Prognostic Implications of Acquired Genetic Changes in Uveal Melanoma

Anna Elizabeth Koopmans

Prognostic Implications of Acquired Genetic Changes in Uveal Melanoma

Thesis, Erasmus University Rotterdam, The Netherlands

The research project was initiated by the Department of Ophthalmology and Clinical Genetics, Erasmus MC, University Medical Centre Rotterdam, The Netherlands, and The Rotterdam Eye Hospital, Rotterdam, The Netherlands. The work described in this thesis was financially supported by Combined Ophthalmic Research Rotterdam (CORR, grant number: #1.1.1) and the Stichting Nederlands Oogheelkundig Onderzoek (SNOO, grant numbers: #2011-17, #2012-12 and #2013-14).

The printing of this thesis was financially supported by: Alcon Nederland BV, Ergra Low Vision BV, Landelijke Stichting voor Blinden en Slechtzienden, Oculenti BV, Oogheelkunde Rijswijk, Prof.dr. Henkes Stichting, Rockmed BV, Rotterdamse Vereniging Blindenbelangen, Stichting Blindenhulp, SWOO - Prof.dr. Flieringa.

ISBN: 978-94-6259-522-4

Cover and layout: Anna Koopmans

Printing: Ipskamp Drukkers BV, Enschede

Prognostic Implications of Acquired Genetic Changes in Uveal Melanoma

Prognostische implicaties van verworven genetische veranderingen in uveamelanomen

Proefschrift

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

Prof.dr. H.A.P. Pols

en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op

4 februari 2015 om 15.30 uur

door

Anna Elizabeth Koopmans

geboren te Stockport, Groot-Brittannië

PROMOTIECOMMISSIE

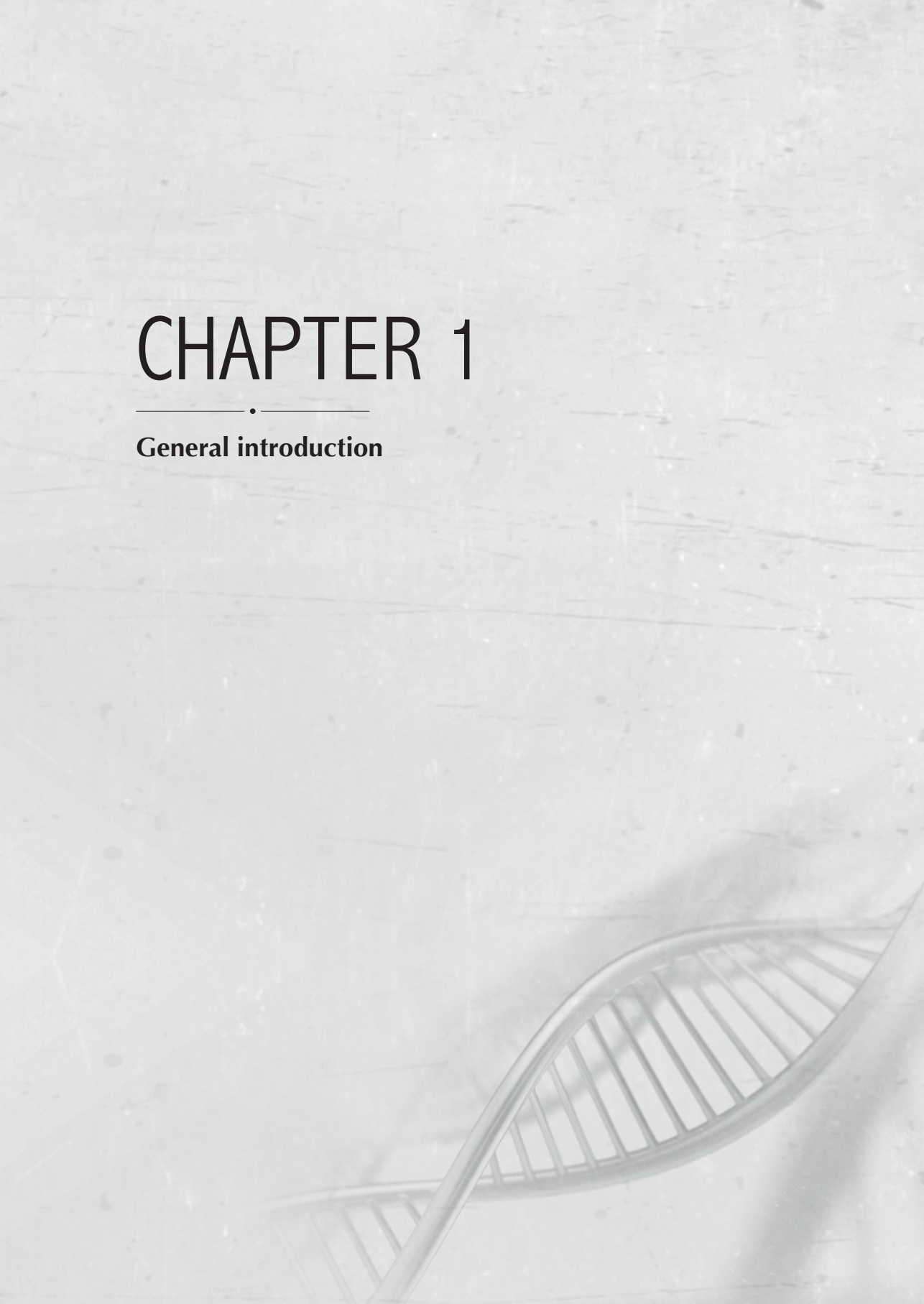
Promotoren:	Prof.dr. J.R. Vingerling
	Prof.dr. D. Paridaens
Overige leden:	Prof.dr. R.M.W. Hofstra
	Prof.dr. S. Sleijfer
	Prof.dr. S. Seregard
Copromotoren:	Dr. J.E.M.M. de Klein
	Dr. E. Kiliç

Voor mijn ouders

CONTENTS

CHAPTER 1

General introduction



CHAPTER 1

General introduction

Partly published in:

Diagnosis and management of uveal melanoma
European Ophthalmic Review. 2013;7(1):56-60

Diagnosis, histopathologic and genetic classification of uveal melanoma
In: Melanoma – From Early Detection to Treatment. 1st edn. Publiser: In-Tech, 2013:137-173

Uveal melanoma (UM) is the most common primary intra-ocular malignancy in adults. The incidence of UM ranges from 4.3 to 10.9 cases per million and has remained stable for the past fifty years.[1] Presentation is at a median age of 60 years, and men and women are equally affected. UM is a neoplasm that arises from melanocytes in the uveal tract, which comprises the choroid, ciliary body, and iris (**Figure 1**). Choroidal melanoma are the most common and display a discoid, dome-shaped or mushroom shaped growth pattern. The tumours have a strong tendency to metastasise to the liver. Over the past decades ocular ophthalmologists have shifted towards more eye conserving treatment with the aim of preserving vision. Despite primary treatment, nearly half of the patients eventually die due to metastatic disease and the patient survival has not improved over the past 30 years (**Figure 2**).[2] Metastasising UM often contain non-random chromosomal aberrations. Therefore, many UM research initiatives focus on finding genetic prognostic markers in UM to select those patients at risk for developing metastatic disease and finding new treatments based on specific gene content.

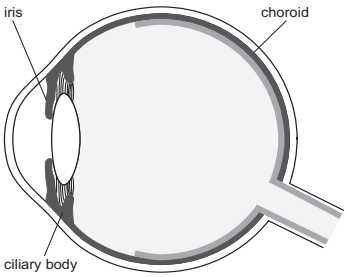


Figure 1. A schematic illustration of the eye depicting the choroid, ciliary body and iris.

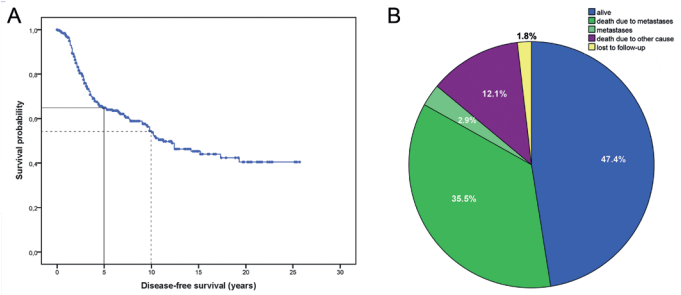


Figure 2. Follow-up data of the Rotterdam Ocular Melanoma Study group (ROMS) uveal melanoma cohort. Kaplan-Meier survival curve of 380 uveal melanoma. The black line and dotted line represents the 5 and 10-year survival, respectively (**A**). Piechart shows that 38.4% of patients have developed metastases thus far (February 2014) and nearly half of patients are still alive (**B**).

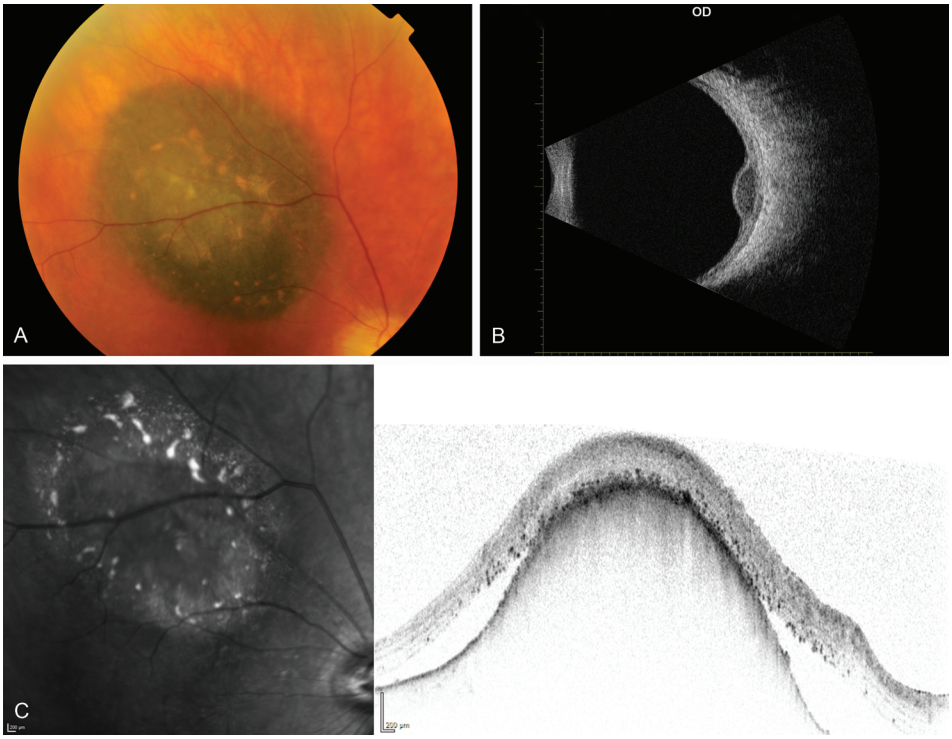


Figure 3. A dark pigmented uveal melanoma with orange pigment (**A**). On B-scan ultrasonography acoustic hollowing and choroidal excavation is present (**B**). Subretinal fluid and retinal pigment epithelial alterations are visible on optical coherence tomography scan at the top of the tumour (**C**).

1.1 CLINICAL PRESENTATION AND MANAGEMENT

UM are often noticed at a routine ophthalmic examination since 30% of the patients have no symptoms.[3] If a patient with UM presents with symptoms, these can include blurred vision, floaters, photopsias and visual loss, depending on the size and location of the tumour. In some cases the patient presents with severe ocular pain secondary to inflammation or neovascular glaucoma. The diagnosis is based on clinical examination with the slit lamp and indirect ophthalmoscope together with ultrasonography (US) of the eye (**Figure 3**). The majority of UM are pigmented lesions (melanotic), only one fourth is amelanotic or relatively non-pigmented. The tumours can grow towards the vitreous displaying a discoid, dome-shaped or mushroom shaped growth pattern. Small melanoma are more difficult to detect than medium and large-sized melanoma. UM are subdivided according to the apical size of the tumour and the diameter. Small melanoma have a diameter of > 5 mm with a thickness of 1.0 to 2.5 mm, medium-sized lesions are ≤ 16 mm in diameter and 2.5 to 10.0 mm in

thickness, and large uveal melanoma are either > 16 mm in diameter with a thickness of > 2.0 mm or have a thickness of > 10.0 mm regardless of the basal diameter.[4] They can appear flat or dome-shaped. It remains challenging for ophthalmologists to differentiate between small choroidal melanoma and choroidal naevi, differential diagnostic the most important alternative. In general, choroidal naevi have a less than 5 mm basal diameter and are minimal in height, less than 2 mm, although several definitions of naevi have been proposed. To differentiate small UM from other choroidal pathologies, including choroidal naevi, Shields et al.[5] constructed the mnemonic 'TFSOM', i.e. 'to find small ocular melanoma' to identify indicators of potential malignancy and predict growth. The letters indicate Thickness > 2 mm, subretinal Fluid, Symptoms, Orange pigment and Margin within 3 mm of the optic disc. If none of the factors are present there is a 4% chance of growth, while if there are one or two factors present the chance of growth rises to 36% and > 45%, respectively.[6] The TFSOM mnemonic was later extended with 'Using Helpful Hints Daily' including three features as Ultrasound Hollowness (or low acoustic profile), absence of a Halo around the tumour and absence of Drusen.[7] Extraocular extension into the orbit can occur at any stage and can be detected with US, computed tomography (CT) and magnetic resonance imaging (MRI). Optical coherence tomography (OCT) and fundus autofluorescence can be useful in differentiating melanoma from other pigmented lesions (e.g. melanocytoma).[8, 9] Small tumours can be measured and subretinal fluid can be observed with an OCT, whereas orange pigment can be visualised using fundus autofluorescence. Spectral domain OCT can be useful in the detection of subretinal deposits, vitreous seeding and transretinal tumour extension.[10] Other ancillary tests include fluorescence angiography, indocyanine green angiography, CT and MRI. The diagnostic value of fluorescence angiography is limited but it can aid in differentiating UM from other lesions. The choroidal vasculature can be visualised with indocyanine green angiography and provides more information than fluorescence angiography. Often late staining is observed because the indocyanine green leaks in the extracellular space of the tumour.[11-13] To detect extrascleral extension, CT and MRI are more sensitive than US.[14, 15] However, these techniques are not routinely used in the diagnostic evaluation of UM since they are quite expensive.

1.2 HISTOPATHOLOGICAL FEATURES

Uveal melanoma develop from melanocytes of the uvea that are derived from neural crest cells. Initially Callender and colleagues[16] described several melanoma cell types, and currently three histopathological UM categories are recognised: spindle, epithelioid and mixed cell type.[17] Haematoxylin and eosin (H&E) staining is used to differentiate between cell

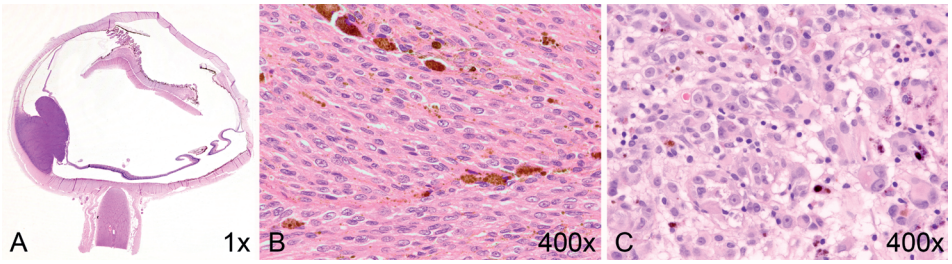


Figure 4. Haematoxylin and eosin staining of formalin fixed and paraffin embedded eye sample with a typical mushroom shaped melanoma (**A**). Uveal melanoma tissue with spindle cell type characterised by elongated nuclei (**B**). Uveal melanoma tissue with epithelioid cells containing large pleomorphic nuclei and prominent eosinophilic nucleoli (**C**).

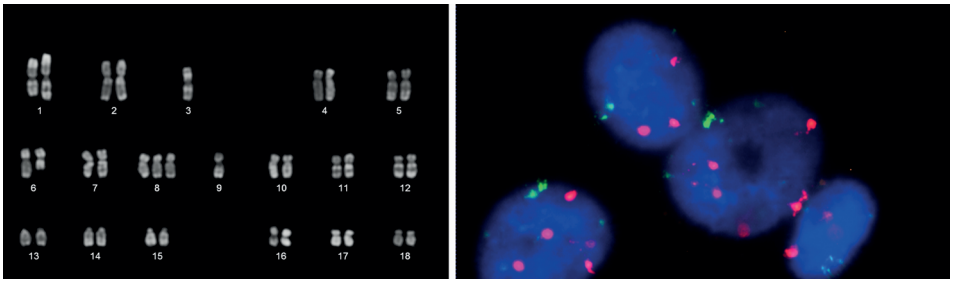


Figure 5. Example of a karyogram showing monosomy 3 and trisomy of chromosome 8 (**A**). Fluorescence *in situ* hybridisation analysis of a tumour demonstrates 1 signal for the probe on centromere 3 (green signals) and 3 to 4 signals of the probe on centromere 8 (red signals) (**B**).

types. Spindle cells exhibit elongated nuclei that may contain eosinophilic nucleoli. In general, tumours containing spindle cells grow slowly and might be associated with better prognosis. On the other hand, tumours consisting of faster growing epithelioid cells, have a more aggressive behaviour, and are therefore associated with poor clinical outcome. Epithelioid cells have more polygonal cytoplasm and contain eccentric placed large pleomorphic nuclei and prominent eosinophilic nucleoli (**Figure 4**). The mixed-cell type melanoma has variable proportion of spindle and epithelioid cells with a minimum of 10% of any one type.[18] Other inter-tumour factors, like the presence of certain extracellular matrix patterns (three closed loops located back to back identified by periodic acicd-Schiff (PAS) staining) and increased mitotic figures (number of mitoses per 50 high-power fields equal to 8 mm^2) can both provide additional adverse prognostic information.[19, 20] Other histological features associated with mortality and metastases are mean diameter of ten largest nucleoli, degree of pigmentation, presence of inflammation and tumour necrosis.[21] Extrascleral extension by perineural, perivascular, intravascular or direct scleral invasion is correlated with a worse prognosis,

especially when the orbital fat resection margin is positive.[22] Immunohistochemistry may be of diagnostic value. S-100 is expressed by cells of neuroectodermal origin. HMB-45 binds to gp100, an antigen expressed by melanocytes that can be useful in differentiating UM from nonmelanocytic tumours.[23]

1.3 GENETIC FACTORS

Cytogenetic studies in solid tumours have been a greater challenge than in haematological malignancies since metaphase chromosome spreads of good quality are more difficult to obtain. Solid tumours frequently have highly complex chromosome alterations and are more heterogeneous. Despite this, UM has been well studied since the late eighties with different techniques. Over the years, we have learned that the majority of UM contain non-random chromosomal anomalies on either the short arm (p) and or long arm (q) of chromosomes 1, 3, 6 and 8, which can serve as prognostic markers.

Cytogenetic and molecular techniques in UM research

To examine chromosomal changes in UM tissue several cytogenetic and molecular techniques are available. UM are quite suitable for cytogenetic analysis because of their relatively simple karyotype. Large chromosomal gains, deletions and translocations can be visualised with conventional karyotyping and spectral karyotyping (**Figure 5A**). However, for the detection of smaller abnormalities other techniques are necessary, such as fluorescent *in situ* hybridisation (FISH) (**Figure 5B**), comparative genomic hybridisation (CGH) or quantitative polymerase chain reaction (qPCR) based techniques. An approach is the multiplex ligation-dependent probe amplification (MLPA), which allows the relative quantification of multiple loci in one single reaction. MLPA can detect patients at risk for metastatic disease using the results for chromosome 3 and 8 with similar accuracy as FISH.[24, 25] MLPA and other qPCR based techniques as multiplex amplicon quantification (MAQ) fill the gap between more expensive genome-wide screening assays and cheaper methods that only provide information on a single locus.[26]

After completion of the human genome project, genome-wide DNA assays became available. Micro-assay based CGH, single nucleotide polymorphism (SNP) analysis and gene expression profiling (GEP) analysis are frequently applied techniques. With the development of Next Generation Sequencing (NGS) technologies, the genome can be analysed at base pair level. Genome-wide mutation analyses of tumour samples led to the discovery of a subset of genes in UM such as *GNAQ* and *BAP1*.

Chromosomal anomalies

Monosomy 3

Monosomy of chromosome 3 is observed in approximately 50% of the cases of UM and is strongly associated with clinical and histopathological prognostic factors and with metastatic death (**Figure 6**).[27-29] Prescher and associates[30] were the first to find a strong correlation between loss of chromosome 3 and a poor prognosis of the patient. Since then several groups have confirmed the prognostic value of monosomy 3.[31-34] Since loss of chromosome 3 often occurs with other chromosomal aberrations such as 1p loss, and gain of 6p and 8q, it is assumed to be a primary event.[35] Mostly one entire copy of chromosome 3 is lost, although in some cases, isodisomy of chromosome 3 or a partial loss of chromosome 3 is acquired.[36-38]

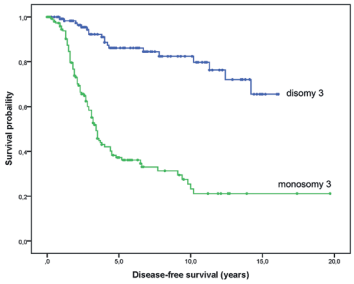


Figure 6. Kaplan-Meier survival curve of 380 uveal melanoma patients showing a lower disease-free survival for patients with monosomy 3 compared with patients with disomy 3.

Chromosome 8

Abnormalities in chromosome 8, and in particular gain of 8q or an isochromosome 8q, are thought to be a secondary event in UM as variable copy numbers can be present in one melanoma.[39, 40] Gain of chromosome 8q is frequently found in tumours that also have loss of chromosome 3, and this is associated with a poor patient outcome.[34-36] A SNP array analysis with this chromosome status is depicted in **Figure 7**. The relationship between the percentages of aberrant copy numbers within UM cells and patient outcome has been investigated. A higher percentage of cells with monosomy 3 and chromosome 8q gain in primary UM shows a strong relation with poor disease-free survival compared to low percentage aberrations.[41]

Chromosome 6

Rearrangements on chromosome 6 affect both arms of the chromosome, resulting in deletions of 6q and gains of 6p. The relative gain of chromosome 6p can occur either through an isochromosome of 6p or a deletion of 6q. Aberrations resulting in a relative increase of 6p have been found to be related with both a longer survival[34] or a decreased survival.[36] The effect of chromosome 6 aberrations on patient outcome is not conclusive.

