levels.[10] Therefore, interference of the p53 pathway could be caused by another mechanism. Kiliç et al. suggested that p73, a p53 homologue located on 1p36, is a possible prognosis-related suppressor gene.[23] Another candidate gene located on 1p36 is *CHD5*, which encodes a protein that functions in the p53 pathway, and was recently shown to function as a tumor suppressor *in vivo*.[24] The *APITD1* gene in the reported neuroblastoma gene cluster is associated with p53 activity and has been shown to inhibit cell growth.[7,9] Because expression of *APITD1* is almost absent in

a variety of tumors[9] and because of its is relation with p53 activity, downregulation 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 whether a reduced *APITD1* copy number 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 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 (Figure 2). This result indicates 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.[9]

The negative effect on prognosis of loss of 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. After the data in Table 1, we could verify for this group of cases that concurrent loss of 1p36 and chromosome 3 has an adverse effect on patient outcome (log-rank p= 0.0259), which shows 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 1p36. 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.

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Chapter 5

Tumors Generated in the Immune Privileged Ocular Microenvironment Escape Elimination by Downregulating Tumor Antigens

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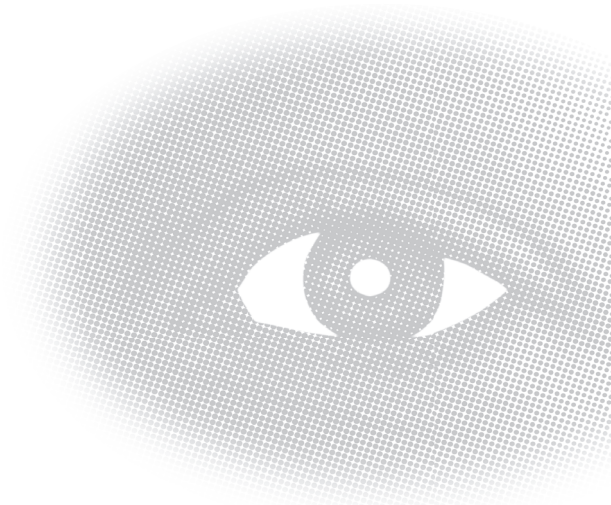


ABSTRACT

Previous studies demonstrated that the immune privileged anterior chamber (AC) of the eye induced a permanent escape phenotype among progressively growing ocular tumors. These eye-derived tumor cells acquired the ability to grow progressively, even when injected into *non*-immune privileged sites in immunized mice. To determine how eye-derived tumors acquired this escape phenotype, we utilized CMS5 fibrosarcomas that express a single tumor-specific antigen (tERK-1) that is recognized by CD8+ Vβ8.3+ T cells from DUC18 transgenic mice. CMS5 tumor cells were inoculated into the AC of BALB/c mice and recovered 10 days later to generate eye-derived CMS5 (eCMS5) cells. Wild type (WT) or eCMS5 cells were injected subcutaneously into the flank of (i) DUC18 or BALB/c naïve mice, or (ii) BALB/c mice adoptively transferred with either tERK-specific, or naïve CD8+ T cells. Expression of MHC Class I was determined by flow cytometry and tERK-1 antigen expression was determined by rt-PCR. WT-CMS5 tumors were universally rejected from the flank of DUC18 mice. Conversely, eCMS5 tumor cells grew progressively in the flanks of (i) BALB/c mice that received DUC18 T cells, as well as (ii) DUC18 mice. The eCMS5 tumor cells expressed normal levels of MHC class I, but failed to express the tERK-1 tumor antigen. We conclude that the ocular environment triggered the loss of the tERK-1 antigen in CMS5 fibrosarcomas, which allowed eye-derived tumor cells to escape immune elimination.

INTRODUCTION

Immune privileged sites, such as the anterior chamber of the eye, harbor an environment in which immunogenic tissues survive for an extended period of time.[1] The anterior chamber establishes immune privilege via: (i) production of soluble factors such as TGFβ2, αMSH, vasointestinal peptide, calcitonin gene related peptide, thrombospondin, somatostatin and indolamine 2,3 deoxygenase (IDO)[2], and (ii) expression of cell surface molecules including Fas Ligand that triggers apoptosis of Fas-expressing lymphocytes[3], B7-2 (CD86) that triggers suppression of CTLA-4 expressing activated lymphocytes[4], PD-L1 that induces apoptosis of PD-1 expressing T cells[5], and complement inhibiting proteins that block activation of the complement cascade.[6, 7] In addition, the ocular environment alters local antigen presenting cells that activate regulatory T cells in the spleen and promote the establishment of a deviant immune response termed anterior chamber associated immune deviation (ACAID).[8-10] Together, these unique ocular immunoregulatory mechanisms allow immunogenic tissue to survive within the anterior chamber of the eye through the suppression of innate and adaptive immunity.

The host benefits from ocular immune privilege by preventing excessive inflammation that could block the visual axis, and destroy irreplaceable ocular tissues. On the other hand, immune privilege may be detrimental to the host by providing a favorable immunosuppressive environment in which pathogens can survive and escape immune elimination.[11, 12] An example of the detrimental aspect of ocular immune privilege is that some tumor cells that are placed within the anterior chamber grow progressively, while a similar inoculation of tumor cells is rapidly rejected from non-privileged sites, such as the flank.[13, 14] The idea that immune privilege is beneficial to cancer cells, and detrimental to the host is supported by data indicating spontaneous tumors forming within *non*-privileged sites establish their own immune privileged microenvironment. Moreover, this tumor-generated privileged environment is similar to the ocular immune privileged environment.[15] Thus, tumors adopt some of the mechanisms used by the eye to escape immune-mediated elimination. In an effort to understand how tumors establish their own immune privilege, we previously examined whether the ocular environment altered tumors growing within the eye. The results demonstrated that P815 tumor cells growing within the anterior chamber developed an escape phenotype that allowed them to grow progressively and escape immune-mediated elimination. Moreover, eye-derived tumor cells that were injected into the flank of immunized mice also escaped immune rejection. Additional experiments demonstrated that eye-derived tumor cells did not trigger proliferation of tumor-specific T cells in vitro and failed to elicit delayed type hypersensitivity in vivo, even though tumor cells expressed high levels of MHC Class I. Together, these data indicate that the ocular environment contains factors that induce an escape phenotype in intraocular tumors by a yet to be determined mechanism.

It is possible that eye-derived P815 tumors escape elimination by downregulating the expression of tumor antigens. However, it was difficult to assess the importance of individual antigens on P815 tumor cells, since they express multiple tumor antigens.[16] Therefore, the current study was performed with CMS5, a methylcholanthrene-induced murine fibrosarcoma that expresses a single tumor-specific antigen recognized by a single population of CD8+ cytotoxic T cells. CMS5 expresses a mutated ERK-2 kinase antigen tERK-1 (tumor-derived extracellular signal-regulated kinase-1), which binds to H-2Kd Class I.[17-19] Fifty to eighty percent of the CD8+ T cells in DUC18 transgenic T cell mice express high levels of the Vβ8.3+ T cell receptor that recognizes tERK-1 presented by H-2Kd Class I. These tumor-specific CTL are capable of rapid and specific elimination of subcutaneously growing CMS5 tumors.[20] Using the CMS5/DUC18 murine tumor model, we determined whether the ocular environment was capable of downregulating expression of tumor antigens and inducing a tumor escape phenotype that was able to avoid elimination outside of the ocular environment.

MATERIALS AND METHODS

Animals

BALB/c mice were purchased from Taconic (Albany, NY). Male and female DUC18 transgenic mice were generated from breeder pairs kindly gifted by Dr. P. Allen (Washington University, St. Louis, MO). Briefly, the V-Jα and V-D-Jβ exon cassettes were cloned into TCR α and β shuttle vectors and were coinjected into BALB/c pronuclei. Resulting DUC18 mice were screened for co-integration of TCR α and β vectors by PCR. TCR expression was screened by FACS analysis. DUC18 transgenic mice were identified by PCR amplification of the C18β transgene from tail DNA and backcrossed to BALB/c mice. All mice were housed and maintained in a specific pathogen free facility at the Schepens Eye Research Institute. All mice were treated according to the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research. The Schepens ACUC committee approved all mouse experiments.

CELL LINES

CMS5 is a methylcholanthrene-induced fibrosarcoma of BALB/c origin with a single base pair mutation in the ERK2 gene resulting in the expression of a unique tumor-rejection antigen (tERK-1) that is presented by H-2K^d class I molecule.[17-19] "*Eye-derived*" CMS5 tumor cells (eCMS5) were generated by inoculating 1x10^6 CMS5 tumor cells into the anterior chamber of BALB/c mice. Ten days post inoculation, tumor-bearing eyes were

enucleated and a single-cell suspension was prepared by mechanical dissociation. All tumor cell lines were cultured in DMEM (BioWithaker), 10% Fetal Bovine Serum, penicillin (100U/ml), streptomycin (100μg/ml) and incubated at 37°C and 10% CO_2.