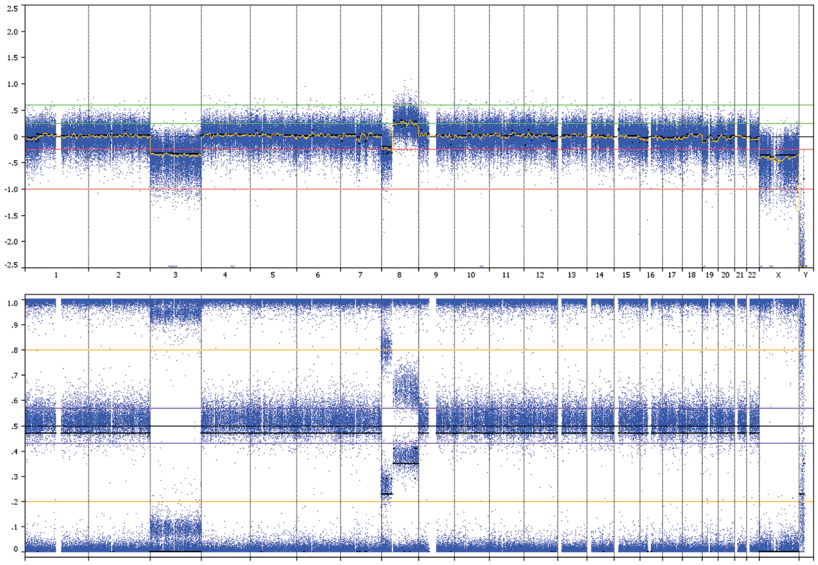


Figure 7. Single nucleotide polymorphism array of an uveal melanoma. The upper panel (LogR ratio) shows loss of chromosome 3, partial loss of chromosome 8p and gain of chromosome 8q. The lower panel depicts the B-allele frequency representing allelic imbalance at these chromosomes.

Chromosome 1

In cutaneous melanoma rearrangements on the short arm of chromosome 1 are a common abnormality, occurring in about 80% of all cases.[42, 43] In UM this region on 1p is also frequently affected, giving rise to a deletion of 1p. However, these anomalies on chromosome 1 are less common than those in skin melanoma with a frequency of approximately 30%.[28, 32, 35, 39, 44] Kiliç and colleagues[45] established that tumours with concurrent loss of chromosome 1p and 3 are at higher risk of metastasising than the tumours with other aberrations.

Aberrations on other chromosomes have been explored, such as chromosome 9p21,[39] chromosome 11q23,[32] chromosome 18q22,[46, 47] and chromosome 16q.[31, 48] The impact on the prognosis, however, remains unclear due to contradictory findings.

Gene expression profiling

Using GEP uveal melanoma can be classified into two classes of tumours that correspond remarkably well with the ability of the tumour to metastasise. In a study of 25 UM, class 1 tumours had a low risk of metastasising and class 2 tumours had a high risk of developing metastasis.[49] This molecular classification strongly predicts metastatic death and outperforms other clinical, histopathological and cytogenetic prognostic indicators.[50-52] Class 1 tumours

predominantly show disomy of chromosome 3, whereas class 2 tumours consist mostly of monosomy 3.[52]

Candidate genes in uveal melanoma

After identifying the non-random chromosomal alterations in UM, the search for potential oncogenes and tumour suppressor genes followed. By narrowing down altered regions on chromosomes, researchers have tried to identify genes involved in tumourigenesis or progression towards metastasis. This way, studies have been conducted on chromosome 8q revealing potential oncogenes such as *MYC*, which is amplified in about 30% of the UMs.[53] Other oncogenes on chromosome 8q have been described, such as *DDEF1* and *NBS1* (now referred to as *ASAP1* and *NBN*, respectively).[54, 55] Yet, no specific oncogenic mutations on this region have been reported thus far. Other candidate genes were proposed, such as *HDM2*, *BCL-2* and *CCND1*. However, the pathogenic significance for any of these genes has not been established. Mutations in certain genes have been well described for cutaneous melanoma. Examples of such genes are the oncogenes *NRAS*, *BRAF* and *AKT3*, and the tumour suppressors *CDKN2A*, *PTEN* and *TP53*. In contrast to skin melanomas, *PTEN* mutations were not observed in a study of nine cell lines.[56] Nevertheless, in 15% of the UM cases mutations in *PTEN* were found resulting in activation of *AKT* and overexpression of the PI3K-PTEN-AKT pathway preventing apoptosis.[57, 58, 59]

In a large proportion of the UM, the RAS-RAF-MEK-ERK pathway or mitogen-activated protein kinase (MAPK) pathway is constitutionally activated, leading to excessive cell proliferation and suggesting the presence of activating mutations upstream in the pathway.[60, 61] Mutation analysis on potential mutation sites in the *BRAF* gene were performed, since a sin-

Table 1. Three known implicating genes in uveal melanoma at the beginning of our study

GNAQ	***GNA11***	***BAP1***
Guanine nucleotide binding protein (G protein), q polypeptide	Guanine nucleotide binding protein (G protein), alpha 11	BRCA1 associated protein-1
Chromosome 9	Chromosome 19	Chromosome 3
Prevalence 46 - 53% UM[68, 69]	Prevalence 34% UM[70]	Prevalence 47% UM[71]
Overall prevalence Gα mutations 85 - 93%		
Involved in MAPK pathway		Involved in cell cyle regulation, chromatin dynamics, DNA damage response
Activating hotspot mutation at codon R183 or Q209		Inactivating mutations located throughout gene
Associated with melanocytic proliferation; also mutations in blue nevi of the skin		Associated with metastasising UM

Abbreviations: UM = uveal melanoma.

gle substitution (p.V600E) in *BRAF* occurs frequently in benign and premalignant cutaneous naevi.[62, 63] However, *NRAS* and *BRAF* mutations have been reported in a few UM but overall these mutations are rare.[64-67] With the recent discovery of activating *GNAQ* and *GNA11* (together Gα) mutations new light has been shed on the MAPK pathway. Gα mutations occur mutually exclusive in a large proportion of UM and blue naevi (**Table 1**).[68-70]

Exome genome sequencing led to the discovery of the *BAP1* gene in UM.[71] BAP1, a nuclearly localised enzyme, was originally identified as an ubiquitin hydrolase that binds to the RING finger domain of BRCA1.[8, 72] *BAP1* is located on chromosome 3p21.1 and is thought to be a tumour suppressor gene.[73] Mutations in this gene first have been reported in a small number of breast and lung cancer cell lines.[8] Recently, inactivating somatic mutations were found in 84% of the metastasising UM.[71] To understand more about the impact of *BAP1* mutations on UM and other types of cancers, more extensive clinical, molecular genetic, and functional studies are ongoing.

1.4 TREATMENT OF PRIMARY UVEAL MELANOMA

The treatment of UM depends on the size and location of the tumour, the secondary effects of the tumour on the eye (for instance, inflammation or neovascular glaucoma), the status of the fellow eye and the patients' choice. Over the years, eye-preserving therapies have proved to be equally effective in terms of overall patient survival and metastasis-free survival compared with radical treatment.[74, 75] Therefore, nowadays, radical treatment or enucleation (removal of the eye) is performed in those cases with a larger or advanced melanoma, or when extraocular extension is present.[76] A summary of the treatment modalities for UM, together with indications and complications, is listed in **Table 2**.

The most common treatment among the conservative modalities is brachytherapy. With brachytherapy or local irradiation, radioactive material is placed on the sclera at the location of the tumour. Ruthenium-106 (Ru-106) and iodine-125 (I-125) are frequently used applicators. The plaque-shaped applicator is sutured to the sclera and removed a few days later once the required dose of at least 80 Gy has been delivered to the tumour. Ru-106 applicators have a limited depth of penetration compared with I-125 applicators. Therefore Ru-106 is usually used for tumours with a maximal thickness of 7.0 mm.[77, 78] Brachytherapy can be combined with transpupillary thermotherapy (TTT) to treat those cases with thicker tumours or to gain more tumour control if there is a suspicion that the tumour margins are not covered with brachytherapy. This is referred to as 'sandwich therapy'.[79, 80] TTT is rarely used as a primary treatment; only those patients with small pigmented choroidal melanoma near to

Table 2. Summarised treatment options for uveal melanoma

Treatment	Indications	Complications	SER
Brachytherapy	Small- and medium-sized tumours Thickness ≤ 7 mm, if maximal thickness is > 7 mm combination with transpupillary thermotherapy	Radiation retinopathy (maculopathy) (24-40%), papillopathy (10-14%), vitreous haemorrhage (9-15%), cataract (13-32%), neovascular glaucoma (8-15%)[83, 85, 89, 91]	10-20%
Proton beam radiotherapy	Small-, medium- and large-sized tumours LTD ≤ 20 mm and thickness ≤ 12 mm	Retinal detachment, vitreous haemorrhage, neovascular glaucoma (38%), cataract (29%), radiation retinopathy (10%), radiation papillopathy (10%), dry eye (24%)[95]	10-15%
Stereotactic radiotherapy	Small- and medium-sized tumours LTD < 16 mm, thickness ≤ 12 mm	Radiation cataract (3-53%), tumour vasculopathy (80%), radiation retinopathy (5-81%), radiation optic neuropathy (11-64%), neovascular glaucoma (3-42%), vitreous haemorrhage (3-33%), retinal detachment (14-37%), conjunctival symptoms (26%), dry eye syndrome (8%), eye lash loss (26%)[98-100]	3-16%
Photodynamic therapy	Amelanotic tumours		
Endoresection	Tumours (> 6-8 mm) preferably without ciliary body infiltration	Retinal detachment (9-33%), entry site tears, cataract, acute glaucoma, choroidal neovascular membranes[104, 106]	6%
Exoresection	Tumours (> 6-8 mm) which are more anteriorly located (with ciliary body infiltration) Tumours that are likely to develop complications with radiotherapy (e.g. neovascular glaucoma)	Retinal detachment, vitreous haemorrhage, subretinal fibrosis, cystoid macular oedema, elevated intraocular pressure, scleral thinning	4%
Enucleation	Large-sized, advanced tumours or if extraocular extension is present LTD > 16mm with thickness > 2.0 mm or thickness of > 10 mm regardless of the LTD	If extraocular extension is present, orbital recurrence can occur (10%)[107]	

Abbreviations: LTD = largest tumour diameter, SER = secondary enucleation rate.

the fovea or optic disc may sometimes receive TTT.[81] After brachytherapy, radiation-induced complications can occur and include radiation retinopathy, radiation maculopathy, radiation opticopathy, vitreous haemorrhage, cataract and iris neovascularisation leading to neovascular glaucoma.[82-84] Local recurrences after brachytherapy have been described in 4 – 28%, depending on tumour size and duration of follow up.[85-88] Ten to 22% of the patients eventually have to be secondary enucleated due to radiation-induced side effects.[89-92]

Heavy particle radiation with proton beam is available in some centres and some administer this treatment to all patients, while others reserve it for patients whose tumour is unsuitable for brachytherapy. Proton beam radiotherapy consists of several steps. First, a clip is sutured to the sclera around the base of the tumour followed by dose admission two weeks later (50-70 Gy relative biological effectiveness in 4-5 fractions).[93, 94] Proton beam radiotherapy has some advantages compared with brachytherapy and fractioned stereotactic radio-

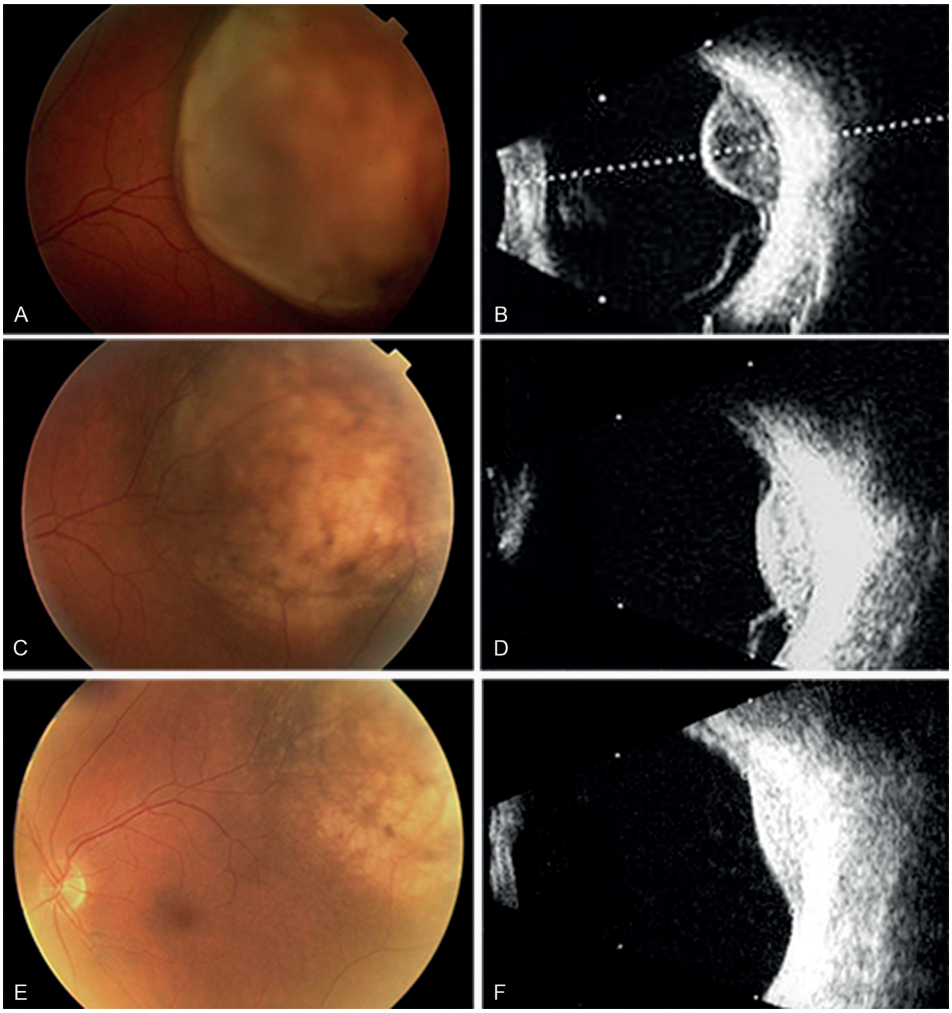


Figure 8. Baseline fundus photograph of an uveal melanoma. The lesion is located in the upper temporal choroid 3 mm from the fovea. The best corrected visual acuity (BCVA) at baseline was 0.7 (**A**). Baseline ultrasonography (US) shows subretinal fluid and a prominence of 6.6 mm (**B**). Fundus 3 months after treatment with stereotactic radiotherapy. The BCVA decreased to 0.4 (**C**). Corresponding US 3 months later showing a decreased prominence of 4.6 mm (**D**). Fundus photograph 12 months after treatment showing a slightly elevated scar. The BCVA is equal to baseline measurement 0.7 (**E**). After 12 months the tumour prominence has decreased to 2.4 mm on US (**F**).

therapy (fSRT, see below) since the dose reaches the tumour homogeneously and healthy tissue surrounding the tumour can be spared.[95] Proton beam radiation enables treatment of choroidal melanoma of all sizes but usually tumours up to 20 mm in diameter and 12 mm thickness are treated. When treating larger or thicker tumours with proton beam, there is a lower chance of preserving vision.[96] The local recurrence rate after treatment is 5% after

10 years and this is in the same range as brachytherapy.[86] Secondary enucleation rates are 10 – 15% due to local recurrences or complications.[97, 98] Complications after proton beam radiotherapy include retinal detachment, neovascular glaucoma, cataract, optic neuropathy, maculopathy, vitreous haemorrhage and dryness.[93]

Another eye-preserving option is fSRT for the treatment of small and medium-sized posteriorly located melanoma and is becoming available in more centres. The effect of fSRT on a medium-sized choroidal melanoma is depicted in **Figure 8**. One advantage of fSRT compared with previously mentioned therapies, is that fSRT requires no surgical procedures to determine the tumour localisation and dimensions. The tumour borders are delineated with CT and/or MRI. Stereotactic irradiation can be performed using a gammaknife, a cyberknife or a linear accelerator. Most centres administer a total dose of 50 Gy in 4-5 fractions, although some centres prescribe higher doses.[99, 100] Reported complications are similar to those of brachytherapy and proton beam radiotherapy and can lead to visual impairment and secondary enucleation (3 – 16%).[99-101] Other radiogenic side effects include conjunctivitis, blepharitis or dry eye syndrome. Studies report similar local tumour control rates of approximately 90% 5 and 10 years after fSRT.[99-101]

Photodynamic therapy is occasionally used as an alternative treatment for UM. The use of photosensitiser verteporfin has been described in several reports and is being more applied in amelanotic tumours.[102-104]

Local resection of small iris and ciliary body melanoma are widely carried out. This is not the case for choroidal melanoma. In the past, ophthalmologists were reluctant to operate on choroidal melanoma as they were concerned about manipulation of tumour cells that could lead to an increased risk of metastasis. Currently, due to improved surgical techniques and more insights on tumour progression towards metastatic disease, surgical excision is considered to be a therapeutic option for choroidal tumours. The tumour can be removed in several ways, through the vitreous and retina with a vitreous cutter, endoresection, or through a scleral opening, exoresection (e.g. iridectomy, iridocyclectomy, cyclochoroidectomy, choroidectomy). This way eyes with large melanoma, which otherwise would be enucleated, can be preserved. Surgical resection can serve as primary treatment for UM or additional to another kind of radiotherapy. Often, radiotherapy is administered prior to endoresection and exoresection, and is followed by treatment with brachytherapy. By using resection as a treatment, radiation-induced problems such as toxic tumour syndrome, a result of radiation-induced necrosis comprising exudative maculopathy, serous retinal detachment, rubeosis and neovascular glaucoma, can be avoided. Another advantage is that, especially with the larger melanoma, tumour tissue is available for prognostification and research.[85, 105, 106]

1.5 TREATMENT OF LIVER METASTASIS

Despite successful eradication of the ocular tumour, about 50% of all UM patients develop metastatic disease.[108] Metastases spread haematogeneously to the liver and death often follows within 1 year if systemic symptoms occur.[109] There are no standardised therapies that improve survival in metastatic disease. Systemic treatment options, such as intravenous chemotherapy and immunotherapy, prolong life only rarely.[110] Systemic therapy may be more effective if administered early after diagnosis treating micrometastatic rather than macrometastatic disease. Targeted systemic therapies in metastatic UM are currently being investigated. Preclinical studies suggest potential benefit when modulating MAPK and phosphatidylinositol 3-kinase/AKT (PI3K/AKT) pathways, or receptor tyrosine kinases. The BRAF inhibitor sorafenib was administered to uveal metastatic patients in a phase II trial (www.clinicaltrials.gov). A new adjuvant immunotherapy protocol has been developed, where clinical, histological and cytogenetic factors are used to identify high risk UM patients and treat these by immunisation with their own trained dendritic cells to prevent future metastatic disease.[111] This multicentre trial is ongoing within our Rotterdam Ocular Melanoma Study group (ROMS) in collaboration with the Department of Tumour Immunology of Radboud University Nijmegen.

For the treatment of liver metastasis there are several locoregional techniques available, for instance, hepatic intra-arterial chemotherapy, chemoembolization, immunoembolization and isolated liver perfusion.[112] A partial hepatectomy can be beneficial in some highly selected patients. Surgery in patients with four or fewer metastatic lesions, more than 24 months from initial diagnosis to liver metastases and absence of miliary disease (multiple, diffuse, small sized, dark punctuate lesions on CT) has been associated with a better outcome.[113] A microscopically complete liver resection can increase the survival time. In a retrospective study of 255 patients who underwent surgical resection, the median overall post-operative survival was 14 months. In patients whom had a microscopically complete liver resection (compared with a microscopically or macroscopically incomplete liver resection) the survival increased to 27 months.

Despite primary therapeutic advances in the treatment of primary UM, the rate of metastatic disease is still not reduced, making it more important to find alternative treatments for metastases in particular. With new technologies, such as NGS, more is learned about the human genome and new cancer-susceptibility genes in UM, which may serve as targets for new interventions.

1.6 SCOPE AND OUTLINE OF THIS THESIS

This thesis aims to find prognostic factors in uveal melanoma facilitating metastasis prediction in UM patients.

In the era of NGS the regions of interest (genetically) have been narrowed down from chromosome to gene level. This part of the thesis concerns mutation analysis in candidate genes to characterise different subsets of tumours. In **Chapter 2.1**, we describe *GNAQ* and *GNA11* mutations occurring in the majority of UM and being an early event in the UM development. Another candidate gene is *BAP1*, located on chromosome 3p21.1, and **Chapter 2.2** stresses the importance of *BAP1* mutations in metastasising tumours. Furthermore, this chapter elaborates on the possibility to replace genetic screening of *BAP1* by immunohistochemistry. A subset of prognostically favourable UM harbour mutations in the *EIF1AX* and *SF3B1* gene which is described in **Chapter 2.3**, and **Chapter 2.4** describes an UM case in which cytogenetic and mutation analyses have been performed on both primary tumour and corresponding metastases tissue. In **Chapter 2.5**, we analysed *TERT* mutations in several types and stages of eye cancer including conjunctival naevi, primary acquired melanosis (PAM) of the conjunctiva, conjunctival melanoma and UM.

New techniques such as NGS in the field of cancer genetics are used to investigate mutations and copy number variations in UM. In the third of chapter of thesis, we present a sequencing platform, HaloPlex, for targeted sequencing of genes and examine whether variant frequencies are a good reflection of the copy number status of the tumour (**Chapter 3.1**).