Adoptive transfer of splenocytes

DUC18 splenocytes from homozygous transgenic mice were prepared in a single suspension with serum-free HBSS. Vβ8.3, CD8 double-positive cells were identified by FACS analysis to calculate the number of transferred tumor specific DUC18 splenocytes in each experiment. Naïve BALB/c splenocytes were used as a negative control. Spleen cells (3×10^4 Vβ 8.3^+ / $CD8^+$ T cells) were suspended in 0.1 ml of serum free HBSS and injected intravenously into the tail vein.

Anterior chamber tumor cell injections

Tumor cell inoculation into the anterior chamber of the eye was performed as previously described.[21] Briefly, mice were anesthetized by a 0.15 ml intraperitoneal injection of a mixture of ketamine, xylazine and saline in a 1:1:6 ratio, respectively. Anesthetized mice were given an anterior chamber injection of $1x10^6$ CMS5 tumor cells in a volume of 2 μl serum-free HBSS. Tumor mass was assessed by slit lamp observation and recorded using an intraocular tumor growth scoring system.

Subcutaneous inoculations

Mice were anesthetized by a 0.1 ml intraperitoneal injection of a mixture of ketamine, xylazine and saline in a 1:1:6 ratio, respectively. The injection site was shaved and $1x10^6$ tumor cells suspended in 0.2 ml HBSS were subcutaneously administered. Tumor mass was measured horizontally and longitudinally and averaged to yield a mean tumor diameter. Growth measurements were taken 3 times per week.

Histology

The growth of CMS5 tumors in the anterior chamber of the eye was documented using clinical observations that were corroborated by histological analysis of tumor growth. Enucleated tumor-bearing eyes were placed in paraformaldehyde and embedded in paraffin. Serial paraffin sections of tumor-bearing eyes were stained with haemotoxylin and eosin.

Flow Cytometry

Single-cell suspensions prepared from spleen and lymph nodes were stained with both 53-6.7-R-PE (rat anti-mouse CD8a antibody; PharMingen) and 1B3.3-FITC (hamster anti-mouse Vβ8.3 antibody; PharMingen) to identify double-positive lymphocytes. Freshly isolated tumor cell suspensions were stained with 553564-Biotin-conjugated (mouse

anti-mouse H-2K^d antibody PharMingen) to determine H-2Kd class I expression. The respective directly conjugated isotype control antibodies were used as negative controls to determine the gates for identifying positively stained cells. 50,000 events were collected per sample to identify cell populations.

Reverse Transcription and Polymerase Chain Reaction

Total RNA from tumor cells was isolated with RNAzol and used as a template for cDNA synthesis using an iScript cDNA kit (BioRad). Tumor cDNA was amplified in a PCR reaction mix consisting of, PCR buffer (50mM KCl, 10mM Tris±HCl, pH 9,1% Triton X-100), 2 mM $MgCl_2$, 0.5 mM dNTP, tERK-1 primers: 0.4 mM of forward (TTGGCATCAATGACAT) and reverse (TGTGGCTACGTACTCTGTC), and 1 unit of Taq polymerase. PCR conditions used were 94°C for 5 min., 35 cycles of (94°C for 30 sec, 55°C for 30 sec. and 72°C for 1 min) with a final extension at 72°C for 5 min. The PCR reaction generated a 320-bp product from both tumor associated ERK (tERK) and normal ERK cDNA.

*Sfc*I restriction endonuclease digestion of PCR-generated tERK-1 / ERK cDNA

To distinguish between tERK and normal ERK DNA, PCR products were digested with the SfcI restriction endonuclease, which cleaves tERK into 161bp and 159bp fragments but not wild type ERK PCR product (320bp). Twenty microliters of PCR product was mixed with 10 units of SfcI restriction endonuclease and incubated at 25°C for 18 hours. Enzyme activity was inactivated by incubation at 65°C for 20 min. Products were separated on a 2% agarose gel by electrophoresis and visualized by GelStar (Cambrex) staining.

RESULTS

Eye-derived CMS5 tumors escape immune elimination

The current study determines whether the ocular environment conferred a tumor escape phenotype to CMS5 fibrosarcomas. In order to obtain eye-derived CMS5 cells, naïve BALB/c mice received an anterior chamber injection of tumor cells. Ten days later, tumor-bearing eyes were enucleated and eye-derived CMS5 tumor cell lines were established in vitro. Protective immunity was adoptively transferred to BALB/c mice by a single intravenous injection of 3 x 10^4 DUC18 (Vβ8.3, CD8+) T cells. As a negative control, mice received a similar number of naïve BALB/c T cells. Mice then received a subcutaneous flank injection of either 1 x 10^6 WT-CMS5 cells or a similar number of eye-derived CMS5 cells. Adoptive transfer of DUC18 T cells eliminated WT-CMS5 flank tumors by day 15, while transfer of naïve BALB/c T cells resulted in progressive tumor growth (Figure 1A). This confirms the adoptive transfer of DUC18 T cells conferred protective immunity. However, a similar number of DUC18 T cells were unable to completely eliminate the

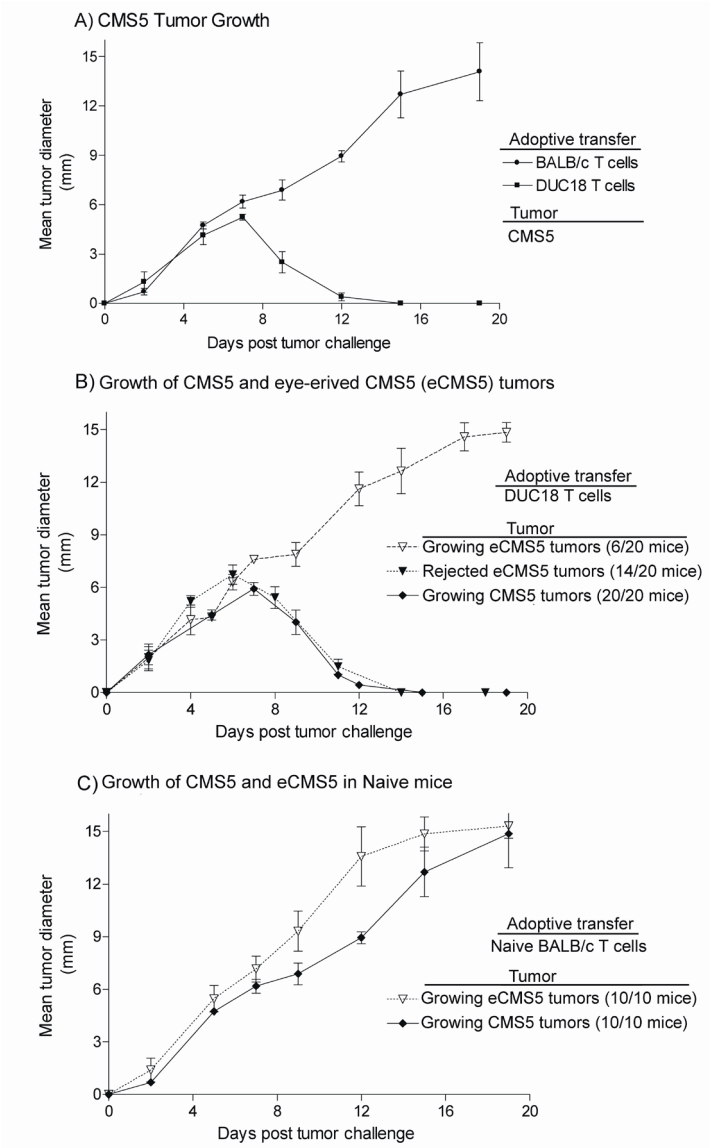


Figure 1. Subcutaneous growth of CMS5 tumor cells in BALB/c mice receiving adoptively transferred T cells. (A) DUC18 T cells confer protective immunity. BALB/c mice received an adoptive transfer of 3 x 10^4 splenic CD8+ T cells from either BALB/c or DUC18 mice. This was followed by a subcutaneous flank injection of WT-CMS5 tumor cells (1x10^6 cells). Tumor mass was measured horizontally and longitudinally and averaged to yield the mean tumor diameter (± SEM). All graphs represent the results of at least two independent experiments. (B) Eye-derived tumor cells escape immune rejection. BALB/C mice received an adoptive transfer of 3 x 10^4 DUC18 (Vβ8.3+, CD8+) T cells followed by a subcutaneous flank injection of either WT-CMS5 or "eye-derived" eCMS5 tumor cells (1x10^6 cells). (C) Growth of WT-CMS5 and eCMS5 in naïve mice. BALB/c mice received an adoptive transfer of 3 x 10^4 naïve splenic CD8+ T cells from BALB/c mice. This was followed by a subcutaneous flank injection of either WT-CMS5, or eCMS5 tumor cells (1x10^6 cells).

eye-derived CMS5 tumors, which grew progressively in thirty percent of the mice (6/20 mice) (Figure 1B). The remaining mice rejected the eye-derived tumors within 15 days. Growing CMS5 tumors in the anterior chamber of the eye did not alter the proliferation of the tumor cells, since eye-derived and wild-type CMS5 tumors grew at equal rates in mice receiving naïve BALB/c T cells (Figure 1C).

A trivial explanation of why the eye-derived tumors grew progressively within the flank of BALB/c mice is that an insufficient number of DUC18 T cells were adoptively transferred into the recipient mice. To determine if complete rejection of eye-derived CMS5 tumors was achieved with a prolonged presence of a higher concentration of DUC18 T cells, we performed a second series of experiments in which either wild-type or eye-derived CMS5 tumor cells were injected directly into the flank of DUC18 mice that possess a high frequency of transgenic DUC18 T cells (50% to 80% of CD8+ T cells). As expected, WT-CMS5 tumor cells were rejected in 100% of the DUC18 mice within 15 days post inoculation (Figure 2). However, once again the eye-derived CMS5 tumor cells grew progressively in the flank of 50% of the mice (15/30 mice). Together, these results indicate that the ocular environment of a naïve BALB/c mouse induces a tumor escape phenotype in CMS5 tumor cells that allows them to avoid elimination by DUC18 CD8+ T cells. Moreover, the escape phenotype of eye-derived CMS5 tumor cells is an intrinsic characteristic of the tumor cells that is stable and permanent even after to the tumor cells are removed from the eye. The frequency of the escape phenotype among eye-derived tumor cells may be a consequence of the clonal advantage of tumor cells expressing this unique phenotype within the eye.

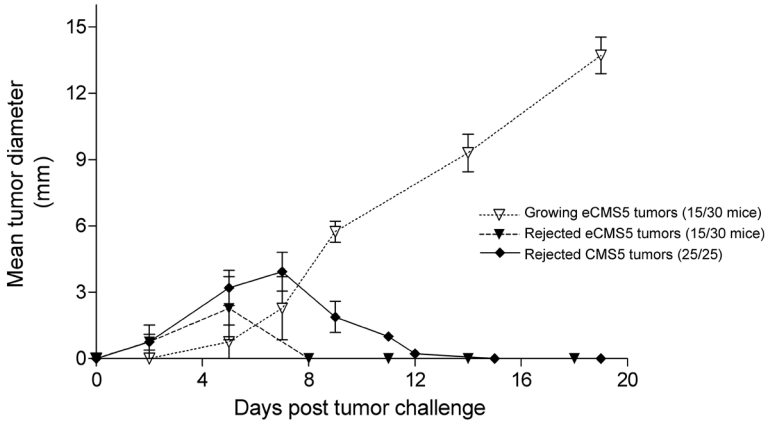


Figure 2. Subcutaneous growth of CMS5 tumor cells in DUC18 mice. DUC18 mice received a subcutaneous inoculation with either WT-CMS5 or eCMS5 tumor cells ($1x10^6$ cells). On the designated days, the tumor mass was measured horizontally and longitudinally and averaged to yield the mean tumor diameter (± SEM).

CMS5 tumors in the anterior chamber of DUC18 mice

Tumor escape mutants are frequently produced when mutant cells benefit from a clonal advantage that is conferred by selective T cell pressure that eliminates wild-type, but not mutant tumor cells. However, in the previous experiments the escape phenotype was produced in eye-derived CMS5 tumors growing in the anterior chamber of *naïve* BALB/c mice that lacked tumor-specific DUC18 T cells. In the next series of experiments, we determined whether the presence of DUC18 T cells provided a selective advantage for the growth of tumor escape mutants within the anterior chamber of the eye. We compared the growth of CMS5 tumor cells (1 x 10^6 cells) injected into the anterior chamber of either DUC18 or BALB/c mice. As a control to assess tumor growth and rejection, an identical number of tumor cells were injected into the flank of DUC18 and BALB/c mice. Tumor growth was measured in the flank by mean tumor diameter. Ocular tumors were assessed by slit lamp examinations using a clinical staging system that was corroborated by histological sections of tumor-bearing eyes (Figure 3 and 4). As expected, by day 16, there was a dramatic difference between the size of flank tumors in DUC18 mice (no tumors detected) and BALB/c mice (12 mm tumors) (Figure 5A). By contrast, at day 16 there was *no difference* between the size of tumors in the anterior chamber of DUC18 mice and BALBc mice (Figure 5B) and both were at Stage IVa/b (Figure 3). Even in the presence of DUC18 T cells, tumor-containing eyes were proptotic and tumor cells filled the anterior chamber. For the next two weeks, ocular tumors in BALB/c mice continued to grow progressively with no signs of rejection. However, ocular tumors in DUC18 mice slowly regressed and were eliminated completely by day 30 post inoculation. Tumor rejection coincided with ocular "phthisis" in which the eye was destroyed and replaced by a fibrotic scar. These results indicate that, while there is a transient tumor escape from DUC18 T cells in the anterior chamber, ultimately ocular tumors are eliminated and there is no immune-mediated selective advantage.

MHC Class I expression by CMS5 tumor cells

One of the most common mechanisms that tumors use to escape adaptive immune-mediated elimination is downregulation of MHC Class I.[22-24] To determine whether escape of eye-derived CMS5 tumor cells is due to loss of MHC Class I, MHC expression on wild type and eye-derived CMS5 tumor cells was analyzed by flow cytometry. Of particular interest was H-2K^d Class I expression, since the tERK tumor antigen is presented exclusively by this Class I molecule. Staining of eye-derived CMS5 and wild-type CMS5 tumor cells with MHC Class I K^d, L^d and D^d molecule specific antibodies demonstrated that wild-type and eye-derived CMS5 tumor cells expressed similar levels of all three MHC Class I molecules (Figure 6A-C). Our results demonstrate that the escape phenotype of eye-derived CMS5 tumors is not due to loss of MHC Class I expression.