The fourth chapter of this thesis will mainly focus on different aspects of histopathological research and associations with chromosomal aberrations and patient survival. Chemokine receptors are expressed widely on different types of cancers and play a role in the transport of cancer cells to secondary sites. In **Chapter 4.1**, we examine the expression levels of chemokine receptors CCR7 and CXCR4/CXCL12 in primary and metastatic UM tissue to see whether these chemokine's are involved in UM metastasis. Several well-established features are associated with the prognosis of UM patients, such as epithelioid cell type, extracellular matrix patterns, extraocular extension, and chromosomal aberrations including monosomy 3 and gain of 8q. The importance of chromosome 8q gain as an additional risk factor for metastasis in patients with extraocular extension is decribed in **Chapter 4.2**. **Chapter 4.3** illustrates a melanoma with unusual clear cell changes.

Finally, in **Chapter 5** our main findings are summarised, and a general discussion and future prospects are presented.

REFERENCES

1. Singh AD, Damato BE, Pe'er J, Murphee AL, Perry JD. *Essentials of Ophthalmic Oncology*. Slack Incorporated: Thorofare; 2009. 288 pp.
2. Singh AD, Turell ME, Topham AK. Uveal melanoma: trends in incidence, treatment, and survival. *Ophthalmology*. 2011;118:1881-1885.
3. Damato B. Does ocular treatment of uveal melanoma influence survival? *Br J Cancer*. 2010;103:285-290.
4. COMS. Comparison of clinical, echographic, and histopathological measurements from eyes with medium-sized choroidal melanoma in the collaborative ocular melanoma study: COMS report no. 21. *Arch Ophthalmol*. 2003;121:1163-1171.
5. Shields CL, Shields JA, Kiratli H, De Potter P, Cater JR. Risk factors for growth and metastasis of small choroidal melanocytic lesions. *Ophthalmology*. 1995;102:1351-1361.
6. Shields CL, Cater J, Shields JA, et al. Combination of clinical factors predictive of growth of small choroidal melanocytic tumors. *Arch Ophthalmol*. 2000;118:360-364.
7. Shields CL, Furuta M, Berman EL, et al. Choroidal nevus transformation into melanoma: analysis of 2514 consecutive cases. *Arch Ophthalmol*. 2009;127:981-987.
8. Jensen DE, Proctor M, Marquis ST, et al. BAP1: a novel ubiquitin hydrolase which binds to the BRCA1 RING finger and enhances BRCA1-mediated cell growth suppression. *Oncogene*. 1998;16:1097-1112.
9. Schaudig U, Hassenstein A, Bernd A, Walter A, Richard G. Limitations of imaging choroidal tumors in vivo by optical coherence tomography. *Graefes Arch Clin Exp Ophthalmol*. 1998;236:588-592.
10. Heindl LM, Mardin CY, Holbach LM, et al. Vitreal seeding from uveal melanoma detected by high-resolution spectral-domain optical coherence tomography. *Arch Ophthalmol*. 2009;127:1062-1064.
11. Frenkel S, Barzel I, Levy J, et al. Demonstrating circulation in vasculogenic mimicry patterns of uveal melanoma by confocal indocyanine green angiography. *Eye (Lond)*. 2008;22:948-952.
12. Guyer DR, Yannuzzi LA, Krupsky S, et al. Digital indocyanine-green videoangiography of intraocular tumors. *Semin Ophthalmol*. 1993;8:224-229.
13. Stanga PE, Lim JI, Hamilton P. Indocyanine green angiography in chorioretinal diseases: indications and interpretation: an evidence-based update. *Ophthalmology*. 2003;110:15-21; quiz 22-13.
14. Hosten N, Bornfeld N, Wassmuth R, et al. Uveal melanoma: detection of extraocular growth with MR imaging and US. *Radiology*. 1997;202:61-67.
15. Scott IU, Murray TG, Hughes JR. Evaluation of imaging techniques for detection of extraocular extension of choroidal melanoma. *Arch Ophthalmol*. 1998;116:897-899.
16. Callender GR. Malignant melanotic tumors of the eye: a study of histologic types in 111 cases. *Trans Am Acad Ophthalmol Otolaryngol*. 1931;36:131-142.
17. Campbell RJ, Sobin LH, Zimmerman LE, World Health Organization. *Histological typing of tumours of the eye and its adnexa*. 2nd ed. Springer: Berlin ; London; 1998. viii, 115 pp.
18. Edge SB, Byrd DR, Compton CC, et al. In: Trotti A, (ed). *AJCC cancer staging manual*. 7th edn. Springer: New York; 2010. pp 547-559.
19. Folberg R, Rummelt V, Parys-Van Ginderdeuren R, et al. The prognostic value of tumor blood vessel morphology in primary uveal melanoma. *Ophthalmology*. 1993;100:1389-1398.
20. Mooy CM, Luyten GP, de Jong PT, et al. Immunohistochemical and prognostic analysis of apoptosis and proliferation in uveal melanoma. *Am J Pathol*. 1995;147:1097-1104.
21. Gill HS, Char DH. Uveal melanoma prognostication: from lesion size and cell type to molecular class. *Canadian journal of ophthalmology Journal canadien d'ophtalmologie*. 2012;47:246-253.

22. COMS. Histopathologic characteristics of uveal melanoma in eyes enucleated from the Collaborative Ocular Melanoma Study. COMS report no. 6. *Am J Ophthalmol*. 1998;125:745-766.

23. Burnier MN, Jr., McLean IW, Gamel JW. Immunohistochemical evaluation of uveal melanocytic tumors. Expression of HMB-45, S-100 protein, and neuron-specific enolase. *Cancer*. 1991;68:809-814.

24. Damato B, Dopierala J, Klaasen A, et al. Multiplex ligation-dependent probe amplification of uveal melanoma: correlation with metastatic death. *Invest Ophthalmol Vis Sci*. 2009;50:3048-3055.

25. Vaarwater J, van den Bosch T, Mensink HW, et al. Multiplex ligation-dependent probe amplification equals fluorescence in-situ hybridization for the identification of patients at risk for metastatic disease in uveal melanoma. *Melanoma Res*. 2012;22:30-37.

26. Kumps C, Van Roy N, Heyrman L, et al. Multiplex Amplicon Quantification (MAQ), a fast and efficient method for the simultaneous detection of copy number alterations in neuroblastoma. *BMC Genomics*. 2010;11:298.

27. Horsman DE, Sroka H, Rootman J, White VA. Monosomy 3 and isochromosome 8q in a uveal melanoma. *Cancer Genet Cytogenet*. 1990;45:249-253.

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

29. Sisley K, Rennie IG, Cottam DW, et al. Cytogenetic findings in six posterior uveal melanoma: involvement of chromosomes 3, 6, and 8. *Genes Chromosomes Cancer*. 1990;2:205-209.

30. Prescher G, Bornfeld N, Hirche H, et al. Prognostic implications of monosomy 3 in uveal melanoma. *Lancet*. 1996;347:1222-1225.

31. Kilic E, van Gils W, Lodder E, et al. Clinical and cytogenetic analyses in uveal melanoma. *Invest Ophthalmol Vis Sci*. 2006;47:3703-3707.

32. Sisley K, Parsons MA, Garnham J, et al. Association of specific chromosome alterations with tumour phenotype in posterior uveal melanoma. *Br J Cancer*. 2000;82:330-338.

33. Sisley K, Rennie IG, Parsons MA, et al. Abnormalities of chromosomes 3 and 8 in posterior uveal melanoma correlate with prognosis. *Genes Chromosomes Cancer*. 1997;19:22-28.

34. White VA, Chambers JD, Courtright PD, Chang WY, Horsman DE. Correlation of cytogenetic abnormalities with the outcome of patients with uveal melanoma. *Cancer*. 1998;83:354-359.

35. Prescher G, Bornfeld N, Friedrichs W, Seeber S, Becher R. Cytogenetics of twelve cases of uveal melanoma and patterns of nonrandom anomalies and isochromosome formation. *Cancer Genet Cytogenet*. 1995;80:40-46.

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

37. Scholes AG, Liloglou T, Maloney P, et al. Loss of heterozygosity on chromosomes 3, 9, 13, and 17, including the retinoblastoma locus, in uveal melanoma. *Invest Ophthalmol Vis Sci*. 2001;42:2472-2477.

38. White VA, McNeil BK, Horsman DE. Acquired homozygosity (isodisomy) of chromosome 3 in uveal melanoma. *Cancer Genet Cytogenet*. 1998;102:40-45.

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

40. Prescher G, Bornfeld N, Becher R. Two subclones in a case of uveal melanoma. Relevance of monosomy 3 and multiplication of chromosome 8q. *Cancer Genet Cytogenet*. 1994;77:144-146.

41. van den Bosch T, van Beek JG, Vaarwater J, et al. Higher percentage of FISH-determined monosomy 3 and 8q amplification in uveal melanoma cells relate to poor patient prognosis. *Invest Ophthalmol Vis Sci*. 2012;53:2668-2674.

42. Fountain JW, Bale SJ, Housman DE, Dracopoli NC. Genetics of melanoma. *Cancer surveys*. 1990;9:645-671.

43. Zhang J, Glatfelter AA, Taetle R, Trent JM. Frequent alterations of evolutionarily conserved regions of chromosome 1 in human malignant melanoma. *Cancer Genet Cytogenet*. 1999;111:119-123.

44. Parrella P, Sidransky D, Merbs SL. Allelotype of posterior uveal melanoma: implications for a bifurcated tumor progression pathway. *Cancer Res*. 1999;59:3032-3037.

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

46. Mensink HW, Kilic E, Vaarwater J, et al. Molecular cytogenetic analysis of archival uveal melanoma with known clinical outcome. *Cancer Genet Cytogenet*. 2008;181:108-111.

47. White JS, McLean IW, Becker RL, Director-Myska AE, Nath J. Correlation of comparative genomic hybridization results of 100 archival uveal melanoma with patient survival. *Cancer Genet Cytogenet*. 2006;170:29-39.

48. Vajdic CM, Hutchins AM, Kricker A, et al. Chromosomal gains and losses in ocular melanoma detected by comparative genomic hybridization in an Australian population-based study. *Cancer Genet Cytogenet*. 2003;144:12-17.

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

50. Petrausch U, Martus P, Tonnies H, et al. Significance of gene expression analysis in uveal melanoma in comparison to standard risk factors for risk assessment of subsequent metastases. *Eye (Lond)*. 2008;22:997-1007.

51. van Gils W, Lodder EM, Mensink HW, et al. Gene expression profiling in uveal melanoma: two regions on 3p related to prognosis. *Invest Ophthalmol Vis Sci*. 2008;49:4254-4262.

52. Worley LA, Onken MD, Person E, et al. Transcriptomic versus chromosomal prognostic markers and clinical outcome in uveal melanoma. *Clin Cancer Res*. 2007;13:1466-1471.

53. Parrella P, Caballero OL, Sidransky D, Merbs SL. Detection of c-myc amplification in uveal melanoma by fluorescent in situ hybridization. *Invest Ophthalmol Vis Sci*. 2001;42:1679-1684.

54. Ehlers JP, Harbour JW. NBS1 expression as a prognostic marker in uveal melanoma. *Clin Cancer Res*. 2005;11:1849-1853.

55. Ehlers JP, Worley L, Onken MD, Harbour JW. DDEF1 is located in an amplified region of chromosome 8q and is overexpressed in uveal melanoma. *Clin Cancer Res*. 2005;11:3609-3613.

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

57. Hoglund M, Gisselsson D, Hansen GB, et al. Dissecting karyotypic patterns in malignant melanoma: temporal clustering of losses and gains in melanoma karyotypic evolution. *Int J Cancer*. 2004;108:57-65.

58. Abdel-Rahman MH, Yang Y, Zhou XP, et al. High frequency of submicroscopic hemizygous deletion is a major mechanism of loss of expression of PTEN in uveal melanoma. *J Clin Oncol*. 2006;24:288-295.

59. Ibrahim N, Haluska FG. Molecular pathogenesis of cutaneous melanocytic neoplasms. *Annu Rev Pathol*. 2009;4:551-579.

60. Weber A, Hengge UR, Urbanik D, et al. Absence of mutations of the BRAF gene and constitutive activation of extracellular-regulated kinase in malignant melanoma of the uvea. *Lab Invest*. 2003;83:1771-1776.

61. Zuidervaart W, van Nieuwpoort F, Stark M, et al. Activation of the MAPK pathway is a common event in uveal melanoma although it rarely occurs through mutation of BRAF or RAS. *Br J Cancer*. 2005;92:2032-2038.

62. Davies H, Bignell GR, Cox C, et al. Mutations of the BRAF gene in human cancer. *Nature*. 2002;417:949-954.

63. Pollock PM, Harper UL, Hansen KS, et al. High frequency of BRAF mutations in nevi. *Nat Genet*. 2003;33:19-20.

64. Cohen Y, Goldenberg-Cohen N, Parrella P, et al. Lack of BRAF mutation in primary uveal melanoma. *Invest Ophthalmol Vis Sci*. 2003;44:2876-2878.

65. Kilic E, Bruggenwirth HT, Verbiest MM, et al. The RAS-BRAF kinase pathway is not involved in uveal melanoma. *Melanoma Res*. 2004;14:203-205.

66. Mooy CM, Van der Helm MJ, Van der Kwast TH, et al. No N-ras mutations in human uveal melanoma: the role of ultraviolet light revisited. *Br J Cancer*. 1991;64:411-413.

67. Saldanha G, Purnell D, Fletcher A, et al. High BRAF mutation frequency does not characterize all melanocytic tumor types. *Int J Cancer*. 2004;111:705-710.

68. Bauer J, Kilic E, Vaarwater J, et al. Oncogenic GNAQ mutations are not correlated with disease-free survival in uveal melanoma. *Br J Cancer*. 2009;101:813-815.

69. Van Raamsdonk CD, Bezrookove V, Green G, et al. Frequent somatic mutations of GNAQ in uveal melanoma and blue naevi. *Nature*. 2009;457:599-602.

70. Van Raamsdonk CD, Griewank KG, Crosby MB, et al. Mutations in GNA11 in uveal melanoma. *N Engl J Med*. 2010;363:2191-2199.

71. Harbour JW, Onken MD, Roberson ED, et al. Frequent mutation of BAP1 in metastasising uveal melanoma. *Science*. 2010;330:1410-1413.

72. Farmer H, McCabe N, Lord CJ, et al. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature*. 2005;434:917-921.

73. Ventii KH, Devi NS, Friedrich KL, et al. BRCA1-associated protein-1 is a tumor suppressor that requires deubiquitinating activity and nuclear localization. *Cancer Res*. 2008;68:6953-6962.

74. Seddon JM, Gragoudas ES, Albert DM, et al. Comparison of survival rates for patients with uveal melanoma after treatment with proton beam irradiation or enucleation. *Am J Ophthalmol*. 1985;99:282-290.

75. Seddon JM, Gragoudas ES, Egan KM, et al. Relative survival rates after alternative therapies for uveal melanoma. *Ophthalmology*. 1990;97:769-777.

76. Spagnolo F, Caltabiano G, Queirolo P. Uveal melanoma. *Cancer Treat Rev*. 2012;38:549-553.

77. Bergman L, Nilsson B, Lundell G, Lundell M, Seregard S. Ruthenium brachytherapy for uveal melanoma, 1979-2003: survival and functional outcomes in the Swedish population. *Ophthalmology*. 2005;112:834-840.

78. Damato B, Patel I, Campbell IR, Mayles HM, Errington RD. Local tumor control after 106Ru brachytherapy of choroidal melanoma. *International journal of radiation oncology, biology, physics*. 2005;63:385-391.

79. Bartlema YM, Oosterhuis JA, Journee-De Korver JG, Tjho-Heslinga RE, Keunen JE. Combined plaque radiotherapy and transpupillary thermotherapy in choroidal melanoma: 5 years' experience. *Br J Ophthalmol*. 2003;87:1370-1373.

80. Pe'er J. Ruthenium-106 brachytherapy. *Dev Ophthalmol*. 2012;49:27-40.

81. Shields CL, Shields JA, Perez N, Singh AD, Cater J. Primary transpupillary thermotherapy for small choroidal melanoma in 256 consecutive cases: outcomes and limitations. *Ophthalmology*. 2002;109:225-234.

82. Gragoudas ES, Li W, Lane AM, Munzenrider J, Egan KM. Risk factors for radiation maculopathy and papillopathy after intraocular irradiation. *Ophthalmology*. 1999;106:1571-1577; discussion 1577-1578.

83. Kinyoun JL, Lawrence BS, Barlow WE. Proliferative radiation retinopathy. *Arch Ophthalmol*. 1996;114:1097-1100.

84. Summanen P, Immonen I, Kivela T, et al. Radiation related complications after ruthenium plaque radiotherapy of uveal melanoma. *Br J Ophthalmol*. 1996;80:732-739.

85. Damato B, Foulds WS. Indications for trans-scleral local resection of uveal melanoma. *Br J Ophthalmol*. 1996;80:1029-1030.

86. Gragoudas ES. 1996 Jules Gonin Lecture of the Retina Research Foundation. Long-term results after proton irradiation of uveal melanoma. *Graefes Arch Clin Exp Ophthalmol*. 1997;235:265-267.

87. Seregard S, aft Trampe E, Lax I, Kock E, Lundell G. Results following episcleral ruthenium plaque radiotherapy for posterior uveal melanoma. The Swedish experience. *Acta Ophthalmol Scand*. 1997;75:11-16.

88. Wilson MW, Hungerford JL. Comparison of episcleral plaque and proton beam radiation therapy for the treatment of choroidal melanoma. *Ophthalmology*. 1999;106:1579-1587.

89. Bell DJ, Wilson MW. Choroidal melanoma: natural history and management options. *Cancer Control*. 2004;11:296-303.

90. Gunduz K, Shields CL, Shields JA, et al. Radiation complications and tumor control after plaque radiotherapy of choroidal melanoma with macular involvement. *Am J Ophthalmol*. 1999;127:579-589.

91. Lommatzsch PK, Werschnik C, Schuster E. Long-term follow-up of Ru-106/Rh-106 brachytherapy for posterior uveal melanoma. *Graefes Arch Clin Exp Ophthalmol*. 2000;238:129-137.

92. Tjho-Heslinga RE, Davelaar J, Kemme HM, et al. Results of ruthenium irradiation of uveal melanoma: the Dutch experience. *Radiother Oncol*. 1999;53:133-137.

93. Desjardins L, Lumbroso-Le Rouic L, Levy-Gabriel C, et al. Treatment of uveal melanoma by accelerated proton beam. *Dev Ophthalmol*. 2012;49:41-57.

94. Gragoudas ES, Lane AM, Regan S, et al. A randomized controlled trial of varying radiation doses in the treatment of choroidal melanoma. *Arch Ophthalmol*. 2000;118:773-778.

95. Weber DC, Bogner J, Verwey J, et al. Proton beam radiotherapy versus fractionated stereotactic radiotherapy for uveal melanoma: A comparative study. *International journal of radiation oncology, biology, physics*. 2005;63:373-384.

96. Conway RM, Poothullil AM, Daftari IK, et al. Estimates of ocular and visual retention following treatment of extra-large uveal melanoma by proton beam radiotherapy. *Arch Ophthalmol*. 2006;124:838-843.

97. Egan KM, Ryan LM, Gragoudas ES. Survival implications of enucleation after definitive radiotherapy for choroidal melanoma: an example of regression on time-dependent covariates. *Arch Ophthalmol*. 1998;116:366-370.

98. Egger E, Zografos L, Schalenbourg A, et al. Eye retention after proton beam radiotherapy for uveal melanoma. *International journal of radiation oncology, biology, physics*. 2003;55:867-880.

99. Krema H, Somani S, Sahgal A, et al. Stereotactic radiotherapy for treatment of juxtapapillary choroidal melanoma: 3-year follow-up. *Br J Ophthalmol*. 2009;93:1172-1176.

100. Muller K, Nowak PJ, de Pan C, et al. Effectiveness of fractionated stereotactic radiotherapy for uveal melanoma. *International journal of radiation oncology, biology, physics*. 2005;63:116-122.

101. Zehetmayer M, Kitz K, Menapace R, et al. Local tumor control and morbidity after one to three fractions of stereotactic external beam irradiation for uveal melanoma. *Radiother Oncol*. 2000;55:135-144.

102. Barbazetto IA, Lee TC, Rollins IS, Chang S, Abramson DH. Treatment of choroidal melanoma using photodynamic therapy. *Am J Ophthalmol*. 2003;135:898-899.

103. Canal-Fontcuberta I, Salomao DR, Robertson D, et al. Clinical and histopathologic findings after photodynamic therapy of choroidal melanoma. *Retina*. 2012;32:942-948.

104. Donaldson MJ, Lim L, Harper CA, Mackenzie J, W GC. Primary treatment of choroidal amelanotic melanoma with photodynamic therapy. *Clin Experiment Ophthalmol*. 2005;33:548-549.

105. Damato BE. Local resection of uveal melanoma. *Dev Ophthalmol*. 2012;49:66-80.

106. Robertson DM. Melanoma endoresection: a perspective. *Retina*. 2001;21:403-407.

107. Garcia-Arumi J, Zapata MA, Balaguer O, et al. Endoresection in high posterior choroidal melanoma: long-term outcome. *Br J Ophthalmol*. 2008;92:1040-1045.

108. Affeldt JC, Minckler DS, Azen SP, Yeh L. Prognosis in uveal melanoma with extrascleral extension. *Arch Ophthalmol*. 1980;98:1975-1979.

109. Zimmerman LE, McLean IW. Do growth and onset of symptoms of uveal melanoma indicate subclinical metastasis? *Ophthalmology*. 1984;91:685-691.

110. Eskelin S, Pyrhonen S, Hahka-Kemppinen M, Tuomaala S, Kivela T. A prognostic model and staging for metastatic uveal melanoma. *Cancer*. 2003;97:465-475.

111. Bol KF, Mensink HW, Aarntzen EH, et al. Long Overall Survival After Dendritic Cell Vaccination in Metastatic Uveal Melanoma Patients. *Am J Ophthalmol*. 2014;158:939-947.

112. Augsburger JJ, Correa ZM, Shaikh AH. Effectiveness of treatments for metastatic uveal melanoma. *Am J Ophthalmol*. 2009;148:119-127.

113. Mariani P, Piperno-Neumann S, Servois V, et al. Surgical management of liver metastases from uveal melanoma: 16 years' experience at the Institut Curie. *Eur J Surg Oncol*. 2009;35:1192-1197.