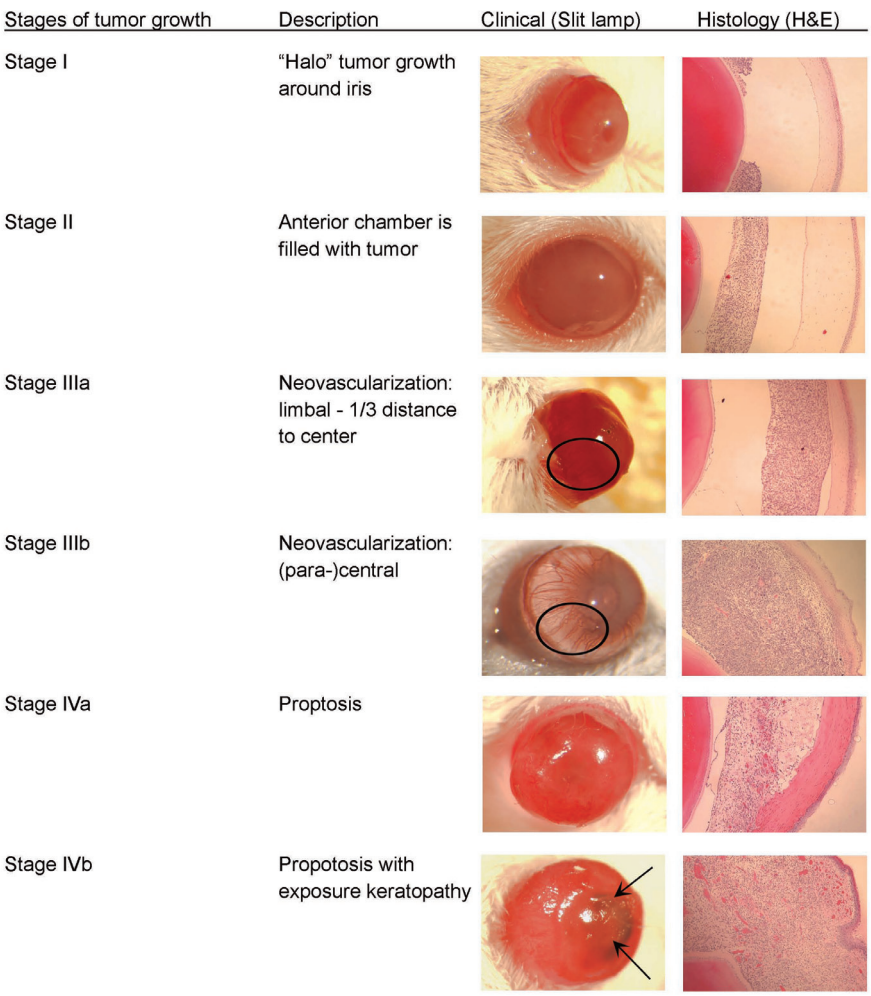


Figure 3. Stages of intraocular tumor growth. A scoring system was developed using slit lamp examination of tumor-containing eyes that was validated by examination of corresponding H&E serial sections. DUC18 or BALB/C mice received an anterior chamber injection of $1x10^6$ CMS5 tumor cells. Data is shown for DUC18 mice, but the results were identical for BALB/c tumor-containing eyes. Stage I: Halo of tumor cells in the anterior chamber, the tumor cells reside on the iris. Stage II: Tumor cells filling the anterior chamber with invasion of lymphocytes and macrophages, plus formation of a few small vessels. Stage IIIa: Scleral neovascularization up to the limbus and invasion of tumor cells into the ciliary body (not shown here) plus accumulation of lymphocytes. Stage IIIb: Central corneal neovascularization with tumor cells invading the cornea. Stage IVa: Proptosis of the bulbus with rearrangement of the corneal layers histologically. Stage IVb: Proptosis with exposure keratopathy, histological destruction of the corneal stroma by tumor and inflammatory cells and vascularization.

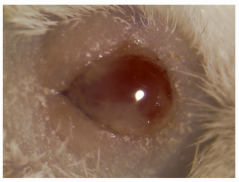
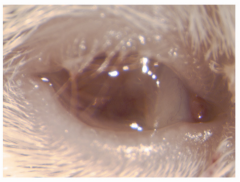
Stages of rejection	Description	Clinical (Slit lamp)
Stage I	Eye lids closed and swollen	
Stage II	Eyelids open and swollen	
Stage III	Phthisis	

Figure 4. Stages of intraocular tumor rejection. A scoring system was developed to document rejection of intraocular tumors. Histological sections were not obtained from these eyes, since it was not technically possible to enucleate enact tumor-containing eyes from these mice. Stage I: Closed eye lids are the first stage of tumor regression, followed by, Stage II: Swollen and thickened eye lids, and ultimately, Stage III: Phthisis, occurs when the eye is completely destroyed and replaced by a fibrotic scar.

tERK-1 tumor antigen is downregulated in eye-derived CMS5 tumor cells

To determine whether eye-derived CMS5 tumors escape elimination through loss of the tERK-1 tumor antigen, we examined the expression of tERK-1 on wild-type and eye-derived CMS5 tumor cells using tERK-1 specific reverse transcription (RT)-PCR as originally described by Matsui et al.[25] To identify tERK-1 gene expression, we relied on the existence of a single base pair mutation in the tERK-1 gene that caused a single amino acid difference between the tERK-1 protein and the original ERK-2 protein. This single point mutation creates a *Sfc*I digestion site in the tERK-1 PCR product, so that treatment of the PCR product with the *Sfc*I restriction endonuclease yields two bands (161 bp and 159 bp) as visualized by agarose gel electrophoresis. If tERK-1 is absent, a single 320 bp band will be present. The results reveal that wild-type CMS5 tumor cells display a single 320 bp band that is cleaved by *Sfc*I into 161bp and 159bp bands to reveal that tERK-1 is present (Figure 6D, lanes A and B). By contrast, in two eye-derived tumor cell lines that grew progressively in the flank of DUC18 mice, the PCR product was not cut by *Sfc*I, indicating that tERK-1 was absent (Figure 6D, lanes D and F). These results demonstrate that eye-derived CMS5 tumor cells that grow progressively in the flank of DUC18 lost expression of the tERK-1 tumor antigen.

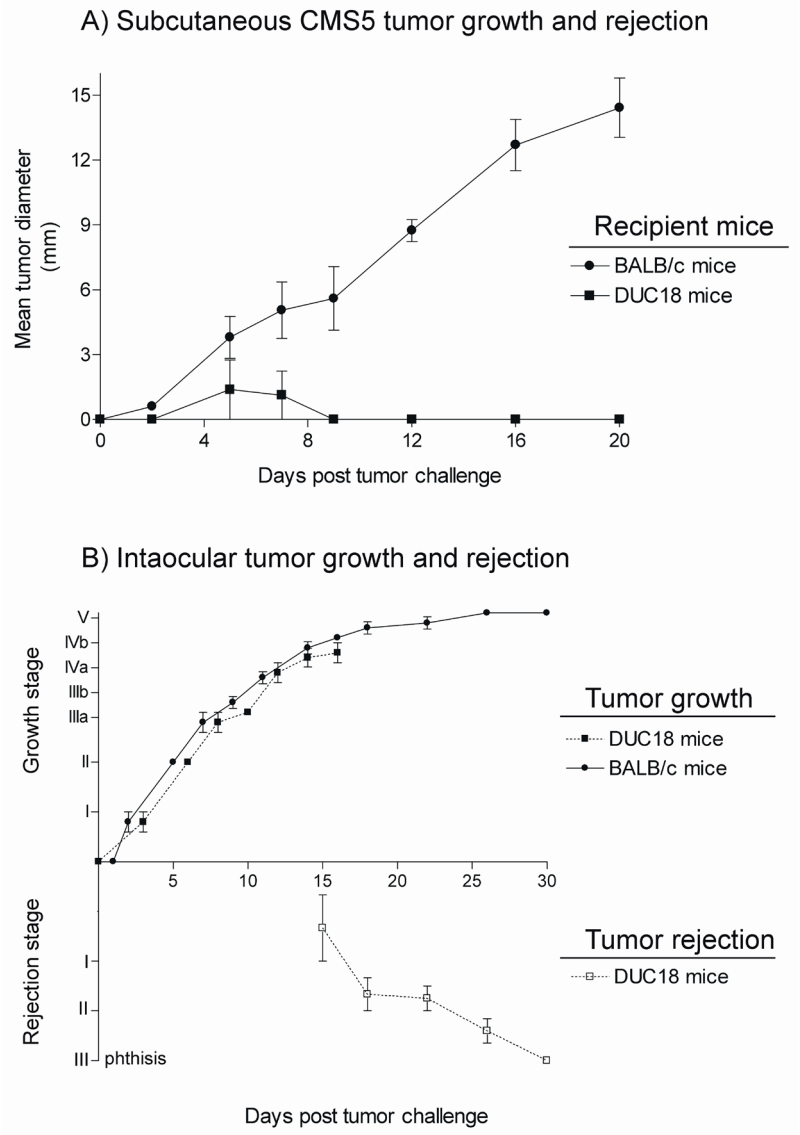


Figure 5. Prolonged growth of WT-CMS5 in the anterior chamber of DUC18 mice. (A) WT-CMS5 tumor cells ($1x10^6$ cells) were injected into the subcutaneous tissues of the flank in either BALB/c, or DUC18 mice. Mean tumor diameter was measured on the designated days post inoculation. (B) WT-CMS5 tumor cells ($1x10^6$ cells) were injected into the anterior chamber of either BALB/c, or DUC18 mice. The stages of tumor growth and rejection were determined via slit lamp examination on the designated days post inoculation.