CHAPTER 2

Candidate genes involved in uveal melanoma tumourigenesis

CHAPTER 2.1

Patient survival in uveal melanoma is not affected by oncogenic mutations in *GNAQ* and *GNA11*

Anna E. Koopmans, Jolanda Vaarwater, Dion Paridaens, Nicole C. Naus, Emine Kiliç and Annelies de Klein

Br J Cancer. 2013 Jul 23;109(2):493-496

ABSTRACT

Background. Mutations in *GNAQ* and *GNA11*, encoding the oncogenic G protein alpha subunit q and 11, respectively, occur frequently in the majority of uveal melanoma.

Methods. Exon 4 and 5 from *GNAQ* and *GNA11* were amplified and sequenced from 92 ciliary body and choroidal melanoma. The mutation status was correlated with disease-free survival and other parameters.

Results. None of the tumours harboured a *GNAQ* exon 4 mutation. A *GNAQ* mutation in exon 5 codon 209 was found in 46/92 (50.0%) of the tumours. Only 1/92 (1.1%) melanoma showed a mutation in *GNA11* exon 4 codon 183, while 39/92 (42.4%) harboured a mutation in exon 5 of *GNA11* codon 209. Six tumours did not show any mutations in exons 4 and 5 of these genes. Univariate analyses showed no correlation between disease-free survival and the mutation status.

Conclusion. *GNAQ* and *GNA11* mutations are, in equal matter, not associated with patient outcome.

INTRODUCTION

Previous studies identified high frequencies of activating somatic mutations in the *GNAQ* and *GNA11* genes in uveal melanoma (UM).[1-4] *GNAQ* and *GNA11* encode the heterotrimeric guanine nucleotide-binding protein G subunit alpha q and 11, respectively. Mutations in *GNAQ*, or its paralog *GNA11* (together Gα genes), occur mutually exclusively in codon 183 (exon 4) or 209 (exon 5), leading to a constitutive activation of the MAP kinase (MAPK) pathway.[4, 5] Limited information is available on correlation of the mutations with survival. We examined to what extent oncogenic *GNAQ* and *GNA11* mutations are correlated with the patient survival.

METHODS

Uveal melanoma were collected from enucleated patients at Erasmus University Medical Centre and the Rotterdam Eye Hospital (Rotterdam, the Netherlands). Informed consent was obtained before the operation and the study was performed according to the tenets of the Declaration of Helsinki. Fresh tumour material was obtained within 1 h of enucleation and processed for fluorescence *in situ* hybridisation (FISH) as described previously.[6] Part of the tumour was snap-frozen and stored in liquid nitrogen. The remainder of the eye was embedded in paraffin. All tumours were histopathologically confirmed. Only tumours located in the ciliary body and choroid were included in this study. FISH analysis was performed on directly fixated tumour cells for chromosome 1, 3, 6 and 8 using centromeric or locus-specific probes.[6] High-resolution whole-genome analysis was performed on tumour-derived DNA, using the Illumina BeadChip HumanCytoSNP-12 v2 (Illumina, San Diego, CA, USA) according to the manufacturer's protocol. Filtering, normalisation and data analysis were done using version 6 of the Nexus software program (BioDiscovery, El Segundo, CA, USA). In total, 92 patients were selected for whom follow-up and clinical, histopathological, and cytogenetic data were available.

DNA isolation

To examine tumour content, haematoxylin and eosin (H&E) staining was conducted on a 5-µm section of snap-frozen tumour. Depending on the size of the tumour, 10 – 15 sections of 20 µm were used for DNA isolation using QIAmp DNA-mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA concentration was measured with the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

GNAQ and GNA11 mutation analysis

In a previous study, our group performed mutation analysis of *GNAQ* exon 5 in 75 samples.[1] In the present study, we amplified *GNAQ* exon 5 in 17 other tumour samples with polymerase chain reaction (PCR) using the primers 5′-ACCATTTTGCTTGGCACAGATAAGG-3′ and 5′-GTAAGTTCACTCCATTCCCCACACC-3′. *GNAQ* exon 4 and *GNA11* exon 4 and 5 were amplified using the primers: 5′-TCTTTTTCTCCCACCCCTTGC-3′ and 5′-TTGTTTTGAAGCCTACACATGATTCC-3′ to examine *GNAQ* exon 4, 5′-GTGCTGTGTCCCTGTCCTG-3′ and 5′-GGCAAATGAGCCTCTCAGTG-3′ to examine *GNA11* exon 4, and 5′-GATTGCAGATTGGGCCTTGG-3′ and 5′-TCTCCTCCATCCGGTTCTGG-3′ to examine *GNA11* exon 5. PCR products were purified using ExoSAP-IT (USB, Staufen, Germany), and sequenced using BigDye Terminator chemistry v3.1 on an ABI Prism 3130xl Genetic Analyser (Applied Biosystems, Foster City, CA, USA). Sequences were aligned and compared with reference sequence hg19 from the Ensemble genome database (ENST00000286548 and ENST00000078429) using SeqScape software version 2.6 (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

The primary endpoint for disease-free survival was defined as the time to the development of metastatic disease, whereby death due to other causes was treated as censored. The influence of single prognostic factors on disease-free survival was assessed using the Kaplan-Meier method (for categorical variables) or the Cox proportional hazard analysis (for continuous variables). To identify the independent value of the prognostic factors on disease-free survival, we used a multivariate Cox proportional hazard analysis with a forward stepwise method based on likelihood ratios. An effect was considered significant if the P-value was ≤ 0.05. The statistical analyses were performed with the SPSS software version 20.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

A total of 92 patients were included in the study. Forty-eight of the patients were male and 44 were female. The median age was 62 years (range 21 – 86); the mean largest tumour diameter was 13.3 mm (range 7.0 – 19.0) and the mean tumour thickness was 8.3 mm (range 1.5 – 22.0). On the basis of cell type, 15 tumours were classified as epithelioid, 38 as mixed, and 39 as spindle-cell tumours. Most tumours were localised in the choroid; only 6 were localised in the ciliary body. The mean follow-up was 74.9 months (range 5.2 – 200.5) and

44 patients developed metastases, from which 39 died. Sixteen patients died due to another cause and 32 patients were still alive at the end of the study.

Molecular genetic analysis

All UM were analysed for *GNAQ* and *GNA11* mutations and for chromosomal aberrations in chromosome 1, 3, 6 and 8. No mutations were found in *GNAQ* exon 4. Forty-six tumours (50.0%) harboured a mutation in *GNAQ* exon 5 codon 209; details are shown in **Table 1**. Although only one mutated case was found in *GNA11* exon 4, 39 tumours (42.4%) harboured a mutation in *GNA11* exon 5. Six out of 92 tumours contained no mutations in exons 4 and 5 of both genes. One tumour (EOM-0179) showed two mutations in *GNA11* exon 5 (resulting in p.Q209L and p.R214M). Tumour sample EOM-0179 was therefore subjected to deep sequencing with a custom designed HaloPlex Target Enrichment kit for Illumina (Agilent Technologies, Santa Clara, California, USA) and both variants were located within the same read (Koopmans et al., manuscript in preparation). No DNA from blood of this patient was available to determine whether variant R214M is a germline variant. Therefore, we isolated DNA from formalin-fixed paraffin-embedded retina tissue, and Sanger sequencing of *GNA11* exon 5 revealed a wild-type status.

Statistical analysis

Univariate analyses showed that the disease-free survival was significantly shorter in patients with tumours with loss of chromosome 3, loss of chromosome 8p and gain of chromosome 8q. The disease-free survival in patients with tumours harbouring *GNAQ* or *GNA11* mutations was not significantly less than that in the wild-type tumours (**Figure 1**). Correlations between the clinical and histopathological parameters, chromosomal parameters, and *GNAQ*

Table 1. Mutations found in *GNAQ* and *GNA11* in detail

Gene	Mutation	No. of cases	Total (%)
GNAQ exon 4	-	0	0
GNAQ exon 5	Heterozygous Q209L	16	50.0
	Heterozygous Q209P	28	
	Homozygous Q209P	1	
	Heterozygous Q209R	1	
GNA11 exon 4	Heterozygous R183C	1	1.1
GNA11 exon 5	Heterozygous Q209L	37	42.4
	Heterozygous Q209P	1	
	Heterozygous Q209L + heterozygous R214M	1	

Abbreviations : GNAQ = G protein alpha subunit q; GNA11 = G protein alpha subunit 11.

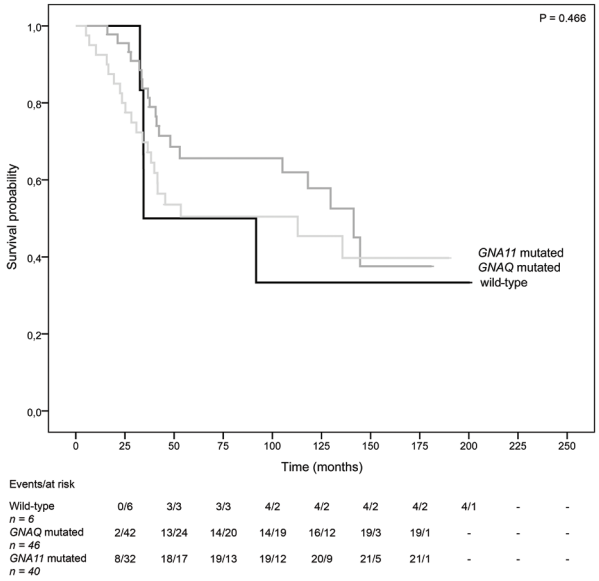


Figure 1. Kaplan-Meier estimate of disease-free survival in patients with tumours harbouring either a *GNAQ* or *GNA11* mutation compared with tumours harbouring no mutation (wild-type). The table shows the number of events and cases at risk over time at the respective time point. Log-rank test was used to compare survival distributions across subgroups.

and *GNA11* mutations using the Fisher's exact test and the Mann-Whitney test showed a weak association between age and both *GNAQ* and *GNA11* mutation status (P = 0.017 and P = 0.004, respectively; **Table 2**). *GNA11* mutation status was also correlated with loss of chromosome 6q (P = 0.045). We examined the possibility that *GNAQ* and *GNA11* mutations may affect the prognosis of patients with monosomy 3 by constructing Kaplan-Meier curves for changes in chromosome 3, stratified for *GNAQ* and *GNA11* mutations. Log-rank tests showed that there was no significant effect on the disease-free survival in tumours with loss of chromosome 3 and the presence of *GNAQ* or *GNA11* mutation (P = 0.745). Multivariate models were constructed for *GNAQ* and *GNA11* separately with positive variables from the univariate analysis. The presence of epithelioid cells, largest tumour diameter, involvement of the ciliary body, chromosome 3 loss, chromosome 8p loss and mutations in *GNAQ* (P = 0.587) or *GNA11* (P = 0.796) were rejected. Only the variable chromosome 8q gain (hazard ratio (HR) 6.562, P = 0.000 for both *GNAQ* and *GNA11* mutation status) and chromosome 6p gain (HR 0.419, P = 0.014 for both *GNAQ* and *GNA11* mutation status) were independent predictors of disease-free survival.

Table 2. Correlations between *GNAQ* and *GNA11* mutations and clinical, histopathological and chromosomal data

	***GNAQ* mutation status**			***GNA11* mutation status**		
	Mutated	**Wild-type**		**Mutated**	**Wild-type**	
Variable	***n* = 46**	***n* = 46**	**P-value**	***n* = 40**	***n* = 52**	**P-value**
Mean age (years)	58.9 ± 1.9	65.0 ± 2.0	**0.017**[a]	66.5 ± 2.0	58.4 ± 1.9	**0.004**[a]
Mean largest tumour diameter (mm)	13.6 ± 0.4	13.0 ± 0.4	0.350[a]	13.3 ± 0.4	13.3. ± 0.4	0.915[a]
Mean tumour thickness (mm)	8.5 ± 0.6	8.0 ± 0.5	0.885[a]	8.1 ± 0.5	8.4 ± 0.5	0.968[a]
Gender, *n* (%)						
Male	25 (27.2%)	23 (25.0%)	0.676[b]	21 (22.8%)	27 (29.3%)	0.956[b]
Female	21 (22.8%)	23 (25.0%)		19 (20.7%)	25 (27.2%)	
Cell type, *n* (%)						
Spindle	17 (18.5%)	14 (15.2%)	0.508[b]	11 (12.0%)	20 (21.7%)	0.270[b]
Mixed/epithelioid	29 (31.5%)	32 (34.8%)		29 (31.5%)	32 (34.8%)	
Involvement of the ciliary body, *n* (%)						
Yes	7 (7.6%)	10 (10.9%)	0.420[b]	8 (8.7%)	9 (9.8%)	0.742[b]
No	39 (42.4%)	36 (39.1%)		32 (34.8%)	43 (46.7%)	
Chromosome 1p loss, *n* (%)						
Yes	16 (17.4%)	15 (16.3%)	1.000[b]	13 (14.1%)	18 (19.6%)	1.000[b]
No	30 (32.6%)	31 (33.7%)		27 (29.3%)	34 (37.0%)	
Chromosome 3 loss, *n* (%)						
Yes	25 (27.2%)	33 (35.9%)	0.130[b]	29 (31.5%)	29 (31.5%)	0.128[b]
No	21 (22.8%)	13 (14.1%)		11 (12.0%)	23 (25.0%)	
Chromosome 6p gain, *n* (%)						
Yes	19 (20.9%)	16 (17.6%)	0.668[b]	14 (15.4%)	21 (23.1%)	0.828[b]
No	27 (29.7%)	29 (31.9%)		25 (27.5%)	31 (34.1%)	
Chromosome 6q loss, *n* (%)						
Yes	12 (13.2%)	19 (20.9%)	0.125[b]	18 (19.8%)	13 (14.3%)	**0.045**[b]
No	34 (37.3%)	26 (28.6%)		21 (23.1%)	39 (42.8%)	
Chromosome 6q gain, *n* (%)						
Yes	3 (3.3%)	4 (4.4%)	0.714[b]	4 (4.4%)	3 (3.3%)	0.456[b]
No	43 (47.3%)	41 (45.0%)		35 (38.5%)	49 (53.8%)	
Chromosome 8p loss, *n* (%)						
Yes	9 (9.8%)	10 (10.9%)	1.000[b]	10 (10.9%)	9 (9.8%)	0.440[b]
No	37 (40.2%)	36 (39.1%)		30 (32.6%)	43 (46.7%)	

Table 2. Correlations between *GNAQ* and *GNA11* mutations and clinical, histopathological and chromosomal data (continued)

	***GNAQ* mutation status**			***GNA11* mutation status**		
	Mutated	**Wild-type**		**Mutated**	**Wild-type**	
Variable	***n* = 46**	***n* = 46**	**P-value**	***n* = 40**	***n* = 52**	**P-value**
Chromosome 8p gain, *n* (%)						
Yes	9 (9.8%)	11 (12.0%)	0.801[b]	9 (9.8%)	11 (12.0%)	1.000[b]
No	37 (40.2%)	35 (38.0%)		31 (33.7%)	41 (44.5%)	
Chromosome 8q gain, *n* (%)						
Yes	26 (28.3%)	34 (37.0%)	0.125[b]	30 (32.6%)	30 (32.6%)	0.122[b]
No	20 (21.7%)	12 (13.0%)		10 (10.9%)	22 (23.9%)	

Abbreviations: GNAQ = G protein alpha subunit q; GNA11 = G protein alpha subunit 11.
The significant correlations (P ≤ 0.05) are shown in bold.
[a] The P-value for the comparison of continuous variables among different subgroups was calculated with the Mann-Whitney test.
[b] The P-value for the comparison of categorical variables among different subgroups was calculated with the Fisher's exact test.

DISCUSSION

In this study, we investigated whether *GNAQ* and *GNA11* mutations in UM are associated with patient survival. We found that these mutations occur mutually exclusive in the majority of UM, up to 93.4%, which is in the same range as reported previously.[1-4] Van Raamsdonk et al.[4] suggested that *GNA11* mutations might have more potent effect on melanocytes than mutations in *GNAQ*. Because the mutations occur in 93.4% of the tumours, it seems to be an early event in the development of a melanoma, and our study demonstrates that mutations in *GNAQ* and *GNA11* do not contribute to the patients' prognosis. Moreover, we conclude that *GNA11* mutations are not more harmful than *GNAQ* mutations in UM patients.

All mutations were localised either in codon 209 (exon 5) for both *GNAQ* and *GNA11* or codon 183 (exon 4) for *GNA11* only. Surprisingly, one tumour harboured a double mutation in *GNA11* codon 209 and 214. The reported heterozygous non-synonymous variant in codon 214 results in arginine to methionine transition. A germline variant was excluded by sequencing normal retinal tissue. Using the *in silico* tool PolyPhen-2 both these transitions seem to be damaging on the structure and function of the protein. The tumour with the double mutation had no chromosomal alterations, and this patient has not developed any metastases at a follow-up time of 154.1 months. To our knowledge, this is the first reported double mutation in *GNA11* exon 5 in UM.

Recently, the Gα genes have been investigated in metastatic lesions showing no dif-

ference in mutation frequency between rapidly progressive and slowly progressive lesions.[7] This is in line with our findings that patient outcome is not influenced by the presence of mutations in *GNAQ* or *GNA11*.

GNAQ and *GNA11* are involved in the MAPK pathway, and mutations in these genes lead to downstream oncogenic signalling.[4, 5] Currently, new therapeutic strategies that inhibit the downstream signalling molecules are being investigated. MEK is a potential target in the MAPK pathway, and the effects of several MEK inhibitors on uveal melanoma cell lines with Gα mutations have been described.[8, 9] In a preclinical study, Gα-mutant UM cells were mildly sensitive to the MEK inhibitor AZD6244, and either moderately or highly sensitive to the MEK inhibitor TAK733. Dual-pathway inhibition of the MAPK and the PI3K/AKT pathway with MEK inhibitor GSK1120212 and PI3K inhibitor GSK2126458 resulted in induction of apoptosis in Gα-mutant UM cells.[10]

In conclusion, we confirm that mutations in *GNAQ* and *GNA11* are, in equal matter, not associated with patient outcome. Also the newly found variant with a double mutation does not affect patient survival. Because the mutations occur in the majority of the tumours, and slowly growing as well as fast growing metastases, targeting of the downstream pathway seems promising. Even though there is no relation with development of metastatic disease, the new therapeutic options would be ideal in stabilising the disease process. At this moment, clinical studies are ongoing and the results have not yet been evaluated.

ACKNOWLEDGEMENTS

We thank Farzia Fakhry for her contributions to the project. This study was supported by grants from the Combined Ophthalmic Research Rotterdam (CORR) and the Stichting Nederlands Oogheelkundig Onderzoek (SNOO).

REFERENCES

1. Bauer J, Kilic E, Vaarwater J, et al. Oncogenic GNAQ mutations are not correlated with disease-free survival in uveal melanoma. *Br J Cancer*. 2009;101:813-815.
2. Onken MD, Worley LA, Long MD, et al. Oncogenic mutations in GNAQ occur early in uveal melanoma. *Invest Ophthalmol Vis Sci*. 2008;49:5230-5234.
3. Van Raamsdonk CD, Bezrookove V, Green G, et al. Frequent somatic mutations of GNAQ in uveal melanoma and blue naevi. *Nature*. 2009;457:599-602.
4. Van Raamsdonk CD, Griewank KG, Crosby MB, et al. Mutations in GNA11 in uveal melanoma. *N Engl J Med*. 2010;363:2191-2199.
5. Van Raamsdonk CD, Barsh GS, Wakamatsu K, Ito S. Independent regulation of hair and skin color by two G protein-coupled pathways. *Pigment Cell Melanoma Res*. 2009;22:819-826.
6. Kilic E, Naus NC, van Gils W, et al. Concurrent loss of chromosome arm 1p and chromosome 3 predicts a decreased disease-free survival in uveal melanoma patients. *Invest Ophthalmol Vis Sci*. 2005;46:2253-2257.
7. Abdel-Rahman MH, Cebulla CM, Verma V, et al. Monosomy 3 status of uveal melanoma metastases is associated with rapidly progressive tumors and short survival. *Exp Eye Res*. 2012;100:26-31.
8. Mitsiades N, Chew SA, He B, et al. Genotype-dependent sensitivity of uveal melanoma cell lines to inhibition of B-Raf, MEK, and Akt kinases: rationale for personalized therapy. *Invest Ophthalmol Vis Sci*. 2011;52:7248-7255.
9. von Euw E, Atefi M, Attar N, et al. Antitumor effects of the investigational selective MEK inhibitor TAK733 against cutaneous and uveal melanoma cell lines. *Mol Cancer*. 2012;11:22.
10. Khalili JS, Yu X, Wang J, et al. Combination Small Molecule MEK and PI3K Inhibition Enhances Uveal Melanoma Cell Death in a Mutant GNAQ- and GNA11-Dependent Manner. *Clin Cancer Res*. 2012;18:4345-4355.

CHAPTER 2.2

Clinical significance of immunohistochemistry for detection of *BAP1* mutations in uveal melanoma

Anna E. Koopmans, Robert M. Verdijk, Rutger W.W. Brouwer, Thierry P.P. van den Bosch, Mike M.P. van den Berg, Jolanda Vaarwater, Christel E.M. Kockx, Dion Paridaens, Nicole C. Naus, Mark Nellist, Wilfred F.J. van IJcken, Emine Kiliç and Annelies de Klein

Modern Pathol. 2014 Oct;27(10):1321-1330

ABSTRACT

Uveal melanoma is a lethal cancer with a strong propensity to metastasise. Limited therapeutic options are available once the disease has disseminated. A strong predictor for metastasis is the loss of chromosome 3. Inactivating mutations in *BAP1* encoding the BRCA1-associated protein 1 and located on chromosome 3p21.1, have been described in uveal melanoma and other types of cancer. In this study, we determined the prevalence of somatic *BAP1* mutations and examined whether these mutations correlate with the functional expression of BAP1 in uveal melanoma tissue and with other clinical, histopathological and chromosomal parameters. We screened a cohort of 74 uveal melanoma for *BAP1* mutations, using different deep sequencing methods. The frequency of *BAP1* mutations in our study group was 47%. The expression of BAP1 protein was studied using immunohistochemistry. BAP1 staining was absent in 43% of the cases. *BAP1* mutation status was strongly associated with BAP1 protein expression ($P < 0.001$), loss of chromosome 3 ($P < 0.001$), and other aggressive prognostic factors. Patients with a *BAP1* mutation and absent BAP1 expression had an almost eightfold higher chance of developing metastases compared to those without these changes ($P = 0.002$). We found a strong correlation between the immunohistochemical and sequencing data and therefore propose that, immunohistochemical screening for BAP1 should become routine in the histopathological work-up of uveal melanoma. Furthermore, our analysis indicates that loss of BAP1 may be specifically involved in the progression of uveal melanoma to an aggressive, metastatic phenotype.