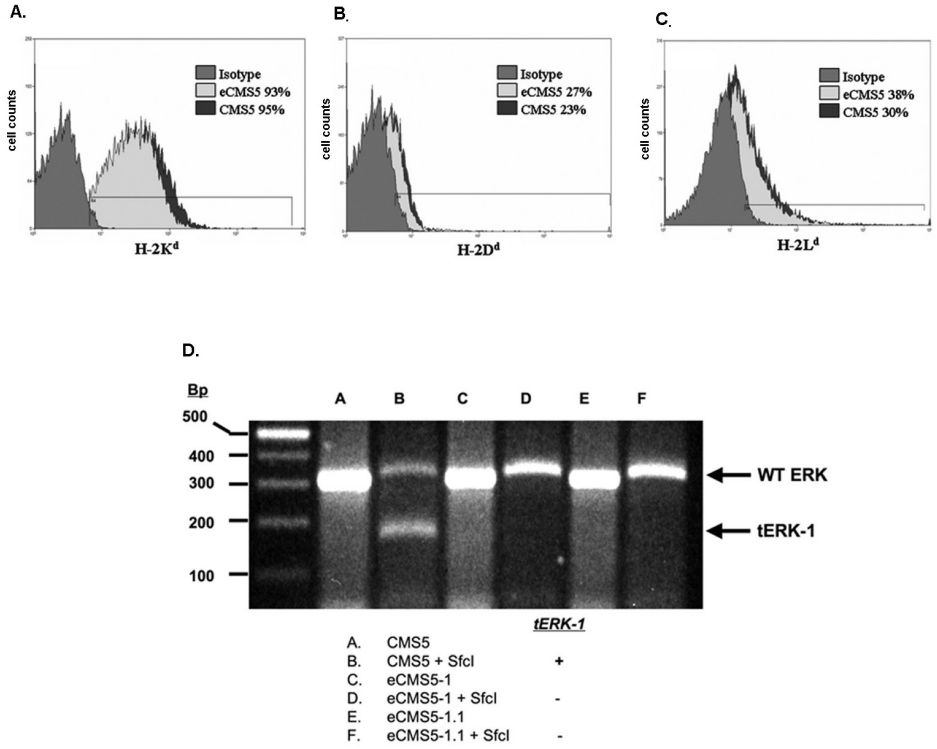


Figure 6. Expression of MHC Class I and tERK-1 tumor antigen in WT-CMS5 and eCMS5 tumor cells. Wild-type and eye-derived CMS5 tumor cells were staining with antibodies specific for either: (A) H-2Kd, (B) H-2Dd, or (C) H-2Ld. Tumor cells were analyzed by flow cytometry. As a negative control to determine the background fluorescence, tumor cells were stained with the appropriate isotype control antibodies. (D) Wild-type and eye-derived CMS5 tumor cell DNA was examined for the presence of either the tERK-1 tumor antigen or the normal ERK gene. To distinguish between tERK-1 and ERK PCR products, PCR products were either (i) undigested, or (ii) digested with the SfcI restriction endonuclease. SfcI cleaves tERK-1 into 161 bp and 159 bp fragments but does not cleave the wild type ERK PCR product (320bp).

DISCUSSION

The development of immune escape mutants is an important process during malignant transformation, since during the earliest stages of tumor formation malignant cells must develop mutations that allow them to escape immune surveillance (i.e. escape mutants).[26-28] However, tumors that avoid surveillance mechanisms and develop into progressively growing tumors are still capable of expressing tumor antigens that are recognized by specific T cells.[29] Development of escape mutant tumor cells is also important in the application of immunotherapies in cancer patients that target tumor-specific antigens. These therapies can become ineffective when, once again, tumors develop immune escape mutants.[30, 31] Therefore, understanding the escape mechanisms used

by tumors is an important issue in cancer immunology. Our current and past studies indicate that the immune privileged environment within the eye possesses a unique ability to trigger the development of immune escape mutants within tumors. Previously, we observed that P815 mastocytoma tumors growing within the immune privileged anterior chamber of the eye acquire immune escape mutants that develop: (i) rapidly within 7 days, (ii) in the absence of tumor-specific T cells, and (iii) express permanent and stable mutations.[21]

In order to study the mechanism of immune escape in more detail, in the present studies we determined whether an immune escape mutant was induced when CMS5 tumor cells that express only a *single* tumor antigen are placed into the anterior chamber of the eye. CMS5 is a methylcholanthrene-induced fibrosarcoma that expresses a mutated ERK-2 kinase antigen, tERK-1 (tumor-derived extracellular signal-regulated kinase-1) that is presented by H-2K^d Class I and recognized by Vβ8.3 CD8+ cytotoxic T cells.[18] DUC18 transgenic T cell mice possess a high frequency of Vβ8.3 CD8+ T cells (50% - 80%).[20] Our results indicate that the ocular environment induces changes in CMS5 tumor cells that grow within the anterior chamber of naïve syngeneic BALB/c mice. When these eye-derived tumor cells were recovered, they now escaped elimination and grew progressively when injected into the flank of BALB/c mice that received an adoptive transfer of DUC18 T cells, indicating that immune-escape mutants had developed. The failure to eliminate the eye-derived tumor cells was not due to an insufficient transfer of protective T cells, since in the presence of the same number of transferred T cells, non-eye derived tumor cells were eliminated from the flank of BALB/c mice. We showed that immune escape was not due to the loss of Class I on the tumor cells, but coincided with a loss of the tERK-1 tumor antigen. Our data indicate the ocular environment is capable of inducing a tumor antigen-loss immune escape mutant.

What was most unexpected about our findings was that the immune escape mutant tumor cells developed in the *absence* of selective immune pressure. In general, there are two necessary stages in the development of an immune escape tumor: (i) acquisition of a mutation that blocks immune elimination, and (ii) immune selective pressure that provides a growth advantage to the escape mutant. Selective pressure is important, since it allows a small subpopulation to become the dominant cell population in the tumor. This is clearly illustrated by in vitro experiments where tumor-specific cytotoxic T cells that eliminate less than 100% of the target tumor cells are capable of providing selective pressure and a growth advantage to tumor escape mutants.[32] In vivo, animal models indicate that immune surveillance provides selective pressure that reduces the immunogenicity of progressively growing tumors.[32] In cancer patients, immunotherapy has also demonstrated clearly that a specific immune response provides selective pressure for the development of tumor immune escape mutants.[30, 31] In all of these examples, the immune escape mutants only developed when an active immune response (either

innate and/or adaptive) provided selective pressure that conveyed a growth advantage to the escape mutant. By contrast, in our experiments immune escape tumor cells developed within the immune privileged anterior chamber of *naïve* BALB/c mice in the *absence* of any selective immune pressure. In addition, the escape mutants developed rapidly, within 10 days after injection into the eye. As far as we know, this is the first example of a tumor antigen-loss escape mutant developing in the absence of immune selective pressure.

It was originally believed that CMS5 tumors were highly resistant to the formation of immune escape mutants, since no antigen-loss variants were detected by either (i) forced passage of high doses of tumor cells in immunized mice, or (ii) repeated in vitro exposure of tumor cells to specific cytotoxic T cells.[18] However, Matsui et al detected tERK-1 loss mutants in mice that received a large dose of tumor cells (6 x 10^6 cells) in the flank of BALB/c mice that eight days later received 1-3 intravenous infusions of 20 x 10^6 DUC18 T cells.[25] Tumors initially regressed, but antigen-loss mutants appeared 20 days later. It was possible to prevent the development of the antigen-loss mutants by increasing the number of DUC18 T cell infusions ($\geq$4 infusions), suggesting that the ratio of tumor cells to specific T cells is critical in determining whether immune escape mutants develop. This is supported by the fact that when CMS5 tumor cells are injected into DUC18 transgenic mice that possess high concentrations of tumor-specific T cells the tumors are always rejected, even when the highest tumor dose possible is injected (30 x 10^6 cells). Rejection is complete and tumors neither recur, nor form escape mutants. By contrast, when we injected a small number of tumor cells (1 x 10^6 cells) into the anterior chamber of DUC18 mice, even though this small tumor burden should be easily eliminated by the high concentration of tumor-specific cytotoxic T cells, there was no evidence of tumor rejection for the first two weeks, suggesting that antigen-loss mutants are present. These tumors were ultimately rejected and we predict this is due to bystander elimination of the antigen-loss mutants.[33] It is interesting to note that previous studies indicated that CD8+ T cells typically mediate rejection of intraocular tumors with minimal destruction of bystander tissue.[34] However, DUC18 rejection of CMS5 tumors triggered phthisis that is typically associated with high bystander killing that coincides with CD4+ T cell mediated inflammation.[34]

How do the immune escape antigen-loss mutants develop within the eye in the absence of immune selective pressure? One possible explanation is that the ocular environment has *non*-immune mediated surveillance mechanisms that exert selective pressure on CMS5 tumor cells. Although there are currently no reports of non-immune surveillance within the eye or other immune privileged sites, there are several mechanisms reported for tumors growing in conventional (non-immune privileged) sites.[35] These surveillance mechanisms include: control of DNA repair[36], regulation of apoptosis[36], cell contact inhibition[37], and epigenetic gene regulation.[38, 39] Feinberg and coworkers provided recent

evidence that loss of an epigenetic regulatory mechanism (parental imprinting) increased the risk of intestinal tumors. Their data suggest there are surveillance mechanisms that monitor the status of epigenetic DNA changes in an effort to reduce the incidence of cancer. On the other hand, the downside of this surveillance mechanism would be that it applies selective pressure for the development of tumor escape mutants that survive by altering epigenetic gene regulation. Recent data from our laboratory supports this hypothesis and suggests that immune escape mutants in eye-derived tumors are due to epigenetic gene regulation. Eye-derived P815 murine mastocytoma tumors display upregulation of de novo methyltransferases and demethylation of tumor cells prevents immune escape (manuscript in preparation).