INTRODUCTION

With an incidence in the Western world of about 5 per million people per year, uveal melanoma (UM) is the most common primary malignancy in the eye.[1] Approximately half of the individuals who were diagnosed with UM will develop metastatic disease, with a 4 – 6-month median survival period when metastasised to the liver.[2] Several prognostic parameters are available to identify patients at risk of developing metastases including cytogenetic aberrations such as loss of chromosome 1p, loss of chromosome 3, gain of chromosome 8 and abnormalities on chromosome 6.[3-7]

Harbour et al.,[8] reported inactivating somatic mutations in *BAP1*, the gene encoding BRCA1-associated protein 1 in the predominantly metastasising (class 2) UM. *BAP1* is located on chromosome 3p21.1, which is frequently deleted in UM. Monosomy 3 is considered to be a relatively early event in UM pathogenesis, and several studies have shown that it strongly correlates with decreased survival.[4-7, 9] BAP1 is a nuclear deubiquitinase which catalysis the removal of single ubiquitin moieties from ubiquitin chains or cleavage of the isopeptide bond between ubiquitin and the substrate protein.[10] It is involved in several biological processes, including chromatin dynamics, the DNA damage response and regulation of the cell cycle and cell growth.[11-13] Inactivating somatic and germline *BAP1* mutations have been identified in a variety of cancers, including malignant pleural mesotheliomas, cutaneous melanoma, atypical cutaneous melanocytic tumours, meningioma, lung adenocarcinoma and renal cell carcinoma.[14-19] The number of reported cancer-prone families with germline *BAP1* mutations is rising and suggesting a *BAP1* cancer syndrome. However, the prevalence of germline *BAP1* mutations in UM patients is low compared to *BAP1* mutations of somatic origin.[8, 16, 20] Although somatic mutations in *BAP1* are highly prevalent in metastasising primary UM, the role of *BAP1* in the progression of UM towards metastatic disease requires further investigation.

The purpose of this study was to identify *BAP1* mutations in UM patients and examine whether these mutations coincide with the protein expression of BAP1 in UM tissue. We also investigated whether *BAP1* mutations in UM were associated with additional clinical, histopathological, and chromosomal parameters.

MATERIAL AND METHODS

Tissue samples

Uveal melanoma specimens were collected from patients who underwent enucleation be-

tween the period 1993 and 2012 at the Erasmus University Medical Centre and the Rotterdam Eye Hospital (Rotterdam, The Netherlands). Clinical and histopathological features, such as tumour location, tumour diameter and thickness, age at time of diagnosis, cell type, and the presence of extracellular matrix patterns were evaluated. Cell type was scored by haematoxylin and eosin (H&E) staining according to the modified Callender classification system. The presence of extracellular matrix patterns were examined with periodic acid-Schiff staining without haematoxylin. The study was performed according to the tenets of the Declaration of Helsinki and an informed consent was obtained before to the operation.

DNA extraction

DNA was isolated from fresh tumour samples using the QIAamp DNA-mini kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. The DNA concentration was measured using the NanoDrop ND-1000 Spectrophotometer (NanoDrop technologies, Wilmington, DE, USA) and Picogreen assay (Molecular Probes, Eugene, OR, USA). DNA was stored at -20˚C.

Copy number analysis

The DNA copy number status of the tumour was examined with single nucleotide polymorphism (SNP) array and fluorescent *in situ* hybridisation (FISH) analysis. Two hundred nanograms of fresh tumour DNA was used as input for whole-genome analysis by SNP array (Illumina 610Q BeadChip, Illumina, San Diego, CA, USA). The data were analysed with version 6 of the Nexus software (BioDiscovery, El Segundo, CA, USA). Chromosomal abnormalities were validated with FISH on directly fixed tumour cells using centromeric or locus-specific probes for chromosome 1, 3, 6, and 8, as described previously.[21]

Sequence data analysis

A 10.2 kilobase (kb) region containing the entire *BAP1* gene was amplified from primary choroidal and ciliary body melanomas by long-range polymerase chain reaction (PCR) kit (Takara Holdings, Kyoto, Japan) using the primers 5'-GGCGCCGCTGTACTGGAGCTTTAGT-3' and 5'-CGGCAGAGGAGAGCGGGACAGAGG-3'. Details of the PCR protocol are available upon request. If no PCR product could be obtained, two additional primers (5'-GGCAGCCTCCCCACAAGCCAAGG-3' and 5'-CGGCAGAGGAGAGCGGGACAGAGG-3') were used to amplify the gene as two overlapping 6.6 kb and 4.2 kb fragments. The amplified DNA was then purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Sample preparation was performed according

to the Illumina TruSeq v3 protocol and the samples were sequenced on the Hiseq2000 with a v3 paired-end flow cell for a read length of two times 100 base pairs (bp) with an index of 7 bp. The reads were aligned against the human reference genome build 19 (hg19) using BWA[22] and the NARWHAL pipeline.[23] Genetic variants were called using tools from the genome analysis toolkit,[24] Picard and samtools.[25] A VCF and Mpileup file for each sample were generated and processed with an in-house variant annotation tool.

The *BAP1* region was captured and a unique index code with a length of 7 bp was incorporated into the sample using a HaloPlex Target Enrichment Kit (Agilent Technologies, Santa Clara, CA, USA). Sample preparation was performed as above and the samples were sequenced on the MiSeq using v2 flow cell for a paired-end read length of 150 bp. Adapter trimming, alignment, variant calling and annotation were performed as above.

Whole exome sequencing was performed using the Agilent version 4 capture kit on at least 1 µg of genomic tumour DNA, followed by sample preparation, sequencing, alignment, variant calling and annotating, as described above (Koopmans et al., manuscript in preparation).

Variants were validated by Sanger sequencing. Oligonucleotide primers were designed from intronic sequences to amplify all coding sequence of *BAP1* with PCR. The primers are listed in the **Supplementary Table S1**, and PCR amplification and Sanger sequencing protocols are available upon request.

Immunohistochemical staining

Immunohistochemistry was performed with an automated immunohistochemistry staining system (Ventana BenchMark ULTRA, Ventana Medical Systems Inc., Tucson, AZ, USA) using the alkaline phosphatase method and a red chromogen. In brief, following deparaffinization and heat-induced antigen retrieval for 64 min, the tissue sections were incubated with a mouse monoclonal antibody raised against amino acids 430 – 729 of human BAP1 (clone sc-28383, 1:50 dilution, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 1 h at 36 ˚C. A subsequent amplification step was followed by incubation with haematoxylin II counter stain for 8 min and then a blue-colouring reagent for 8 min according to the manufacturer's instructions (Ventana). Liver, tonsil, breast tissue, and the retinal pigment epithelium were used as positive controls for BAP1 expression. An ophthalmic pathologist independently evaluated the histopathological characterisation of the tissue sections and the immunohistochemistry stainings. In some cases with suspected clonal subpopulations, multiple staining and double staining of BAP1 and HMB-45 and/or CD45 was conducted using the 3,3′-diaminobenzidine method. The samples were scored positive or negative by masked screening.

Statistical analysis

The co-occurrence of *BAP1* mutations with absent BAP1 expression and other clinical, histopathological, and genetical data were calculated using either the χ^2-test or Fisher's exact test (categorical variables) and the Mann-Whitney test (continuous variables). The influence of these variables on survival was determined with the Kaplan-Meier method for categorical variables and using the Cox regression method for continuous variables. Survival analysis was performed on the basis of disease-free survival, defined as the time period from enucleation until the development of metastasis or death due to metastasis. Death due to another cause or lost to follow-up was treated as censored. Subsequently, Cox multivariate proportional hazards regression (forward logistic regression method) was used to confirm that the variables were independent predictors of survival. All tests were two-sided. An effect was considered significant if the P-value was 0.05 or less. The statistical analyses were performed with the SPSS-20 software package.

RESULTS

Tumour samples

A total of 74 patients with histopathologically proven uveal melanoma were included in our study. There were 34 men and 40 women, and the mean age was 63 years (range 37 – 86). The mean disease-free survival was 52 months (5 – 209). Thirty-five patients were alive at the last follow-up, 29 patients developed metastatic disease of which 26 died, 9 patients died due to another cause and 1 patient was lost during follow-up (with a survival of 69 months). Detailed clinicopathological data are provided in **Table 1** and **Supplementary Table S2**. The majority of UM were of choroidal origin (n = 64), only 10 tumours (14%) originated from the ciliary body. From the 64 choroidal tumours, 8 invaded the ciliary body. Most cases displayed a spindle or mixed cell morphology (n = 64) and only 10 cases (14%) revealed a pure epithelioid phenotype. The tumours ranged from 5 to 21 mm in diameter (a mean value of 13.6 mm) and from 1.5 to 15 mm thick (a mean value of 7.8 mm). According to the 7th edition of the American Joint Committee on Cancer TNM classification (TNM7) for UM, we classified UM on the basis of the anatomic extent of the primary tumour (T).[26] Seven tumours (10%) were classified as T1, 26 (35%) as T2, 35 (47%) as T3, and 6 (8%) as T4. Extracellular matrix patterns were present in 36 UM (49%).

Table 1. Clinicopathological and chromosomal features of the study cohort (n = 74)

Clinicohistopathological data	**Mean (range)**
Age, years	62 (37 - 86)
Largest basal tumour diameter, mm	13.6 (5 - 21)
Tumour thickness, mm	7.8 (1.5 - 15)
Disease-free survival, months	52 (5 - 209)
	***n* (%)**
Follow-up	
Alive	35/74 (47%)
Metastasis present	3/74 (4%)
Died due to metastatic disease	26/74 (35%)
Died due to other disease cause	9/74 (12%)
Lost to follow-up	1/74 (1%)
Gender	
Male	34/74 (46%)
Female	40/74 (54%)
Localisation tumour	
Choroid	64/74 (87%)
Ciliary body	10/74 (13%)
Tumour invasion ciliary body	
No invasion	56/74 (76%)
Invasion	18/74 (24%)
Cell type	
Spindle	29/74 (39%)
Mixed	35/74 (47%)
Epithelioid	10/74 (14%)
Epithelioid cells	
Absent	25/74 (34%)
Present	49/74 (66%)
Size category according to TNM7	
1	7/74 (10%)
2	26/74 (35%)
3	35/74 (47%)
4	6/74 (8%)
Extracellular matrix patterns	
Absent	38/74 (51%)
Present	36/74 (49%)
Chromosomal data	***n* (%)**
Ploidy status	
Disomy	66/74 (89%)
Triploidy	5/74 (7%)
Tetraploidy	3/74 (4%)
Chromosome 3 loss	46/74 (62%)

Genetic and histopathological analyses

FISH and SNP array analysis

Cytogenetic analysis was performed using a SNP array (*n* = 59) and FISH (*n* = 66). The study group contained 46 tumours with loss of chromosome 3 (62%). Three tumours appeared to have a loss of heterozygosity (LOH) region ranging from 0.57 to 4.1 megabase (Mb) around the *BAP1* gene. Therefore, we coded them as LOH for chromosome 3p21.1. A SNP array of one case with LOH is shown in **Figure 1A**. Other chromosomal aberrations included loss of chromosome 1p (*n* = 23, 31%), gain of chromosome 6p (*n* = 37, 51%), loss of chromosome 6q (*n* = 29, 40%), loss of chromosome 8p (*n* = 16, 22%), gain of chromosome 8p (*n* = 15, 20%), and gain of chromosome 8q (*n* = 43, 58%). Eight tumours were polyploid with either a triploid (*n* = 5, 7%) or tetraploid (*n* = 3, 4%) status. Seven out of these eight tumours showed relative loss of chromosome 3 compared with their baseline chromosome status.

Mutation analysis

In all, 57 tumour samples were sequenced using the long-range PCR approach, generating 2,992,269 – 46,389,045 mapped reads per sample. Six samples were sequenced using the HaloPlex method. Of these, two samples were also sequenced with the long-range approach. Nineteen tumours were subjected to whole exome sequencing, in which the coding region of the entire genome was sequenced. Of these, seven were also sequenced with the long-range approach. Two groups of untypical UM were selected for the exome sequencing: seven UM with monosomy 3 and a follow-up of more than 60 months without any metastasis, and 10 disomy 3 tumours who did develop metastasis. Two samples were polyploid with relative chromosome 3 loss and metastatic disease. For all 19 samples, a mean coverage over 68 × was reached for the target regions (Koopmans et al., manuscript in preparation). For the current study, we only investigated the *BAP1* gene in the samples subjected to exome sequencing.

A *BAP1* mutation was detected in 35 UM samples (47%). These included 7 missense mutations, 3 nonsense mutations, 12 deletions and 2 insertions leading to a frameshift, 1 in-frame deletion and 10 mutations located at a splice site. The mutations were located throughout the gene (**Figure 2A**). Thirty-three out of the 35 variants were validated using Sanger sequencing.

Immunohistochemistry

We assessed BAP1 expression by immunohistochemistry. In 31 of the 73 UM investigated (43%), BAP1 expression was below the level of detection in the tumour. In these samples, the retinal pigment epithelium stained positive. One of the paraffin slides could not be examined due to insufficient material (sample S20). In the upper panel of **Figure 1**, a UM is shown

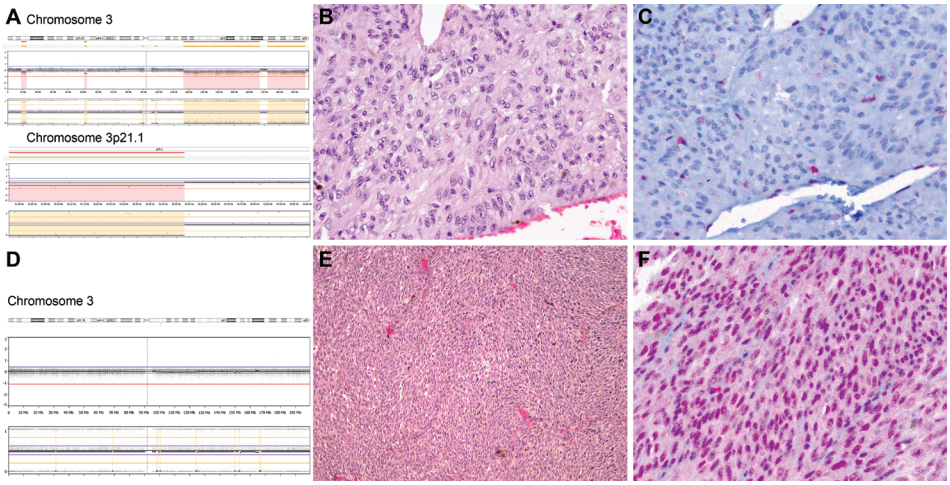


Figure 1. Histopathological and genetic features of two uveal melanoma cases. In case S42, loss of heterozygosity (LOH) of 3p21.1 was observed on single nucleotide polymorphism (SNP) array (**A**). The tumour shows mainly spindle cells in a haematoxylin and eosin (H&E) staining (400x) (**B**). Immunohistochemistry revealed no expression of BAP1 in the tumour cells and positive expression in endothelium and inflammatory cells (400x) (**C**). In case S4, the SNP array displays a disomy 3 (**D**). Spindle tumour cells in the uveal melanoma are shown in a H&E staining (200x) (**E**). Immunohistochemistry revealed a strong nuclear positivity for BAP1 (400x) (**F**).

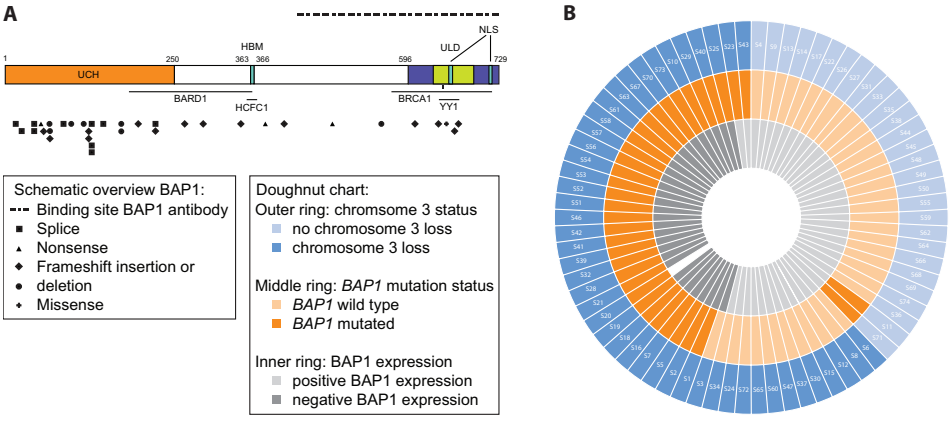


Figure 2. Schematic overview of sequence data and immunohistochemical analyses. A schematic overview of the *BAP1* gene and its functional domains. BAP1 is composed of an N-terminal UCH domain (orange; amino acid (aa) 1 – 250), an HCF1-binding domain (HBM)-like motif (blue; aa 363 – 366), an UCH37-like domain (ULD) (green; aa 634 – 693), and a nuclear localisation signal (NLS) consisting of two parts (blue; aa 656 – 661 and aa 717 – 722). BAP1 has been reported to interact with BARD1 (aa 182 – 365), HCF1 (aa 365 – 385), BRCA1 (aa 596 – 721), and YY1 (aa 642 – 686).[27] The binding site for BAP1 antibody is depicted with a dashed line (aa 430 – 729). The found mutations and indels are shown below, classified according to their type and position (**A**). A multilevel doughnut chart was constructed for all samples (n = 74) regarding the chromosome 3 status (outer ring), *BAP1* mutations status (middle ring) and BAP1 expression (inner ring) (**B**).

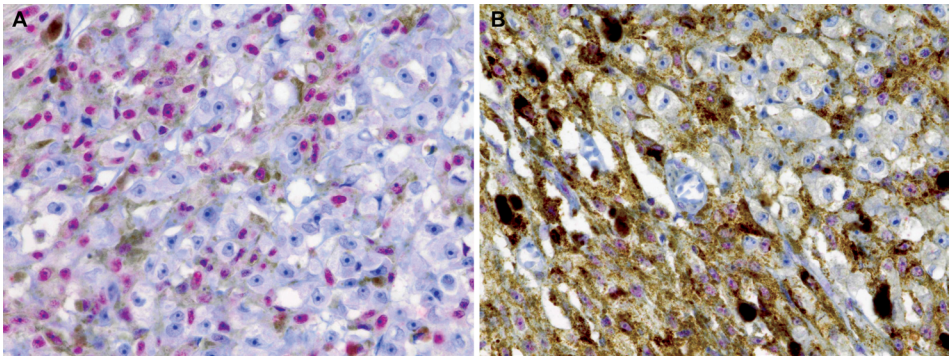


Figure 3. Histopathological features of a uveal melanoma case with heterogeneous expression of BAP1. In case S7, a heterogeneous distribution of BAP1 expression was observed throughout the tumour with immunohistochemistry (400x) (**A**). Staining with HMB-45 revealed strong positivity (3,3'-diaminobenzidine staining, brown colour) concluding that the cells investigated were melanoma cells (400x) (**B**).

with LOH of the *BAP1* gene and no detectable BAP1 staining (**Figures 1A-C**). In the lower panel, a UM with a disomy 3 and positive BAP1 expression is shown (**Figures 1D-F**). In four tumours, a subpopulation of cells, ranging from 20 to 80% of the total, was observed, that were negative for BAP1 staining. The remaining tumour cells (corresponding 80 to 20% of the total), stained positive for BAP1 (**Supplementary Table S2**). In these four samples, a staining with HMB-45 was performed. We observed that both the BAP1-positive and -negative cells stained positive for HMB-45 (**Figure 3**).

The majority of the BAP1-negative tumours also harboured a *BAP1* mutation (**Figure 2B**). More specifically, 30 out of the 31 UM that did not show any BAP1 staining had a *BAP1* mutation (SNPs, base insertions, or deletions). Four of the 42 BAP1-positive tumours harboured a *BAP1* mutation (**Table 2**). As shown in **Figure 2B**, two of the 28 tumours possessing a normal chromosome 3 copy number had a *BAP1* sequence variant (sample S11 and S71; **Supplementary Table S2**). In the group of UM with loss of chromosome 3 (including the polyploid tumours with a relative chromosome 3 loss), 33 of the 46 tumours harboured a *BAP1* mutation. We did not observe any BAP1 staining in 30 of the corresponding samples. Immunohistochemistry could not be conducted for sample S20. Two tumours, S23, and S43, stained positive for BAP1 despite their monosomy 3 and *BAP1* mutation status. Tumour S23 contained a heterozygous deletion of 22 bp in exon 16 (p.R666fs) leading to a frameshift in 74% of the reads. In this sample, heterogeneous levels of BAP1 expression were observed. We estimated that BAP1 expression was absent in 20% of the tumour, whereas the remaining 80% of the tumour cells did stain positive for BAP1. Tumour S43 had a 7 bp frameshift deletion in exon 16 (p.E673X) in 54% of the reads. However, in this tumour 100% of the tumour tissue showed normal BAP1 expression. Lastly, there was one *BAP1* wild-type UM (sample

Table 2. Correlations between *BAP1* mutation and clinicopathological and genetical data

	***BAP1* mutation status**		
	Mutated	**Wild-type**	
Clinicohistopathological data	***n* = 35**	***n* = 39**	**P-value**
Mean age, years	66	60	0.060[a]
Mean largest basal diameter, mm	14.1	13.2	0.289[a]
Mean tumour thickness, mm	8.0	7.6	0.574[a]
Gender			
Male	15	19	0.613[b]
Female	20	20	
Localisation tumour			
Choroid	29	35	0.502[c]
Ciliary body	6	4	
Ciliairy body involvement			
Absent	25	31	0.420[b]
Present	10	8	
Epithelioid cells			
Absent	5	20	**0.001**[b]
Present	30	19	
Extracellular matrix patterns			
Absent	12	26	**0.005**[b]
Present	23	13	
BAP1 expression			
Negative	30	1	**< 0.001**[b]
Positive	4	38	
Chromosomal data	***n***	***n***	**P-value**
Ploidy status			
Diploid	31	35	1.000[c]
Polyploid	4	4	
Chromosome 3 loss			
Yes	33	13	**< 0.001** [b]
No	2	26	

[a] Associations for continuous variables were calculated with the Mann-Whitney test. [b] Associations for categorical variables were calculated with the χ^2-test if the expected count was greater than 5. [c] Associations for categorical variables were calculated with the Fisher's exact test if the expected count was less than 5. A P-value of 0.05 or less was considered significant and is shown in bold.

S3) that did not stain positive for BAP1. In this case, BAP1 staining was absent in ~ 50% of the tumour cells.

Statistical analysis

BAP1 mutations strongly coincided with an absent BAP1 expression (P < 0.001) and monosomy 3 (P < 0.001, **Table 2**). The presence of epithelioid cells (P = 0.001) and extracellular matrix patterns (P = 0.005) were also significantly overrepresented in UM with *BAP1* mutations. The sensitivity and specificity for the detection of BAP1 depletion by immunohistochemistry compared with mutation analysis were 88% and 97%, respectively. For survival analysis, we included only the unselected UM samples (*n* = 55) and excluded UM specimens that were selected for exome sequencing. Univariate analyses showed that the disease-free survival was significantly shorter in patients with a *BAP1* mutation (32 versus 133 months, P < 0.001, **Figure 4A**). We examined whether *BAP1* mutations influenced the prognosis of monosomy 3 patients by constructing Kaplan-Meier curves and performing the Log-rank test. Patients with monosomy 3 UM and a *BAP1* mutation seem to have a worse prognosis, although this was not statistically significant (P = 0.122, **Figure 4B**). Patients with tumours with a negative BAP1 staining also had a significant shorter disease-free survival compared to tumours with a positive BAP1 staining (31 versus 133 months, P < 0.001). Other factors that affected the survival in UM patients were: age at time of diagnosis (P = 0.040), largest basal tumour diameter (P = 0.005), the presence of epithelioid cells (P = 0.003), presence of

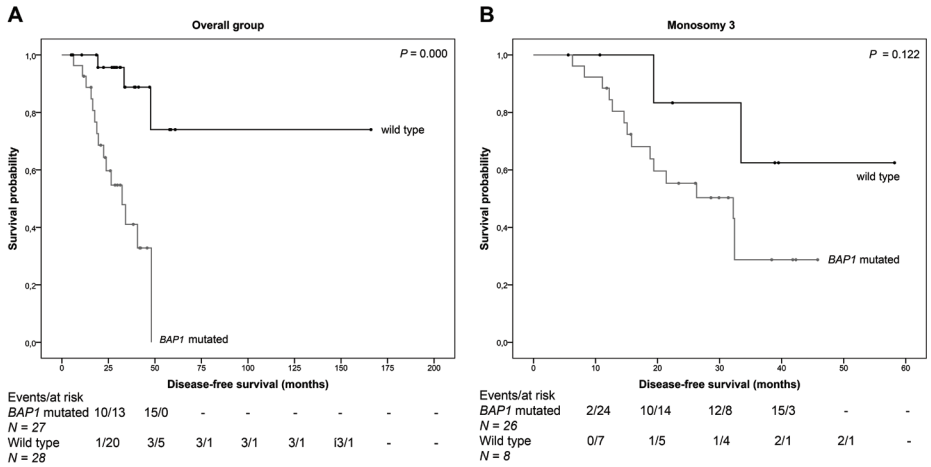


Figure 4. Kaplan-Meier estimate of disease-free survival in uveal melanoma patients. Kaplan-Meier survival curves displaying melanoma-related mortality for 55 patients based on the *BAP1* mutation status (**A**) and the survival curves for 34 patients with loss of chromosome 3 (**B**). The table shows the number of events and cases at risk overtime at the respective time point. Log-rank tests were used to compare survival distributors across subgroups.

Table 3. Multivariate Cox regression of disease-free survival in 55 patients

Variable	Beta	SE	Wald	Hazard ratio	95% CI	P-value
BAP1 mutation with negative BAP1 staining	2.045	0.644	10.076	7.731	2.187 – 27.322	0.002
Largest basal diameter	0.205	0.085	5.847	1.228	1.040 – 1.450	0.016
Age	-	-	-	-	-	-
Epithelioid cells	-	-	-	-	-	-
Extracellular matrix patterns	-	-	-	-	-	-
Monosomy 3	-	-	-	-	-	-

Abbreviations: CI = confidence interval.

extracellular matrix patterns (P = 0.001), and chromosome 3 loss (P < 0.001). Considering the strong interaction between *BAP1* mutation status and BAP1 expression, we validated whether this concurrent inactivation of the gene and protein is an independent parameter for disease-free survival. The possible confounding variables were analysed in a multivariate model. After correcting for these variables, we found that patients with a concurrent *BAP1* mutation and a negative BAP1 expression have an 7.7 times greater chance of developing metastases compared with those without these aberrations (P = 0.002, **Table 3**). The largest basal diameter of the tumour was also an independent predictor for disease-free survival (P = 0.016). The age at time of diagnosis, presence of epithelioid cells, extracellular matrix patterns, and loss of chromosome 3 did not reach significance and were rejected.

DISCUSSION

In this study, we found that nearly half of the investigated UM tumours harboured an inactivating *BAP1* mutation and that this was strongly associated with the absence of BAP1 staining, monosomy 3, and other prognostic features of aggressive tumours, such as the presence of epithelioid cells and extracellular matrix patterns. Nonetheless, a few discrepancies were observed between *BAP1* mutation status and BAP1 immunohistochemistry. For two samples (S11 and S71), the immunohistochemistry results can be explained by the fact that both tumours were disomic for chromosome 3 and harboured a heterozygous *BAP1* mutation. Thus, presumably the remaining wild-type allele led to a normal positive staining. As mentioned previously, LOH of a small region containing the *BAP1* gene was found in a three tumours by SNP array analysis. These tumours (S21, S37, and S42) were classified as loss of chromosome 3p21.1 although chromosome 3 was not entirely deleted. In addition, S21 and S42 harboured a hemizygous *BAP1* mutation (**Figures 1A-C** and **Figure 2B**). Our study confirms

that biallelic inactivation of *BAP1* in UM tumour tissue is required to prevent BAP1 protein expression, through loss of one copy of chromosome 3 and a *BAP1* mutation in the remaining copy. Two UM (S23 and S43) with monosomy 3 had a positive BAP1 staining, despite harbouring an out-of-frame deletion in exon 16. One possibility for the positive staining could be that the truncated proteins (p.R666fs and p.E673X) are still detected by the BAP1 antibody. Interestingly, both deletions were heterogeneous for hemizygous mutant suggesting that a normal population of cells is still present in the tumour, which could have led to a positive staining. However, this is not supported by the observation that 80% and 100%, respectively, of the S23 and S43 tumour cells stained positive for BAP1. In two cases with evident heterogeneous subpopulations of cells with and without BAP1 expression within the same UM (S5 and S7), the percentage of BAP1-negative UM cells was equal to the percentage of chromosome 3 loss. Even though in our study, the percentage of *BAP1* mutation does not always correlate with the percentage of absent expression, in most cases, these percentages are high enough to classify the tumours in the correct category. In one tumour sample (S3), 50% of the cells did not stain positive for BAP1 even though no *BAP1* mutation was detected by exome sequencing. To be sure that the investigated clonal subpopulation were melanoma cells, we carried out a staining with HMB-45 and confirmed that this was the case. In this tumour S3, LOH of chromosome 3 was detected by FISH and SNP array analysis. Possibly, intronic variants which cannot be detected with exome sequencing prevent BAP1 expression in some of the cells in this tumour. Alternatively, the apparent LOH might reflect a more complex genetic rearrangement, where BAP1 is lost in a proportion of the cells comprising the tumour.

Somatic *BAP1* mutations have been described in other cancers, such as malignant pleural mesotheliomas and cutaneous melanoma, and the absence of BAP1 expression in mesotheliomas has been demonstrated by immunohistochemistry.[15] In contrast to our UM cohort, 25% of the mesotheliomas without a *BAP1* mutation did not display any immunohistochemistry staining for BAP1.

In two UM, a mutation was detected by one of the Next Generation Sequencing (NGS) approaches but could not be validated with Sanger sequencing (S58 and S63). In both cases, the percentage of reads with the mutation was quite low (5% and 4%, respectively). A limitation of conventional Sanger sequencing is that low mosaicism variants are difficult to detect below a level of ~ 20%[28] and this is likely to be the reason why S58 and S63 could not be validated. Both tumour samples stained BAP1-negative.

A recent study suggested that BAP1 inactivation might be more characteristic of epithelioid mesotheliomas.[29] We also found a correlation between *BAP1* inactivation and UM with an epithelioid cell type suggesting that BAP1 deficiency may be particularly involved in the pathogenesis of uveal melanoma with an aggressive phenotype.

Previous research has shown that *BAP1* mutations are present in 47% (27/57) of the primary UM and 84% (26/31) of class 2 UM.[8] Our findings support the hypothesis that somatic *BAP1* mutations promote metastases. In the overall study group, we found a *BAP1* mutation in 62% (18/29) of metastasising UM, and it is important to note that this could be an underestimation, since in a few patients, limited follow-up data was available. Selection bias could have occurred because untypical UM were selected for exome sequencing. Therefore, we excluded these tumours from our survival analysis. Nonetheless, it would be interesting to enlarge the study group with random, nonselected UM with a longer follow-up. After excluding the exome sequencing samples, there were six patients without metastasis after a follow-up of 4 years or longer. None of these individuals had a *BAP1* mutation. Of the 18 patients who developed metastases, 15 UM harboured a *BAP1* mutation.

In the current study, we only investigated the BAP1 expression in tumours from enucleated eyes. Over the years, eye-sparing therapies have proved to be equally effective in terms of patient survival compared with radical treatment.[30, 31] With eye-sparing therapies biopsies can be taken for prognostication and it is also possible to perform BAP1 immunohistochemistry on biopsy specimens in our institute. This technique has an additive value in determining the patients' prognosis.

Recent work of Matatall and associates[32] demonstrated that BAP1 depletion induces a primitive, stem-like phenotype and these findings implicate BAP1 in the maintenance of melanocyte identity in UM cells. Therapeutic strategies that target these specific pathways in UM are urgently needed. Currently, therapeutic agents targeting BAP1 deficiency are being investigated. Histone deacetylase (HDAC) inhibitors have shown to reverse the effects of BAP1 depletion in UM cells.[33] As therapeutic options emerge, it is important to be able to rapidly identify the patients, enucleated and as conservatively treated patients, who would benefit from a specific intervention. Given the costs of *BAP1* mutation analysis, immunohistochemistry offers an economical and fast alternative. In our study, we demonstrated that there is a strong association between BAP1 staining and *BAP1* mutation status with a sensitivity of 88% and a specificity of 97%. We propose that, the BAP1 immunohistochemistry should be implemented in the routine histopathological examination of UM.

ACKNOWLEDGMENTS

This study was supported by a grant of the Combined Ophthalmic Research Rotterdam (CORR) and Stichting Nederlands Oogheelkundig Onderzoek (SNOO).

REFERENCES

1. Singh AD, Turell ME, Topham AK. Uveal melanoma: trends in incidence, treatment, and survival. *Ophthalmology*. 2011;118:1881-1885.
2. Woodman SE. Metastatic uveal melanoma: biology and emerging treatments. *Cancer J*. 2012;18:148-152.
3. Kilic E, Naus NC, van Gils W, et al. Concurrent loss of chromosome arm 1p and chromosome 3 predicts a decreased disease-free survival in uveal melanoma patients. *Invest Ophthalmol Vis Sci*. 2005;46:2253-2257.
4. Prescher G, Bornfeld N, Hirche H, et al. Prognostic implications of monosomy 3 in uveal melanoma. *Lancet*. 1996;347:1222-1225.
5. Sisley K, Parsons MA, Garnham J, et al. Association of specific chromosome alterations with tumour phenotype in posterior uveal melanoma. *Br J Cancer*. 2000;82:330-338.
6. Sisley K, Rennie IG, Parsons MA, et al. Abnormalities of chromosomes 3 and 8 in posterior uveal melanoma correlate with prognosis. *Genes Chromosomes Cancer*. 1997;19:22-28.
7. White VA, Chambers JD, Courtright PD, Chang WY, Horsman DE. Correlation of cytogenetic abnormalities with the outcome of patients with uveal melanoma. *Cancer*. 1998;83:354-359.
8. Harbour JW, Onken MD, Roberson ED, et al. Frequent mutation of BAP1 in metastasising uveal melanomas. *Science*. 2010;330:1410-1413.
9. Kilic E, van Gils W, Lodder E, et al. Clinical and cytogenetic analyses in uveal melanoma. *Invest Ophthalmol Vis Sci*. 2006;47:3703-3707.
10. Nishikawa H, Wu W, Koike A, et al. BRCA1-associated protein 1 interferes with BRCA1/BARD1 RING heterodimer activity. *Cancer Res*. 2009;69:111-119.
11. Farmer H, McCabe N, Lord CJ, et al. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature*. 2005;434:917-921.
12. Jensen DE, Proctor M, Marquis ST, et al. BAP1: a novel ubiquitin hydrolase which binds to the BRCA1 RING finger and enhances BRCA1-mediated cell growth suppression. *Oncogene*. 1998;16:1097-1112.
13. Ventii KH, Devi NS, Friedrich KL, et al. BRCA1-associated protein-1 is a tumor suppressor that requires deubiquitinating activity and nuclear localisation. *Cancer Res*. 2008;68:6953-6962.
14. Abdel-Rahman MH, Pilarski R, Cebulla CM, et al. Germline BAP1 mutation predisposes to uveal melanoma, lung adenocarcinoma, meningioma, and other cancers. *J Med Genet*. 2011;48:856-859.
15. Bott M, Brevet M, Taylor BS, et al. The nuclear deubiquitinase BAP1 is commonly inactivated by somatic mutations and 3p21.1 losses in malignant pleural mesothelioma. *Nat Genet*. 2011;43:668-672.
16. Njauw CN, Kim I, Piris A, et al. Germline BAP1 inactivation is preferentially associated with metastatic ocular melanoma and cutaneous-ocular melanoma families. *PLoS One*. 2012;7:e35295.
17. Pena-Llopis S, Vega-Rubin-de-Celis S, Liao A, et al. BAP1 loss defines a new class of renal cell carcinoma. *Nat Genet*. 2012;44:751-759.
18. Testa JR, Cheung M, Pei J, et al. Germline BAP1 mutations predispose to malignant mesothelioma. *Nat Genet*. 2011;43:1022-1025.
19. Wiesner T, Obenauf AC, Murali R, et al. Germline mutations in BAP1 predispose to melanocytic tumors. *Nat Genet*. 2011;43:1018-1021.
20. Aoude LG, Vajdic CM, Kricker A, Armstrong B, Hayward NK. Prevalence of germline BAP1 mutation in a population-based sample of uveal melanoma cases. *Pigment Cell Melanoma Res*. 2013;26:278-279.
21. Naus NC, Verhoeven AC, van Drunen E, et al. Detection of genetic prognostic markers in uveal melanoma biopsies using fluorescence in situ hybridization. *Clin Cancer Res*. 2002;8:534-539.

22. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics*. 2009;25:1754-1760.

23. Brouwer RW, van den Hout MC, Grosveld FG, van Ijcken WF. NARWHAL, a primary analysis pipeline for NGS data. *Bioinformatics*. 2012;28:284-285.

24. McKenna A, Hanna M, Banks E, et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Res*. 2010;20:1297-1303.

25. Li H, Handsaker B, Wysoker A, et al. The Sequence Alignment/Map format and SAMtools. *Bioinformatics*. 2009;25:2078-2079.

26. Edge SD, Byrd DR, Compton CC, et al. Malignant melanoma of the uvea, In: Edge SD, Byrd DR, Compton CC, Fritz AG, Green FL, Trotti A, (eds). *AJCC Cancer Staging Manual*. 7th edn. Springer: New York; 2010. pp 547-559.

27. Eletr ZM, Wilkinson KD. An emerging model for BAP1's role in regulating cell cycle progression. *Cell Biochem Biophys*. 2011;60:3-11.

28. Rohlin A, Wernersson J, Engwall Y, et al. Parallel sequencing used in detection of mosaic mutations: comparison with four diagnostic DNA screening techniques. *Hum Mutat*. 2009;30:1012-1020.

29. Yoshikawa Y, Sato A, Tsujimura T, et al. Frequent inactivation of the BAP1 gene in epithelioid-type malignant mesothelioma. *Cancer Sci*. 2012;103:868-874.

30. Seddon JM, Gragoudas ES, Albert DM, et al. Comparison of survival rates for patients with uveal melanoma after treatment with proton beam irradiation or enucleation. *Am J Ophthalmol*. 1985;99:282-290.

31. Seddon JM, Gragoudas ES, Egan KM, et al. Relative survival rates after alternative therapies for uveal melanoma. *Ophthalmology*. 1990;97:769-777.

32. Matatall KA, Agapova OA, Onken MD, et al. BAP1 deficiency causes loss of melanocytic cell identity in uveal melanoma. *BMC Cancer*. 2013;13:371.

33. Landreville S, Agapova OA, Matatall KA, et al. Histone deacetylase inhibitors induce growth arrest and differentiation in uveal melanoma. *Clin Cancer Res*. 2012;18:408-416.

Supplementary Table S1. The primer sequences used to amplify the exons of *BAP1*

BAP1	Forward	Reverse
Exon 1	5′-TCCCGACCCTCCCCTTCG-3′	5′-GGGTGGGGCGACAAGAGG-3′
Exon 2 and 3	5′-GGAGAGCGACCCAGGTGAGG-3′	5′-GGGTTCCTGGCACTGTCTTCC-3′
Exon 4	5′-CATCCTTGGCCCTCAGTTCC-3′	5′-TCCATTTCCACTTCCCAAGC-′3
Exon 5	5′-TTTAGAGTAGGAGGGTGTCTGAGTCC-3′	5′-CCCTCCCTAGGCCTGTGTCC-3′
Exon 6 and 7	5′-GCTCTCTGAAGCTTTGCCTTCC-3′	5′-TGCCACTGGGTACCACATACC-3′
Exon 8	5′-CCCGACCAGCTCCTGATTCC-3′	5′-CAGATTCACCATATGGCCTTGC-3′
Exon 9	5′-GTTGGGGTGGGGCCTATACC-3′	5′-ACAAATGCTGTGGGGGAAGG-3′
Exon 10	5′-GGTAGAGCCAAGGCCATTATTCC-3′	5′-TGACGGGGGAAGAACACTGC-3′
Exon 11	5′-GCCGGGGAGACTGTGAGC-3′	5′-CATGGGAAAATTGCCTGTTGC-3′
Exon 12	5′-CGCTGACTCAGTCTGGAAAACC-3′	5′-CCCAGGGCCCCAAACTCC-3′
Exon 13a	5′-CTGCAGCTGTCAGAACTTGATGC-3′	5′-AAGCACTGCCGATCTCAGAGG-3′
Exon 13b	5′-TCAATTCCTCTGTCCATCAAGACTAGC-3′	5′-TCAGAGTGCAGGACACTTTGTGG-3′
Exon 14	5′-GCCTTGGACTGGCTCACTGG-3′	5′-CCAGCCACCAATCTTCACACC-3′
Exon 15 and 16	5′-CATGGACTCGCTGCTCATCC-3′	5′-GAGGGGAGCTGAAGGACACG-3′
Exon 17	5′-TGAGGCTTGAGCAGACCTTGG-3′	5′-CCAGATGCTGCCTCCTGAGC-3′

Supplementary Table S2. Summary clinicohistopathological and genetic data on uveal melanoma tumour samples in the study

Sample	Age at diagnosis	Follow-up	Alive / dead	Metastases	Monosomy 3	*BAP1* mutation (in cDNA or gDNA, Hg19)	Mutation type	BAP1 IHC	Other chromosomal abnormalities*	Copy number
S1	86	110.4	dead	no	yes	c.1265-1266delGT	deletion (out-of-frame)	-	1p loss, 8q gain	diploid
S2	47	32.4	dead	yes	yes	g.chr3:52,443,761A>G	splice site (out-of-frame)	-	8p loss, 8q gain	diploid
S3	77	209.1	alive	no	yes			-	1p loss, 6p gain, 6q gain	diploid
S4	38	135.7	dead	yes	no			+	1p loss, 6p gain, 6q loss, 8p gain, 8q gain	diploid
S5	84	22.5	dead	yes	yes	c.766C>G	missense	-	1p loss, 8q gain	diploid
S6	74	191.5	alive	no	yes			+	1p loss, 6p gain, 6q gain, 8q gain	triploid
S7	82	68.9	alive	no	yes	c.1065-1066insGG	insertion (out-of-frame)	-	1p loss, 6p gain, 6q gain	diploid
S8	72	167.0	dead	no	yes			+		diploid
S9	37	170.7	dead	yes	no			+	6p gain, 6q loss	diploid
S10	58	87.0	dead	yes	yes	c.678insA	insertion (premature termination)	-	6p gain, 8p gain, 8q gain	triploid
S11	59	34.4	dead	yes	no	c.990A>G	missense	+	6p gain, 8p gain, 8q gain	diploid
S12	73	112.7	dead	yes	yes			+	1p loss, 6p gain, 8q gain	diploid
S13	60	166.1	alive	no	no			+		diploid
S14	52	148.4	dead	yes	no			+	6p gain, 6q loss	diploid
S15	80	19.4	dead	yes	yes			+	1p loss, 6q loss, 8p gain, 8q gain	diploid
S16	72	151.0	dead	no	yes	g.chr3:52,441,477G>C	splice site (out-of-frame)	-	1p loss, 8p gain, 8q gain	diploid
S17	46	45.5	dead	yes	no			+	6p gain, 6q loss, 8q gain	diploid
S18	68	42.3	dead	no	yes	g.chr3:52,441,414G>C	splice site (out-of-frame)	-	8p loss, 8q gain	diploid
S19	64	48.1	dead	yes	yes	c.843delC	deletion (out-of-frame)	-	1p loss, 8q gain	diploid
S20	62	142.0	alive	no	yes	g.chr3:52,441,977G>C	splice site (out-of-frame)	N/A		diploid
S21	84	52.9	dead	no	yes	c.1518-1534delACCCCACTCCCATTGT	deletion (premature termination)	-		diploid
S22	49	47.7	dead	yes	no			+	1p loss, 6p gain, 8p gain, 8q gain	diploid