In summary, this report describes the development of CMS5 immune escape mutants that develop within tumors that grow within the immune privileged anterior chamber of the eye. Exposure to the ocular environment triggers formation of tERK-1 antigen-loss mutants that cannot be lysed by specific cytotoxic T cells. The eye represents an extreme environment where immune privilege severely inhibits the induction and expression of innate and adaptive immunity.[40] This environment may similarly affect the behavior of non-immune cells when placed in the eye. Therefore, the eye provides a unique opportunity to study the environmental factors that promote immune and non-immune regulated tumor-cell escape.

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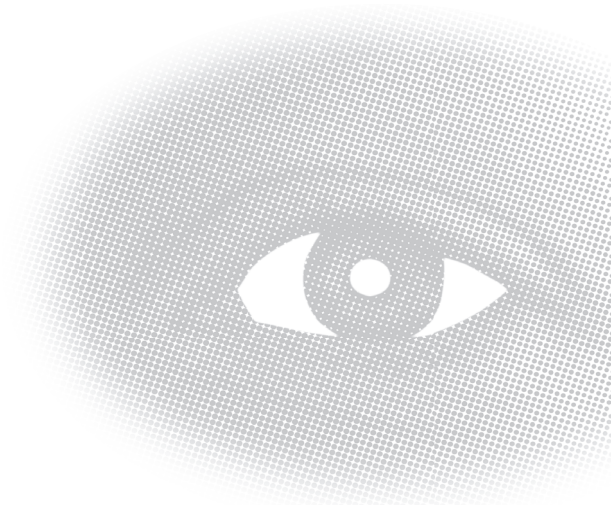
Chapter 6

General Discussion



DISCUSSION

Insights in the molecular changes occurring in uveal melanomas have provided us with the opportunity to assess the risk of metastatic disease for individual patients. Despite these improvements in diagnosis and improved treatment of intraocular melanomas, there has not been an increase in survival in the past decades. Various hypotheses have been put forward to explain the substantial rate of metastasis,[1] but most experts agree that this is attributable to micro-metastases that circulate prior to primary treatment and remain dormant for prolonged periods of time before emerging as clinically detectable metastases.[1, 2] New treatment modalities as adjuvant therapy to eliminate micro-metastases are currently being tested but also improvement in patient selection and the problem of immune-escape of these uveal tumors need to be addressed. The studies described in this thesis shed new light on some of these problems.

1. GENETICS OF UVEAL MELANOMAS

Prognostic parameters

Prognostic factors to identify uveal melanoma patients at risk for metastasizing disease include clinical (tumor location, tumor diameter), histological (cell type, vascular mimicry) and genetic (chromosomal aberrations, gene expression profiling) parameters.[3-5] The chromosomal aberrations will be described in detail below.

It was thought that uveal melanomas have simple karyotypes, but recent work denotes a more complex puzzle of chromosomal aberrations. The chromosomes and chromosomal regions most frequently involved in uveal melanomas are 1p, 3, 6 and 8. Loss of chromosome 3 is the most frequent chromosomal aberration in uveal melanoma and is predominantly found in metastasizing tumors.[6] It is considered to be a primary event, because it is seen in combination with all other chromosomal aberrations in uveal melanomas such as loss of chromosome 1p, gain of 6p and gain of 8q.[7] In the majority of tumors with chromosome 3 loss there is complete monosomy, although in 5-10% of the uveal melanomas isodisomy of this chromosome is acquired (one copy of the chromosome is lost and the remaining copy is duplicated).[5, 8, 9] Single nucleotide polymorphism (SNP)-arrays have elucidated the role of chromosome 3 in hyperdiploid melanomas. In chapter 2 we describe that hyperdiploid melanomas have a high metastatic potential, and SNP-arrays have revealed loss of heterozygosity of chromosome 3, indicating that although genomic instability results in duplication of the genome, one of the allelic copies of chromosome 3 is lost during this process. This might explain why monosomy 3 as well as hyperdiploidy correlate with a poor prognosis, because in both cases only one type allele of the chromosome is present.

In chapter 3b iris melanomas with loss of chromosome 3 are described. Iris melanomas are known to have a low metastatic rate of 7% at 10 years.[10] Surprisingly, loss of chromosome 3 was present in 9/20 iris melanomas, but a correlation with patient survival could not be established. This contrasting result may be attributed -in part- to the differential influence of aqueous and vitreous humor on tumor growth.[11] Tumor location itself is also an independent prognostic factor, with the best survival for iris melanomas and the worst survival for melanomas located in the ciliary body.

Loss of the chromosomal region 1p36 is often found in UM with loss of chromosome 3.[8, 12] Previously, our group described that concomitant loss of chromosome 1p and chromosome 3 correlates with poor prognosis.[13] In the search for candidate genes responsible for the adverse effect of chromosome 1p loss on survival, we encountered a tumor suppressor gene, APITD1. Expression of this gene is affected in neuroblastoma. In uveal melanoma the expression of APITD1 was found not to relate to survival (Chapter 4b). Therefore, the search for candidate genes on the chromosomal region 1p36 is ongoing. An interesting gene in this region is TP73, a p53-homologue, which revealed increased expression of the p73Δex2 trancript in uveal melanomas that lost a copy of chromosome 1p36.[14]

Abnormalities on other chromosomes have also been detected, although they often lead to contradictory results regarding the prognostic impact. Chromosome 18q11 and 21q11 have been suggested to play a prognostic role,[15] but this could not be confirmed by our group as described in chapter 4a. Chromosome 9p21[9, 16] and chromosome 16q[17] have been described to be important in uveal melanoma as well. In chapter 3b chromosome 9p21, a region harboring the tumor suppressor gene CDKN2A that is frequently involved in cutaneous melanoma, has been studied in iris melanoma. Chromosome 9p21 is often lost in iris melanoma, but a correlation with patient survival could not be established. This might be the consequence of small sample size, thus to be sure about the prognostic influence of chromosome 9p in iris melanoma elaboration of the study would be necessary.

Heterogeneity

The majority of human tumors display widespread heterogeneity in many morphological and physiological features, such as expression of cell surface receptors, proliferative and angiogenic potential.[18] Tumor development is often associated with genomic instability and acquisition of genomic heterogeneity,[19] generating both clonal and non-clonal tumor cell populations.[20] Tumor cells mutate to gain a selective advantage in order to survive. In uveal melanoma, morphologic heterogeneity is well recognized with variable proportions of epithelioid and spindle cells. However, there have been few studies on cytogenetic heterogeneity in uveal melanoma.[21, 22] They do confirm the presence of different cytogenetic clones in the tumors. In chapter 3a of this thesis, intra-tumor

heterogeneity of chromosome 3 in uveal melanomas is studied. In some of the tumors, subclones with different percentages of monosomy 3 could be identified. Correlation of the subclones with patient survival did not alter between the clones within the tumor. Our study implies that intra-tumor heterogeneity is present clearly in uveal melanoma and is important to take into account when taking tumor biopsies for diagnostic and prognostic purposes.

Biopsies

One of the challenges of studying clonal heterogeneity in human malignancies is the issue of sampling. The most important aspect of this problem is the availability of representative biopsies. A core biopsy, obtained for diagnostic purposes, samples only small regions of the tumor, and is not likely to be informative about the clonal composition of each tumor as a whole.[18] Adequate analysis of the tumor as a whole -taking into account possible intra-tumor heterogeneity- relies on access to different locations within the tumor. In uveal melanoma, studies reveal that fine-needle aspiration biopsies provide sufficient material for identifying chromosomal aberrations and is a reliable method.[23-25] Depending on the location of the melanoma a transvitreal or transscleral approach can be considered.

2. IMMUNOLOGY OF TUMOR ESCAPE

Patients with large primary uveal melanomas frequently develop lethal metastatic disease. As stated previously, the high metastatic rate of uveal melanomas is probably attributable to micro-metastases that circulate prior to treatment of the intraocular tumor. Molecular testing in uveal melanomas facilitates the identification of high-risk patients for metastases. Yet, it does not really help us understand the tumor behavior. Why do tumors suddenly become aggressive? Why does one tumor metastasize and the other does not? Tumors that grow progressively escape elimination by the immune system, but the mechanisms used by these tumors are still largely unknown.

The understanding of the escape mechanisms used by tumors is an important issue in the immunological field of cancer research. The anterior chamber of the eye is an immune privileged site, in which immunogenic tissues survive for an extended period of time.[26] This environment possesses a unique ability to trigger the development of immune escape mutants within tumors.