Supplementary Table S2. Summary clinicohistopathological and genetic data on uveal melanoma tumour samples in the study (continued)

Sample	Age at diagnosis	Follow-up	Alive / dead	Metastases	Monosomy 3	*BAP1* mutation (in cDNA or gDNA, Hg19)	Mutation type	BAP1 IHC	Other chromosomal abnormalities*	Copy number
S23	57	122.8	dead	yes	yes	c.2470-2491delAAGGACCCACAAC-TACGATGA	deletion (out-of-frame)	+	6q loss, 8p loss, 8q gain	diploid
S24	74	6.3	dead	yes	yes			+	1p loss, 6q loss, 8p loss	tetraploid
S25	64	18.8	dead	yes	yes	c.996C>G	missense	-	6q loss, 8p loss	tetraploid
S26	65	23.5	dead	yes	no			+	6p gain, 6q loss, 8p loss	diploid
S27	42	122.5	alive	yes	no			+	6p gain, 8q gain	diploid
S28	66	13.0	dead	yes	yes	c.1625C>T	nonsense	-	6p gain, 6q loss, 8p loss, 8q gain	diploid
S29	72	23.8	dead	yes	yes	c.1926C>A	nonsense	-	8q gain	triploid
S30	70	38.9	alive	no	yes			+	1p loss, 6p gain, 6q loss, 8q gain	diploid
S31	62	60.9	alive	no	no			+	6p gain, 6q gain	diploid
S32	60	16.6	dead	yes	yes	c.628G>A	nonsense	-	8p loss, 8q gain	diploid
S33	78	27.9	dead	no	no			+	6p gain, 6q loss, 8q gain	diploid
S34	73	58.2	alive	no	yes			+	1p loss, 6q loss	tetraploid
S35	53	57.9	alive	no	no			+	1p loss, 6p gain, 6q gain	diploid
S36	43	58.6	alive	no	no			+	1p loss, 6p gain, 6q loss, 8p gain, 8q gain	triploid
S37	42	33.5	dead	yes	yes			+	6p gain, 6q loss, 8q gain	diploid
S38	66	47.2	dead	no	no			+	6p gain, 6q loss	diploid
S39	56	40.6	dead	yes	yes	g.chr3:52,441,972T>G	splice site (out-of-frame)	-	1p loss, 8p gain, 8q gain	diploid
S40	57	34.2	alive	yes	yes	c.2390-2455delGAGGCTGAGATTG-CAAACTATGAGGCGTGCCTCAAG-GAGGAGGTAGAGAAGAGGAAGAA-GTTCAAGgtgggtgatttctccagttgcctg	deletion (out-of-frame)	-	1p loss, 6p gain, 6q gain	triploid
S41	73	17.7	dead	yes	yes	c.2289-2308delCCACGGACAGCA-GAGAGAAG	deletion (premature termination)	-	6p gain, 6q loss, 8p loss, 8q gain	diploid
S42	47	41.8	alive	no	yes	g.chr3:52,442,095A>C	splice site (out-of-frame)	-	8p gain, 8q gain	diploid

Sample	Age at diagnosis	Follow-up	Alive / dead	Metastases	Monosomy 3	*BAP1* mutation (in cDNA or gDNA, Hg19)	Mutation type	BAP1 IHC	Other chromosomal abnormalities*	Copy number
S43	85	15.7	dead	no	yes	c.2491-2497delGTTCATC	deletion (premature termination)	+	6q loss, 8q gain	diploid
S44	57	39.5	alive	no	no			+	6p gain, 6q loss	diploid
S45	78	41.2	alive	no	no			+	6p gain, 6q gain	diploid
S46	61	45.8	alive	no	yes	c.1364-1372delTCCCCGCTGGTGCT-GGAA GCAAACAG	deletion (premature termination)	-	1p loss, 8q gain	diploid
S47	43	39.5	alive	no	yes			+	6p gain	diploid
S48	73	34.0	alive	no	no			+	6p gain, 6q loss	diploid
S49	55	31.2	alive	no	no			+	6p gain, 6q loss, 8p gain, 8q gain	diploid
S50	56	28.6	alive	no	no			+		diploid
S51	76	11.1	dead	yes	yes	c.675A>G	missense	-	8p loss, 8q gain	diploid
S52	56	38.4	alive	no	yes	g.chr3:52,442,622delA	splice site (out-of-frame)	-	8p loss, 8q gain	diploid
S53	80	26.5	dead	yes	yes	c.847delGGTAGGCTG	deletion (out-of-frame)	-	6p gain, 6q loss, 8p gain, 8q gain	diploid
S54	66	31.4	alive	no	yes	c.660C>G	missense	-	6p gain, 6q loss, 8p gain, 8q gain	diploid
S55	58	31.5	alive	no	no			+	6q loss	diploid
S56	80	15.7	dead	yes	yes	c.2426-2446delGAGGAGGTA-GAGAAGAG GAAG	deletion (in-frame)	-	6p gain	diploid
S57	58	26.1	alive	no	yes	c.1706-1707delAC	deletion (premature termination)	-	8q gain	diploid
S58	74	29.9	alive	no	yes	g.chr3:52,443,626A>T	splice site (out-of-frame)	-	1p loss, 8p gain, 8q gain	diploid
S59	78	33.8	alive	no	no			+	8p loss	diploid
S60	42	22.4	alive	no	yes			+		diploid
S61	72	6.3	dead	yes	yes	c.647-650delCGGC	deletion (premature termination)	-	8p loss, 8q gain	diploid
S62	50	29.5	alive	no	no			+		diploid
S63	50	28.6	alive	no	yes	c.1670T>A	missense	-	8p loss, 8q gain	diploid
S64	64	5.03	dead	no	no			+	6p gain, 6q loss	diploid
S65	56	10.7	alive	no	yes			+	1p loss, 6p gain	diploid
S66	47	26.9	alive	no	no			+		diploid

Supplementary Table S2. Summary clinicohistopathological and genetic data on uveal melanoma tumour samples in the study (continued)

Sample	Age at diagnosis	Follow-up	Alive / dead	Metastases	Monosomy 3	*BAP1* mutation (in cDNA or gDNA, Hg19)	Mutation type	BAP1 IHC	Other chromosomal abnormalities*	Copy number
S67	61	19.6	alive	yes	yes	c.1141-1184delCCACGACATC-CGCTTCAACCTGATGGCAGTG-GTGCCCGACCGCA	deletion (premature termination)	-	8q gain	diploid
S68	79	18.6	alive	no	no			+		diploid
S69	50	19.4	alive	no	no			+	6p gain	diploid
S70	50	23.4	alive	no	yes	g.chr3:52,441,973G>A	splice site (out-of-frame)	-	8q gain	diploid
S71	38	20.9	alive	no	no	c.815A>G	missense	+	6p gain, 6q loss, 8q gain	diploid
S72	73	5.6	alive	no	yes			+	1p loss, 6p gain, 6q loss, 8p loss, 8q gain	diploid
S73	76	11.8	alive	no	yes	g.chr3:52,443,626A>T	splice site (out-of-frame)	-	8p loss, 8q gain	diploid
S74	46	5.9	alive	no	no			+	6p gain, 6q loss	diploid

* Only specific uveal melanoma chromosome alterations were recorded (chromosome 1, 3, 6 and 8).
Abbreviations: IHC = immunohistochemistry; del = deletion; ins = insertion; N/A = not available.

CHAPTER 2.3

SF3B1 mutations predispose to late metastases whereas *EIF1AX* mutations prevent metastases in disomy 3 uveal melanoma

Anna E. Koopmans*, Serdar Yavuzyigitoglu*, Robert M. Verdijk, Alice J.E. van Bodegom, Jolanda Vaarwater, Dion Paridaens, Emine Kiliç and Annelies de Klein

Submitted

* These authors contributed equally to this work

CHAPTER 2.4

Metastatic disease in uveal melanoma: importance of a genetic profile

Jackelien G.M. van Beek, Anna E. Koopmans, Jolanda Vaarwater, Robert M. Verdijk, Annelies de Klein, Nicole C. Naus and Emine Kiliç

Submitted

ABSTRACT

Importance. In this study, we compare chromosomal aberrations and gene mutations between a primary uveal melanoma (UM) and it's metastatic hepatic and peripancreatic metastases.

Observations. DNA was isolated from a large primary UM after fractionated stereotactic radiotherapy and three distinct metastases (two liver samples and one peripancreatic lymph node) to perform single nucleotide polymorphism array and fluorescent *in situ* hybridisation. We analysed mutations in UM target genes *BAP1*, *GNAQ*, *GNA11*, *SF3B1* and *EIF1AX*. The primary tumour revealed no abnormalities in chromosome 3, whereas metastases showed deletion of at least 3q12.1-q24 while the *BAP1* gene was not mutated. All samples revealed the following consistent chromosomal aberrations: loss of 1p, gain of 6p and gain of 8q. Subsequently, heterozygous *SF3B1* and *GNA11* mutations were observed.

Conclusions and Relevance. The metastases showed more genetic aberrations than the primary tumour and therefore may represent the genetic status of the tumour prior to irradiation, whereas the current primary tumour shows presumably irradiation artefacts. An early occurring mutation in *GNA11* was observed in all samples. The *SF3B1* mutation seems to predispose for late metastatic disease in absence of a *BAP1* mutation.

INTRODUCTION

Uveal melanoma (UM) are known to spread to the liver and less frequently to lungs, bones, skin and brain.[1] Metastatic disease occurs in half of the UM patients and predominantly in those with monosomy 3 tumours. Recent studies have identified *BAP1* mutations in metastatic UM. Mutations of *SF3B1* at codon 625 and *EIF1AX* have been identified in low-grade UM with a good prognosis.[2, 3, 4] *GNAQ* and *GNA11* mutations occur in the majority of UM patients and are not associated with survival.[5]

We present a patient with an UM and late occurring metastases in the liver and peripancreatic lymph node. We performed single nucleotide polymorphism (SNP) array analysis and fluorescent *in situ* hybridisation (FISH) on the primary tumour and metastatic tissue. Additionally, we analysed UM target genes *BAP1*, *GNAQ*, *GNA11*, *SF3B1* and *EIF1AX*.

CASE REPORT

In March 2001, a 45-year-old male presented with a decreased vision in his right eye (20/60) and normal vision left (20/20), with normal intraocular pressure. Indirect ophthalmoscopy and ultrasonography (US) of the right eye showed a dome shaped UM located temporal superior (**Figure 1A**) with a low to medium internal reflectivity, choroidal excavation and a largest basal diameter of 15.2 x 14.6 mm and a thickness of 7.8 mm.

The gamma-glutamyl transpeptidase (gamma-GT) was high, compatible with a pre-existent alcohol abuse. Metastatic workup, including abdominal ultrasonography (US), revealed no abnormalities. Despite the large tumour, the patient opted to have fractionated stereotac-

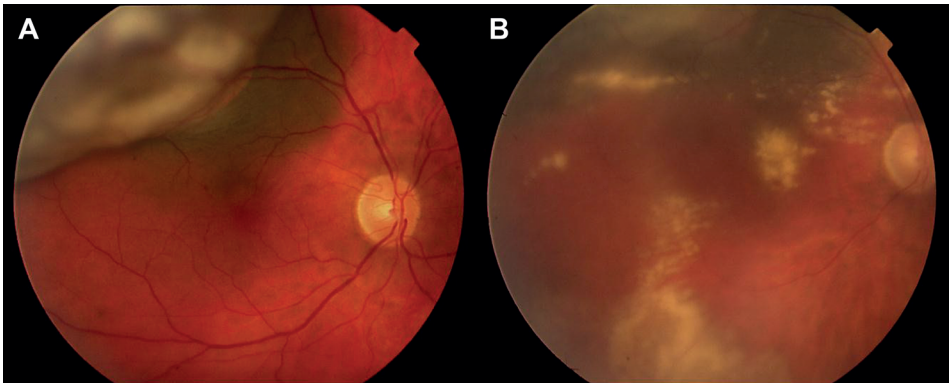


Figure 1. A dome shaped uveal melanoma of the right eye in 2001 (**A**). In 2005, ischemic irradiation retinopathy developed after fractionated stereotactic radiation therapy (**B**).

tic radiotherapy (fSRT) with a total dose of 50 Gray, and according to protocol a magnetic resonance imaging and computed tomography (CT) scan was performed for fSRT treatment planning. In 2004, the patient developed angle-closure glaucoma due to lens swelling and underwent cataract surgery.

In January 2005, the tumour had regressed and a thickness of 2.9 mm was measured on US. Eventually, he developed therapy-resistant neovascular glaucoma due to ischemic retinopathy (**Figure 1B**) and the eye was enucleated. Histopathologic analysis showed a mixed-cell malignant melanoma without ciliary body and scleral invasion, absence of extracellular matrix patterns, and mitotic figures in 4 per 50 high power fields. The centre and base of the tumour were necrotic without any obvious signs of inflammation.

During follow-up visits, only gamma-GT values were elevated between 65 U/L and 150 U/L. In the meantime, the patient requested referral to his primary ophthalmologist. In October 2009, the patient presented with abdominal pain and the CT scan showed several liver metastases and a peripancreatic lymph node metastasis of 5.9 mm. The patient died one month later and metastatic tissue became available with patients' consent. Autopsy revealed that 70% of the liver was infiltrated with UM metastases. As in the primary tumour, mixed-cells were found in the liver metastasis and in the peripancreatic lymph node.

Table 1. An overview of chromosomal alterations and mutations of a primary uveal melanoma and multiple hepatic and peripancreatic metastases.

	Primary tumour	**Liver metastasis 1**	**Liver metastasis 2**	**Peripancreatic lymph node metastasis**
Chromosome 1p	Loss p36.33-p13.2	Loss p36.33-p34.3	Loss p36.33-p34.3	Loss p36.33-p11.2 (whole p-arm)
Chromosome 1q	-	Gain q21.1-q44 (whole q-arm)	Gain q21.1-q44 (whole q-arm)	Gain q21.1-q44 (whole q-arm)
Chromosome 3p	-	Gain p26.3-p11.1	Gain p26.3-p14.3	-
Chromosome 3q	-	Loss q11.2-q24, gain q24-q29	Loss q11.2-q24, gain q24-q29	Loss q12.1-q24
Chromosome 6p	Gain p25.3-12.1	Gain p25.3-12.1	Gain p25.3-12.1	Gain p25.3-12.1
Chromosome 6q	-	Loss q12-q27	Loss q12-q27	Loss q12-q27
Chromosome 8p	Gain p23.3-p12	Loss p23.3-p11.1 (whole p-arm)	Loss p23.3-p11.1 (whole p-arm)	-
Chromosome 8q	Gain q12.3-q24.3	Gain q11.1-q24.3 (whole q-arm)	Gain q11.1-q24.3 (whole q-arm)	Gain q12.3-q24.3
GNA11 mutation	p.Q209L (exon 5)	p.Q209L (exon 5)	p.Q209L (exon 5)	p.Q209L (exon 5)
GNAQ mutation	-	-	-	-
BAP1 mutation	-	-	-	-
SF3B1 mutation	p.R625C (exon 14)	p.R625C (exon 14)	p.R625C (exon 14)	p.R625C (exon 14)
EIF1AX mutation	-	-	-	-

METHODS

Fresh tumour tissue was harvested and DNA was isolated as described previously.[5, 6] Autopsy was performed within 4 days after death and metastatic tissue of liver and peripancreatic lymph node was fresh frozen. To determine copy number variations, the Illumina Human CytoSNP12 Beadchip (Illumina San Diego, California, USA) was used and data were analysed with Nexus version 6 (Nexus BioDiscovery, El Segundo, California, USA). Chromosomal abnormalities were validated with FISH analysis on directly fixed tumour cells using centromeric or locus-specific probes: 1p36.33, 1p12, 1q21.1, 3p13, centromere 3, 3q11.2, 3q13.3, 3q22, 3q25.1, 3q26, 6p22, 6q21, 8p21.3, centromere 8, 8q22 and 22q11.22. Chromosome region 5q21.1 was used as a control. Exon 4 and 5 of *GNAQ* and *GNA11*, and the entire *BAP1* gene were sequenced as described previously.[3, 5] Exon 14 of *SF3B1* (including hotspot R625), and exon 1 and 2 of *EIF1AX* were analysed with Sanger sequencing (protocols available upon request).

RESULTS AND DISCUSSION

Chromosomal alterations and gene mutations as (incomplete and complete) loss of 1p, gain of 6p, gain of 8q, a *GNA11* mutation and a heterozygous mutation in *SF3B1* were observed in all samples. The metastases contained more chromosomal aberrations when compared to the primary tumour, such as gain of 1q, loss of region 3q11.2-3q24 and loss of 6q (**Table 1** and **Figure 2**).

The primary tumour as well as metastatic tissue harboured a mutation in exon 5 of the *GNA11* gene. Most of the UM contain mutations in *GNAQ* and *GNA11* and these mutations are considered to occur early in UM development.[7] Estimates of tumour doubling times range between 30 to 80 days and support the hypothesis that UM with the propensity to metastasise do this when they are small, and prior to detection and treatment of the primary tumour.[8]

Even though several studies on UM metastases describe that most metastases reflect the primary tumour, the patient's primary tumour was irradiated and shows differences from its metastases (**Table 1**).[9] In metastatic samples, we found a gain of 8q and partial loss of chromosome 3q. In the pathogenesis of UM, monosomy 3 is considered to be an early event and gain of 8q a secondary hit.[10] A large tumour diameter, such as the primary tumour, is associated with a high percentage gain of chromosome 8q.[11] Trolet et al.[9] also observed a higher level of 8q gain in monosomy 3 metastatic tumours compared to monosomy 3 non-metastatic UM. Although abnormalities of chromosome 6 have been associated with a good prognosis,

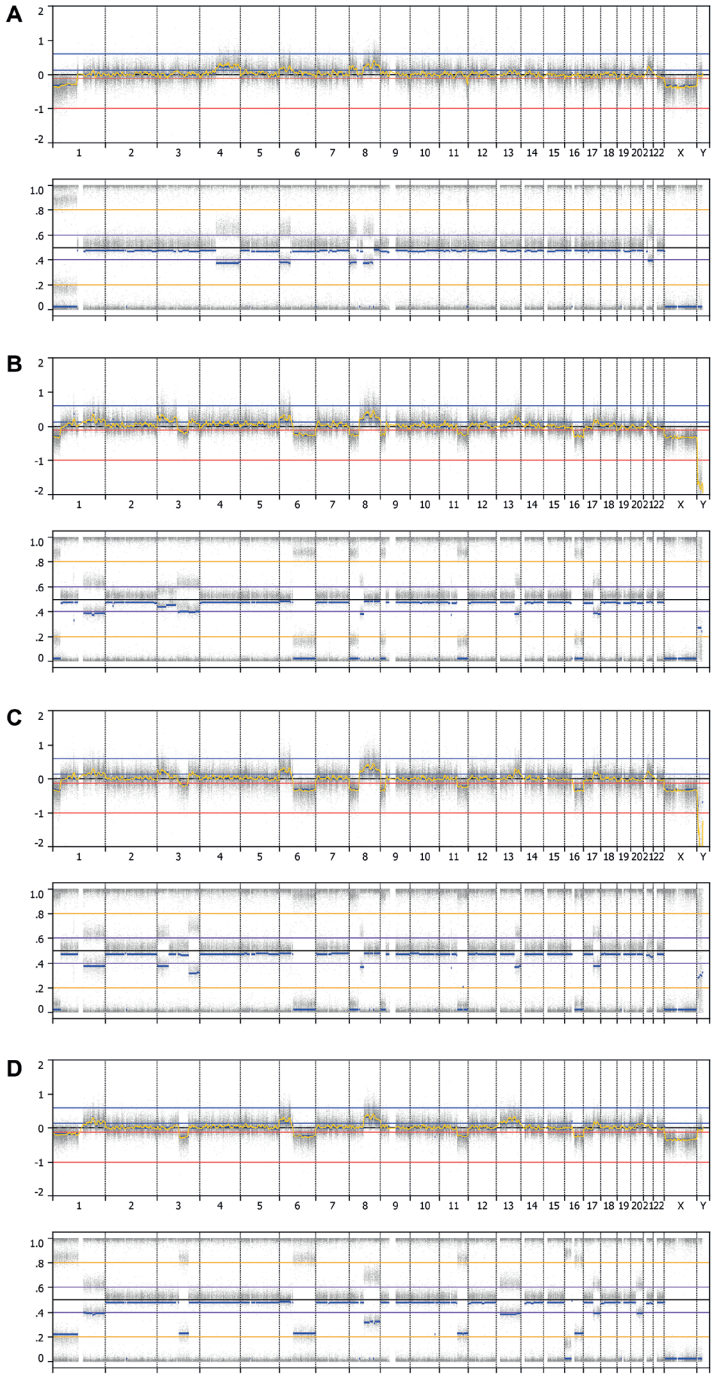


Figure 2. Whole-genome single nucleotide polymorphism array analyses in the primary tumour (**A**), liver metastasis 1 (**B**), liver metastasis 2 (**C**), and the peripancreatic lymph node (**D**). Each panel includes plots of log R ratio (upper panel) and B allele frequency (lower panel).

loss of chromosome 6q has been correlated with decreased survival and has been considered to be a secondary event.[10,12]

The metastases revealed more chromosomal abnormalities compared to the primary tumour. With radiation applied to the primary tumour, cell killing is assumed to occur through DNA cluster damage. This leads to chromosome aberrations and eventually to clonogenic inactivation.[13] Nevertheless, early mutations, such as *GNA11*, were present in all cells. *BAP1* mutations, which are assumed to occur later and predispose for metastatic disease, were absent in the primary tumour, and also in the metastases (**Table 1**). Monosomy 3 and *BAP1* mutations are associated with poor prognosis. However, a subset of UM with a monosomy 3 do not have a *BAP1* mutation.[2,3] If patients develop metastases, they occur much later than metastases in the patients with a *BAP1* mutated tumour.[3] *SF3B1* mutations have been associated with good prognostic parameters.[4, 14] We observed a *SF3B1* mutation in the primary tumour and metastases in absence of a *BAP1* mutation. This implies that a *SF3B1* mutation does not protect for metastases, and that there are different mechanisms involved in developing metastatic disease in UM.