During tumor formation malignant cells develop mutations that allow them to escape the cellular immune response.[27-29] The tumors gain heterogeneity with clonal advantages for cell survival. However, tumors that avoid surveillance mechanisms and develop into progressively growing tumors are still immunogenic and capable of expressing tumor

antigens that are recognized by immune effector cells.[30] Therefore, tumors create micro-environments that prevent them from being eliminated, so called immune privileged environments.

The development of immune escape mutants is an important process that allows tumors to grow and it is essential to realize while developing immunotherapies that target tumor-specific antigens in cancer patients. These therapies can become ineffective when tumors develop immune escape mutants.[31, 32]

In cancer patients, immunotherapy has demonstrated clearly that a specific immune response provides selective pressure that induces the development of tumor escape mutants.[31, 32] By contrast, in the animal study described in chapter 5 immune escape tumor cells developed in the absence of any selective immune pressure.

One possible explanation is that the ocular environment has non-immune mediated surveillance mechanisms that exert selective pressure on the tumor cells. Such surveillance mechanisms include: control of DNA repair,[33] regulation of apoptosis,[33] cell contact Inhibition,[34] and epigenetic gene regulation.[35, 36]

These surveillance mechanisms could also apply selective pressure for the development of tumor escape mutants, for example by altering epigenetic gene regulation.

3. TOWARDS PREVENTION OF METASTATIC DISEASE

The combination of genetic information together with studies on cellular immunity will aid in understanding the oncogenesis and metastatic spread of uveal melanomas. Identifying high-risk patients is achieved excellently with genetic parameters such as loss of chromosome 3 or expression class II. Gene expression and SNP arrays are the most sensitive tools for selecting high-risk patients. The immunological aspect of uveal melanomas is of utmost importance in the light of immunotherapies. There are few treatments available for metastasized uveal melanoma and in a relatively short timeframe patients die of their metastases. Improving survival will most likely require adjuvant systemic therapy for patients who are likely to harbor micro-metastasis in order to delay or prevent the progression of preclinical micro-metastases to untreatable macro-metastatic disease.[37]

Intra-tumor heterogeneity is again an important issue. While the primary melanoma can be irradiated or removed by surgery, its metastases cannot. The tumor cells acquire mutations that allow them to escape immune elimination, thereby generating clonal advantages and heterogeneity. Adjuvant therapies need to deal with this heterogeneity of metastatic tumor cell populations.[18]

For more than a decade, clinical expertise with immunotherapy in the treatment of cancer patients is available. Numerous clinical studies have been performed, which include

the use of IL-2, IL-6, IL-12, M-CSF, TNFα, IFNα, and IFNγ. More and more T cell-based and dendritic cell-based vaccines are becoming available.[38, 39]

Immunotherapy aims to activate the immune system to eradicate the tumor cells and to induce specific and long lasting immunity to protect against recurrent disease. The success of immunotherapy relies on several factors. The therapy must be specific, targeting the tumor cells without creating (too much) bystander damage. Multiple targets or a combination of therapies have to be considered in the light of intra-tumor heterogeneity. An overwhelming immune reaction needs to be elicited to overcome tumor-induced immunosuppression and prevent the development of tumor escape mutants. For this reason, the therapies are likely to be more effective in patients with smaller tumor burdens.

Much can be learnt from experience with treating and investigating other cancers such as skin melanomas and solid tumors arising in immune privileged sites. The focus of research in uveal melanoma might need to shift towards adjuvant therapies for high-risk uveal melanoma patients in order to prevent metastatic disease.

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Summary

Uveal melanoma (UM) is the most common primary intraocular malignancy in adults and has a predilection for hematogenous spread to the liver. There is no effective treatment for liver metastases resulting in tumor-related death in about 45% of UM patients. The melanomas can be classified into high-risk and low-risk tumors. High-risk melanomas are aggressive tumors that metastasize to the liver within 5 years, whereas low-risk melanomas have very low metastatic potential. High-risk uveal melanoma patients probably need different management than low-risk patients, with more frequent follow-up visits to be able to detect and treat metastasis at an early stage. In the past decades, survival of uveal melanoma patients has not changed significantly.

There are a number of clinical, histological and cytogenetic parameters that correlate with metastatic disease and thus predict patient survival. Tumor location in the ciliary body, larger size, the presence of epithelioid cells, closed vascular patterns and scleral invasion all correlate with progressive disease. The most important classic genetic parameters that correlate with metastatic disease are loss of the chromosomal region 1p36, loss of chromosome 3 and gain of the chromosomal region 8q. Expression profiling resulted in two different gene-expression signature classes. A class I gene-expression signature correlates with disease free survival, whereas the expression array class II correlates with early metastatic disease. Although this concerns a more expensive investigation, it is more accurate and this expression array classification outperforms all classic predictors of poor patient survival. These prognostic factors as well as the techniques to detect chromosomal aberrations are described in **Chapter 1**.
Genetic as well as immunological studies on uveal melanomas help to get a better understanding of disease progression and aid to develop targets for future therapies. However, molecular diagnostic testing has raised some questions and in this thesis some of these questions are addressed, for example the importance of intratumor heterogeneity in tumor biopsies.

In **Chapter 2,** we have investigated hyperdiploidy as a possible prognostic marker in choroidal melanomas. Hyperdiploid melanomas have an overall gain of chromosomes (>46 chromosomes), but they do seem to have lost one allele of chromosome 3, and the remaining allele has sometimes duplicated itself. Nevertheless, this results in actual loss of genetic information on chromosome 3. This might explain the correlation with a poor prognosis that is similar to patients with melanomas that have simply lost a copy of chromosome 3. In **Chapter 3** intra- and inter-tumor heterogeneity, structural variations within one tumor and between different tumors, are described. Intra-tumor heterogeneity is present in choroidal melanomas, with differential percentages of chromosome

3 loss throughout the tumor. A consistency in the differences between the tumor base and apex could not be determined. More importantly, the different subclones with chromosome 3 loss in the tumors did not result in different predictions of patient survival. Inter-tumor heterogeneity is described as well. Iris melanomas are known to have a good prognosis and metastasize rarely. Chromosomal aberrations in iris melanomas are compared with those in choroidal and skin melanomas. Although the group of iris melanomas is small, we can conclude that loss of chromosome 3 occurs frequently, but in contrast to choroidal and ciliary body melanomas without a correlation to the patients prognosis. Additionally, loss of the chromosomal region 9p21, a common event in skin melanomas, occurs often in iris melanomas, without prognostic significance.

In **chapter 4,** candidate regions and genes associated with melanoma progression are analyzed. Different cytogenetic techniques were used to analyze newly identified interesting chromosomal regions, such as 18q and 21q, and their role as prognostic parameters. Our study could not confirm the role of chromosomal regions 18q and 21q as prognostic parameters. Furthermore, the role of tumor suppressor gene APITD1, located on the chromosomal region 1p36 which is frequently lost in uveal melanomas, was evaluated. Patients with concurrent loss of chromosome 1p36 and chromosome 3 have a high risk to develop metastases. This notion led to the search for tumor suppressor genes in this critical region on chromosome 1p. Our study indicates that the expression of APITD1 does not play a role in uveal melanomas.

The last part of this thesis, **Chapter 5,** focuses on immunological aspects of tumor growth and progressive disease. In a mouse model, the role of immune privilege in the eye on tumor growth and the induction of tumor escape mechanisms in progressively growing tumors are described. The immune privileged micro-environment induces tumor escape mutants without selective pressure by the immune system.

The major results and implications of the research described in this thesis are discussed in **chapter 6**. The tools to identify high-risk melanoma patients are available and can be applied for therapeutic considerations. The challenge now lies in improving patient survival by trying to prevent and control metastatic disease. Clinical studies with adjuvant immunotherapies that specifically target tumor antigens have started for patients with high-risk uveal melanomas. For patients with liver metastases, new chemotherapeutic agents, in combination with surgery and/or immunotherapies are in development.

Samenvatting

Het uveamelanoom is de meest voorkomende vorm van kanker in het oog bij volwassenen. De uvea (het druivenvlies) bestaat uit de iris (het regenboogvlies), het corpus ciliare (het straallichaam) en de choroidea (het vaatvlies). De tumor wordt behandeld met enucleatie (verwijdering van het oog) of een vorm van radiotherapie (bestraling). Er is helaas nog geen effectieve behandeling voor metastasen (uitzaaiingen) van dit type melanoom. Deze uitzaaiingen zijn vrijwel altijd in de lever gelokaliseerd. De overleving van oogmelanoompatiënten is in de afgelopen decennia niet significant verbeterd: bijna 45 procent van de oogmelanoompatiënten overlijdt aan uitzaaiingen.
De melanomen kunnen worden ingedeeld in hoog- en laagrisico tumoren. Hoogrisico melanomen zijn agressieve tumoren die binnen vijf jaar uitzaaien naar de lever. Laagrisico melanomen zaaien vrijwel nooit uit. De patiënten met een hoogrisico uveamelanoom hebben waarschijnlijk vaker controles nodig om bij hen metastasen in een vroeg stadium te kunnen opsporen en behandelen.

Er is een aantal factoren die samenhangen met progressieve tumorgroei en daarmee de overleving van een patiënt kunnen voorspellen. Lokalisatie van de tumor in het corpus ciliare, de grootte van de tumor, de aanwezigheid van een bepaald type cellen (epitheloïd type), gesloten vaatpatronen en invasieve groei in de sclera (witte oogrok) zijn klinische en weefselspecifieke factoren die de kans op het krijgen van uitzaaiingen doen toenemen.
De belangrijkste genetische parameters die samenhangen met uitzaaiingen zijn het niet aanwezig zijn (verlies) van een deel van chromosoom 1 (1p36), verlies van chromosoom 3 en het dubbel voorkomen (winst) van een deel van chromosoom 8 (8q) in de tumorcellen.
Met nieuwere technieken kan worden gekeken naar verhoogde en verlaagde genexpressie in een tumor. Deze technieken hebben twee verschillende genexpressieklassen opgeleverd. Een klasse I-genexpressieprofiel is geassocieerd met ziektevrije overleving; een klasse II-genexpressieprofiel correleert met uitzaaiingen in de lever.
Genexpressieprofilering van tumoren is nauwkeuriger dan de andere parameters voor metastasering, maar deze vorm van profilering is ook duurder. Alle factoren die de kans op uitzaaiingen en daarmee de overleving van de patiënt voorspellen, zijn samen met de technieken om chromosomale afwijkingen en genetische expressieveranderingen te detecteren beschreven in **hoofdstuk 1**.
Zowel genetische als immunologische studies naar uveamelanomen helpen om de ontwikkeling en progressie van deze vorm van kanker beter te begrijpen, en bovendien dragen ze bij aan de ontwikkeling van nieuwe behandelingen. Het aanbieden van genetische testen in de dagelijkse diagnostiek van uveamelanomen levert een aantal nieuwe

obstakels op. In dit proefschrift worden sommige van deze problemen aangekaart, zoals het belang van heterogeniteit binnen een tumor bij het nemen van biopten uit een tumor.

In **hoofdstuk 2** hebben we hyperdiploïdie als mogelijke prognostische marker in choroïdale melanomen onderzocht. Bij hyperdiploïde melanomen bezitten de tumorcellen meer dan het gebruikelijke aantal chromosomen, dus meer dan 46. Interessant is dat deze tumorcellen waarschijnlijk toch één kopie (allel) van chromosoom 3 hebben verloren en dat de achtergebleven kopie zichzelf soms heeft vermenigvuldigd. Dit impliceert een wezenlijk verlies van genetische informatie op chromosoom 3. Dat de prognose van de hyperdiploïde melanomen net zo slecht is als die van patiënten met melanomen met verlies van een kopie van chromosoom 3 zou hierdoor kunnen worden verklaard.
In **hoofdstuk 3** worden intra- en intertumor heterogeniteit, structurele verschillen binnen één tumor en tussen verschillende tumoren, beschreven. Intra-tumor heterogeniteit is aanwezig in choroïdale melanomen, met verschillende soorten cellen en verschillende percentages van chromosoom 3 verlies in andere delen van de tumor. Er bestaat geen consistent verschil tussen de basis en de top van de tumoren. Als belangrijkste bevinding is gevonden dat de verschillende subklonen met (verschillende percentages) chromosoom 3-verlies de voorspelling op uitzaaiingen en daarmee de overleving van de patiënt niet veranderden. Verderop in dit hoofdstuk beschrijven we ook inter-tumor heterogeniteit. Irismelanomen hebben over het algemeen een goede prognose en zullen zelden uitzaaien. Chromosomale afwijkingen in irismelanomen werden vergeleken met die in choroïdale en huidmelanomen. Alhoewel het aantal onderzochte irismelanomen beperkt was, konden we concluderen dat verlies van chromosoom 3 vaak voorkomt, maar in tegenstelling tot choroïdale en corpus ciliare melanomen geen relatie heeft met de prognose van de patiënt. Daarnaast komt verlies van een deel van de korte arm van chromosoom 9 (9p21) vaak voor in irismelanomen, maar ook dit heeft geen voorspellende waarde voor agressieve tumorgroei met uitzaaiingen.
In **hoofdstuk 4** worden kandidaatregio's en -genen die overeenkomen met tumorprogressie geanalyseerd. Verschillende cytogenetische technieken werden gebruikt om recent ontdekte interessante chromosomale regio's, zoals chromosoom 18q and 21q, te onderzoeken. Onze studie kon de voorspellende waarde van deze chromosomale regio's op het krijgen van uitzaaiingen niet bevestigen. In het tweede deel van dit hoofdstuk wordt de rol van een tumorsuppressorgen, APITD1, geëvalueerd. APITD1 ligt op chromosoom 1p36, en verlies van deze regio treedt vaak op in uveamelanomen. Patiënten met gelijktijdig verlies van chromosoom 1p36 en chromosoom 3 hebben een hoog risico op het ontwikkelen van uitzaaiingen. Deze bevinding heeft geleid tot het zoeken naar tumorsuppressorgenen op dit stuk van chromosoom 1. Uit onze studie kan worden geconcludeerd dat expressie van APITD1 geen rol speelt in uveamelanomen.

Het laatste deel van dit proefschrift, **hoofdstuk 5,** gaat in op immunologische aspecten van tumorgroei en -progressie tot ongebreidelde groei (o.a. uitzaaiingen). De invloed van 'immune privilege' in het oog op de groei van tumorcellen wordt beschreven. Tevens wordt beschreven hoe agressieve tumoren manieren ontwikkelen om het afweersysteem te omzeilen. Het 'immune privileged' milieu in de voorste oogkamer van het oog induceert, zonder selectieve druk van het afweersysteem, mutaties in tumorcellen zodat deze een aanval van het afweersysteem overleven.

De belangrijkste resultaten en implicaties van het onderzoek dat in dit proefschrift is beschreven, worden in **hoofdstuk 6** becommentarieerd. De gereedschappen en technieken om hoogrisico oogmelanoom patiënten te identificeren zijn beschikbaar en kunnen worden ingezet voor therapeutische doeleinden. De nieuwe uitdaging bestaat uit het verbeteren van de overleving van patiënten door te proberen uitzaaiingen te voorkomen en, als ze toch aanwezig zijn, te zorgen dat ze niet verder uitbreiden. Recent zijn bij hoogrisico oogmelanoompatiënten klinische studies gestart met aanvullende immunotherapie, die zich richten op eiwitten op het tumorceloppervlak. Voor patiënten met uitzaaiingen in de lever zijn nieuwe chemokuren, in combinatie met chirurgische verwijdering en/of immunotherapie in ontwikkeling.

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Curriculum Vitae

The author was born on May 8, 1978, in Groningen, the Netherlands. She graduated from secondary school at the Praedinius Gymnasium in Groningen in 1996 and in the same year she moved to South Africa to study Liberal Arts at Stellenbosch University.
In 1997 she started Medical School at Leiden University and from her second year she worked as a medical student nurse at the thoracic intensive care unit of the Leiden University Medical Center. In 2001 she travelled for 2 months to Calcutta, India, to carry out primary health care as a volunteer for the Institute for Indian Mother and Child.
In 2001 and 2002 the author conducted her scientific graduation project at the Laboratory of dr. B.R. Ksander, Schepens Eye Research Institute, Harvard Medical School in Boston, USA. This project considered the role of cytotoxic T lymphocytes in tumor immunology and part of the results are included in this thesis.
Subsequently, the author followed clinical rotations at the Leiden University Medical Center and affiliated hospitals. Ophthalmology and Otolaryngology rotations were followed at the Rotterdam Eye Hospital and the St. Elisabeth Hospital in Willemstad, Curaçao, respectively. In 2004 she obtained her MD degree and started her research on uveal melanoma at the departments of Ophthalmology and Clinical Genetics of the Erasmus Medical Center and Rotterdam Eye Hospital shortly thereafter (dr. A. de Klein, dr. D. Paridaens and prof.dr. G. van Rij). She presented her work at several national and international meetings.
In 2005 the author commenced her residency in Ophthalmology at the Rotterdam Eye Hospital (prof.dr. J.C. van Meurs), which she will complete in 2010.

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