This case report describes an UM with its corresponding metastases which occurred many years after primary treatment. Besides loss of 1p and gain of 8q, a *SF3B1* mutation seems to predispose for late metastatic disease in absence of a *BAP1* mutation.

ACKNOWLEDGEMENTS

We would like to thank S. Broeders for her technical assistance.

REFERENCES

1. Lorigan JG, Wallace S, Mavligit GM. The prevalence and location of metastases from ocular melanoma: imaging study in 110 patients. *AJR Am J Roentgenol*. 1991;157:1279-1281.
2. Harbour JW, Onken MD, Roberson ED, et al. Frequent mutation of BAP1 in metastasizing uveal melanomas. *Science*. 2010;330:1410-1413.
3. Koopmans AE, Verdijk RM, Brouwer RW, et al. Clinical significance of immunohistochemistry for detection of BAP1 mutations in uveal melanoma. *Mod Pathol*. 2014;27:1321-1330.
4. Harbour JW, Roberson ED, Anbunathan H, et al. Recurrent mutations at codon 625 of the splicing factor SF3B1 in uveal melanoma. *Nat Genet*. 2013;45:133-135.
5. Koopmans AE, Vaarwater J, Paridaens D, et al. Patient survival in uveal melanoma is not affected by oncogenic mutations in GNAQ and GNA11. *Br J Cancer*. 2013;109:493-496.
6. Naus NC, van Drunen E, de Klein A, et al. Characterization of complex chromosomal abnormalities in uveal melanoma by fluorescence in situ hybridization, spectral karyotyping, and comparative genomic hybridization. *Genes Chromosomes Cancer*. 2001;30:267-273.
7. Van Raamsdonk CD, Griewank KG, Crosby MB, et al. Mutations in GNA11 in uveal melanoma. *N Engl J Med*. 2010;363:2191-2199.
8. Eskelin S, Pyrhonen S, Summanen P, Hahka-Kemppinen M, Kivela T. Tumor doubling times in metastatic malignant melanoma of the uvea: tumor progression before and after treatment. *Ophthalmology*. 2000;107:1443-1449.
9. Trolet J, Hupe P, Huon I, et al. Genomic profiling and identification of high-risk uveal melanoma by array CGH analysis of primary tumors and liver metastases. *Invest Ophthalmol Vis Sci*. 2009;50:2572-2580.
10. Hoglund M, Gisselsson D, Hansen GB, et al. Dissecting karyotypic patterns in malignant melanomas: temporal clustering of losses and gains in melanoma karyotypic evolution. *Int J Cancer*. 2004;108:57-65.
11. van den Bosch T, van Beek JG, Vaarwater J, et al. Higher percentage of FISH-determined monosomy 3 and 8q amplification in uveal melanoma cells relate to poor patient prognosis. *Invest Ophthalmol Vis Sci*. 2012;53:2668-2674.
12. Aalto Y, Eriksson L, Seregard S, Larsson O, Knuutila S. Concomitant loss of chromosome 3 and whole arm losses and gains of chromosome 1, 6, or 8 in metastasizing primary uveal melanoma. *Invest Ophthalmol Vis Sci*. 2001;42:313-317.
13. Ballarini F, Altieri S, Bortolussi S, et al. The BIANCA model/code of radiation-induced cell death: application to human cells exposed to different radiation types. *Radiat Environ Biophys*. 2014;53:525-533.
14. Martin M, Masshofer L, Temming P, et al. Exome sequencing identifies recurrent somatic mutations in EIF1AX and SF3B1 in uveal melanoma with disomy 3. *Nat Genet*. 2013;45:933-936.

CHAPTER 2.5

Prevalence and implications of *TERT* promoter mutation in uveal and conjunctival melanoma and in benign and premalignant conjunctival melanocytic lesions

Anna E. Koopmans, Kimberley Ober, Hendrikus J. Dubbink, Dion Paridaens, Nicole C. Naus, Stephan Belunek, Bart Krist, Edward Post, Ellen C. Zwarthoff, Annelies de Klein and Robert M. Verdijk

Invest Ophthalmol Vis Sci. 2014 Aug;55(9):6024-6030

ABSTRACT

Purpose. Hotspot mutations in the promoter region of telomerase reverse transcriptase (*TERT* promoter mutations) occur frequently in cutaneous and conjunctival melanoma and are exceedingly rare in uveal melanoma (UM). No information is available on the presence of these mutations in the conjunctival melanocytic precursor lesion primary acquired melanosis (PAM). We tested a cohort of uveal and conjunctival melanomas as well as conjunctival benign and premalignant melanocytic lesions for *TERT* promoter mutations in order to elucidate the role of these mutations in tumour progression.

Methods. *TERT* promoter mutation analysis on fresh tumour DNA and DNA from formalin-fixed, paraffin-embedded specimens was performed by SNaPshot analysis in 102 UM, 39 conjunctival melanomas, 26 PAM with atypia, 14 PAM without atypia, and 56 conjunctival naevi.

Results. Mutations of the *TERT* promoter were not identified in conjunctival naevi or PAM without atypia, but were detected in 2/25 (8%) of PAM with atypia and 16/39 (41%) of conjunctival melanomas. A single *TERT* promoter mutation was detected in 102 UM (1%).

Conclusion. We present the second documented case of *TERT* promoter mutation in UM. In comparison with other types of melanoma, *TERT* promoter mutations occur at extremely low frequency in UM. *TERT* promoter mutations are frequent in conjunctival melanoma and occur at lower frequency in PAM with atypia but were not detected in benign conjunctival melanocytic lesions. These findings favour a pathogenetic tumour progression role for *TERT* promoter mutations in conjunctival melanocytic lesions.

INTRODUCTION

Melanoma is one of the most deadly malignancies of the eye and ocular surface. The incidence of uveal melanoma (UM) (5.6 per million) has remained unchanged for decades,[1] but the incidence of conjunctival melanoma, although rare (0.45 – 0.8 per million),[2] shows a trend of increasing frequency of diagnosis in Europe and the United States.[3, 4] Telomerase reverse transcriptase (*TERT*) promoter mutations occur frequently in cutaneous melanoma[5, 6] and in conjunctival melanoma[7] but rarely in cutaneous acral melanomas[8] and UM.[6, 9] The frequency of *TERT* promoter mutations in primary acquired melanosis (PAM) and conjunctival naevi has not yet been described. Here, we investigated the presence of *TERT* promoter mutations in UM, conjunctival melanoma, PAM with atypia, PAM without atypia, and conjunctival naevi in order to elucidate the role of these mutations in tumour progression.

MATERIALS AND METHODS

Patients and samples

Tissue specimens were obtained from patients with a conjunctival melanocytic lesion or UM who had undergone biopsy, excision, or enucleation between 1972 and 2013 at the Erasmus University Medical Centre or The Rotterdam Eye Hospital, the Netherlands. Fresh tissue samples were obtained from enucleation specimens of 102 primary UM. Conjunctival tissue of all patients diagnosed with conjunctival melanoma or PAM (n = 205), as well as 56 patients with conjunctival naevi, was selected from the electronic archives of the Department of Pathology. Paraffin-embedded tissues were retrieved from the archive, and the amount of tissue present in the blocks was assessed. Samples of 125 patients contained enough material for further study. Next the original glass slides were retrieved from the archive, and the initial diagnoses were reassessed by a pathologist specialised in ophthalmic pathology (RMV), as well as mitosis count, Breslow's thickness, presence of leukocyte infiltration and necrosis (although rare in melanoma). After confirming diagnosis and assessment, 56 conjunctival naevi samples, 14 PAM without atypia samples, 26 PAM with atypia samples, and 39 conjunctival melanoma samples were used for DNA isolation. Clinical details were obtained from patient records. The study was performed accordance with the tenets of the Declaration of Helsinki.

DNA isolation

DNA from UM was extracted directly from fresh tumour tissue or frozen tumour using the QIAmp DNA-mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

The DNA isolation procedure for the conjunctival naevi, PAM and conjunctival melanoma samples was performed on formalin-fixed paraffin-embedded (FFPE) specimens. Criteria for DNA collection was the presence of more than 70% tumour cells. For PAM samples this percentage was not feasible, and a minimum of 20% tumour cells was used for these cases. Microdissection was performed by scraping the tumour cells manually from haematoxylin-stained sections on a glass slide. DNA was extracted from the tissue fragments by incubation overnight at 56°C in lysis buffer (A7933; Promega, Madison, Wisconsin, USA) containing 2 mg/ml proteinase K and 5% Chelex 100 resin. Proteinase K was inactivated at 100°C for 10 min, and the DNA was separated from cell debris by centrifugation at maximum speed in a microcentrifuge for 5 min.

Chromosomal and gene analyses in uveal melanoma

Copy number variations in UM on chromosome 1, 3, 6, and 8 were investigated by fluorescence *in situ* hybridisation (FISH) and single nucleotide polymorphism analysis, as described previously.[10] The *GNAQ* and *GNA11* mutation status was determined with Sanger sequencing,[11] and BAP1 expression was examined as reported previously.[12]

Mutation analysis

SNaPshot analysis to determine the presence of mutations in three mutational hotspots, C228, C242, and C250 (chr5:1,295,228C>T; chr5:1,295,242-243CC>TT; chr5:1,295,250 C>T, respectively; hg19), in the promoter region of *TERT* was performed essentially as described previously.[13] A 155-bp DNA segment was amplified with upstream primer 5′-AGCGCTGCCTGAAACTCG-3′ and downstream primer 5′-CCCTTCACCTTCCAGCTC-3′ as described above. Subsequently, a single nucleotide probe extension assay was performed with probes designed to anneal to the sites of interest (228 probe: 5′-T23 GGCTGGGAGGGCCCGGA-3′; 242-243 probe: 5′-T27 GGAGGGGGCTGGGCGG-3′; 250 probe: 5′-T39 CTGGGCCGGGGACCCGG-3′). These probes were adapted with poly T tails of different lengths to allow separation of the extended products by size. SNaPshot analysis was performed with the SNaPshot multiplex kit (Life Technologies, Bleiswijk, the Netherlands) according to manufacturer's instructions. Thermal cycler conditions were as follows: 35 cycles of 10 s at 96°C and 40 s at 58.5°C. Snapshot products were analysed on an ABI Prism 3730xl genetic analyser (Applied Biosystems, Bleiswijk, the Netherlands).

Statistical analyses

All statistical analyses were performed with the Statistical Package for Social Science (IBM SPSS Statistics 20, Chicago, IL, USA). P-values equal or below 0.05 were considered to be

statistically significant. Correlations were calculated with either the χ^2-test or Fisher's exact test (categorical variables) or the Mann-Whitney U test (continuous variables). Kaplan-Meier survival curves were made for survival analysis by using the Log rank statistic.

RESULTS

Study cohort

SNaPshot analyses were obtained from the tumour samples of 102 UM, 39 conjunctival melanoma, 26 PAM with atypia, 14 PAM without atypia, and 56 conjunctival naevi patients. The samples were primary or recurrent tumours, and seven cases were tested from both primary tumour and recurrences, with identical results. In two PAM cases no reliable results could be obtained because of insufficient quality of the DNA isolated from FFPE tissue. The UM group consisted of 55 men and 47 women with a mean age at diagnosis of 61.5 years (range, 21 – 86 years). Ninety-one tumours were localised in the choroid and 11 in the ciliary body. The mean largest tumour diameter and prominence was 13.4 and 8.2 mm., respectively. Thirty-two of 76 (32.4%) investigated UM showed an absent BAP1 expression, and 93 of 99 (91.2%) tumours harbored a mutation in either *GNAQ* or *GNA11*. The clinical, histopathologic, and molecular characteristics of the UM are shown in **Table 1**. The clinical and histopathologic characteristics of the conjunctival melanocytic lesions are listed in **Table 2**. The mean age at diagnosis was 41.3, 48.0, 64.0 and 62.5 years for conjunctival naevi, PAM without atypia, PAM with atypia, and conjunctival melanoma, respectively. Recurrences of the melanocytic lesions occurred predominantly in the PAM with atypia and conjunctival melanoma group (57.7% and 48.7%, respectively). No metastases were observed in patients with conjunctival naevi or PAM without atypia. Conjunctival naevi were located at different sites on the conjunctiva, while PAM without atypia was located in the bulbar conjunctiva in 71% of cases and more specifically at the limbus in another 21%. Of the PAM lesions with atypia, 17 were located at the limbus (65%), 3 on the palpebral conjunctiva, 1 on the fornix, 1 on the bulbar conjunctiva, and 1 on the caruncula lacrimalis, and 3 were multifocal. Eighteen (46%) conjunctival melanomas were on located at the limbus, 7 on the bulbar conjunctiva, 9 on the palpebral conjunctiva, and 2 in the fornix. The mean Breslow thickness of conjunctival melanoma was 2.3 mm (range, 0.3 – 17.0 mm).

SNaPshot TERT promoter mutation analysis

One of 102 UM (1.0%) showed a 250C>T *TERT* promoter mutation. This tumour had a disomy of chromosomes 1p and 3, and a gain of chromosomes 6 and 8. In addition, this sample

Table 1. Clinical, pathologic, and genetic characteristics of the uveal melanoma cohort (*n* = 102)

	Uveal melanoma
Clinical features	***n* = 102**
Gender	
Male, *n* (%)	55 (53.9)
Female, *n* (%)	47 (46.1)
Mean age at diagnosis, years (range)	61.5 (21 – 86)
Metastasis	
Present, *n* (%)	46 (45.1)
Not present, *n* (%)	56 (54.9)
Survival	
Alive, *n* (%)	35 (34.3)
Death due to metastasis, *n* (%)	44 (43.1)
Death due to other cause, *n* (%)	23 (22.5)
Mean metastasis-free survival, months (range)	78.3 (0.8 – 209.1)
Histopathological features	
Location	
Choroid, *n* (%)	91 (89.2)
Ciliary body, *n* (%)	11 (10.8)
Mean largest tumour diameter, mm (range)	13.4 (6.0 – 22.0)
Mean prominence, mm (range)	8.2 (1.5 – 22.0)
Cell type	
Spindle, *n* (%)	45 (44.1)
Mixed / epithelioid, *n* (%)	57 (55.9)
Extracellular matrix patterns	
Present, *n* (%)	49 (48.0)
Not present, *n* (%)	52 (51.0)
Lymphocytic infiltration	
Present, *n* (%)	6 (5.9)
Not present, *n* (%)	31 (30.4)
Not examined, *n* (%)	65 (63.7)
Necrosis	
Present, *n* (%)	22 (21.6)
Not present, *n* (%)	16 (15.7)
Not examined, *n* (%)	64 (62.7)
Extraocular extension	
Present, *n* (%)	12 (11.8)
Not present, *n* (%)	90 (88.3)
BAP1 expression	
Negative, *n* (%)	32 (31.4)
Positive, *n* (%)	44 (43.1)

Table 1. Clinical, pathologic, and genetic characteristics of the uveal melanoma cohort (continued)

Genetic features	
Chromosome 3	
Loss, *n* (%)	52 (51.0)
Normal, *n* (%)	49 (48.0)
Chromosome 8p	
Loss, *n* (%)	21 (20.6)
Normal, *n* (%)	61 (59.8)
Gain, *n* (%)	19 (18.6)
Chromosome 8q	
Loss, *n* (%)	1 (1.0)
Normal, *n* (%)	37 (36.3)
Gain, *n* (%)	63 (61.8)
GNAQ or *GNA11* mutation	
Mutated, *n* (%)	93 (91.2)
Wild type, *n* (%)	6 (5.9)

harboured a heterozygous *GNA11* mutation in exon 5 (p.Q209L) and had a normal BAP1 staining, compliant with a primary low-risk UM signature. Blood of this patient was available for *TERT* analyses revealing a wild-type status. A total of 16 *TERT* promoter mutations, three 228C>T, two 242CC>TT, and eleven 250C>T, were found in the 39 conjunctival melanoma (41.0%); and 2 of 25 (8.0%) PAM with atypia showed a mutation, one 228C>T (**Figure 1**) and one 250C>T (**Figure 2** and **Table 3**). In one case a conjunctival naevus and a conjunctival melanoma were analysed from the same patient as unrelated lesions. In seven cases, concurrent PAM and conjunctival melanoma were analysed from the same patient. In none of these cases did the lesion contain a *TERT* promoter mutation. In the two cases of PAM with atypia that carried *TERT* promoter mutations, one recurred once as a PAM with atypia and was then radically excised; the other case also had one recurrence as PAM with atypia, which was irradically excised. Subsequently, both these patients received successful additional treatment with cryotherapy and topical mitomycin C with no recurrences. No *TERT* promoter mutations were identified in 13 PAM without atypia and 56 conjunctival naevi patients (16 stromal and 40 compound naevi).

Correlations of TERT promoter mutation status with clinicopathologic patient characteristics

The mean age of patients with conjunctival naevi did not differ significantly from that of patients with PAM without atypia (41.3 versus 48.0 years, P = 0.284). Patients with PAM with

Table 2. Clinical, pathologic, and molecular characteristics of the conjunctival lesion cohort (*n* = 135)

	Conjunctival naevi	PAM without atypia	PAM with atypia	Conjunctival melanoma
Clinical features	***n* = 56**	***n* = 14**	***n* = 26**	***n* = 39**
Gender				
Male, *n* (%)	27 (48.2)	3 (21.4)	13 (50.0)	26 (66.7)
Female, *n* (%)	29 (51.8)	11 (78.6)	13 (50.0)	13 (33.3)
Mean age at diagnosis, years (range)	41.3 (5 - 87)	48.0 (17 - 85)	64.0 (34 - 87)	62.5 (16 - 85)
Recurrence				
Yes, *n* (%)	0	1 (7.1)	15 (57.7)	19 (48.7)
No, *n* (%)	56 (100.0)	12 (85.7)	10 (38.5)	20 (51.3)
Metastasis				
Present, *n* (%)	0	0	2 (7.7)	8 (20.5)
Not present, *n* (%)	52 (92.9)	13 (92.9)	22 (84.6)	25 (64.1)
Survival				
Alive, *n* (%)	49 (87.5)	11 (78.6)	17 (65.4)	24 (61.5)
Death due to metastasis, *n* (%)	0	0	1 (3.8)	3 (7.7)
Death due to other cause, *n* (%)	2 (3.6)	0	6 (23.1)	4 (10.3)
Lost to follow-up, *n* (%)	5 (8.9)	3 (21.4)	2 (7.7)	8 (20.5)
Mean follow-up, months (range)	60.6 (6.9 - 249.5)	142.0 (2.6 - 219.5)	83.0 (1.2 - 225.5)	66.6 (1.0 - 322.8)
Histopathological features	***n***	***n***	***n***	***n***
Location				
Palpebral conjunctiva, *n* (%)	7 (12.5)	1 (7.1)	3 (11.5)	9 (23.1)
Fornix conjunctiva, *n* (%)	1 (1.8)	0	1 (3.8)	2 (5.1)
Bulbar conjunctiva, *n* (%)	23 (41.1)	10 (71.4)	1 (3.8)	7 (17.9)
Limbus, *n* (%)	7 (12.5)	3 (21.4)	17 (65.4)	18 (46.2)
Caruncula lacrimalis, *n* (%)	14 (25.0)	0	1 (3.8)	0

	Conjunctival naevi	PAM without atypia	PAM with atypia	Conjunctival melanoma
Tumour size				
Mean Breslow thickness, mm (range)	-	-	-	2.3 (0.3 - 17.0)
Cell type				
Stromal, *n* (%)	16 (28.6)	-	-	-
Compound, *n* (%)	40 (71.4)	-	-	-
Spindle, *n* (%)	-	-	-	9 (23.1)
Mixed (spindle/epithelioid), *n* (%)	-	-	-	8 (20.5)
Epithelioid, *n* (%)	-	-	-	17 (43.6)
Ulceration				
Present, *n* (%)	-	-	-	12 (30.8)
Not present, *n* (%)	-	-	-	20 (51.3)
Erosion				
Present, *n* (%)	-	-	-	3 (7.7)
Not present, *n* (%)	-	-	-	29 (74.4)
Necrosis				
Present, *n* (%)	-	-	-	2 (5.1)
Not present, *n* (%)	-	-	-	30 (76.9)
Vasoinvasive				
Yes, *n* (%)	-	-	-	1 (2.6)
No, *n* (%)	-	-	-	30 (76.9)
Inflammation				
Present, *n* (%)	-	-	-	22 (56.4)
Not present, *n* (%)	-	-	-	10 (25.6)
Focality				
Unifocal lesion, *n* (%)	11 (19.6)	8 (57.1)	6 (23.1)	14 (35.9)
Multifocal lesion, *n* (%)	0	2 (14.3)	15 (57.7)	17 (43.6)

Abbreviations: PAM = primary acquired melanosis.

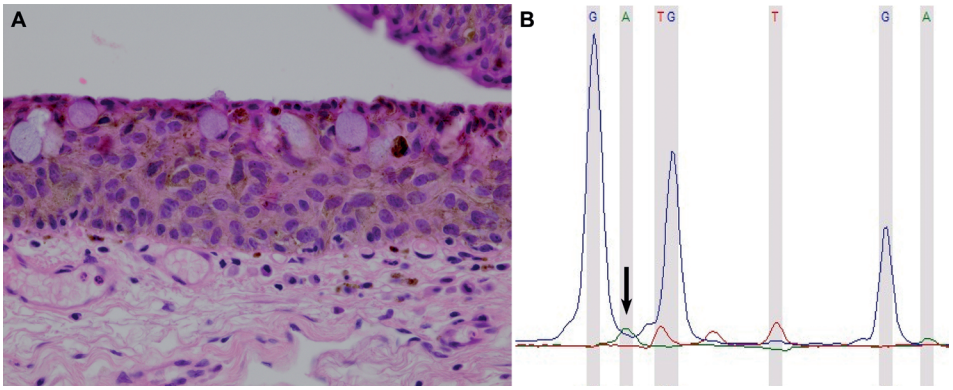


Figure 1. Histology and SnaPShot analysis results of case 15, primary acquired melanosis with severe atypia (**A**), 228C>T mutation (**B**) indicated by arrow.

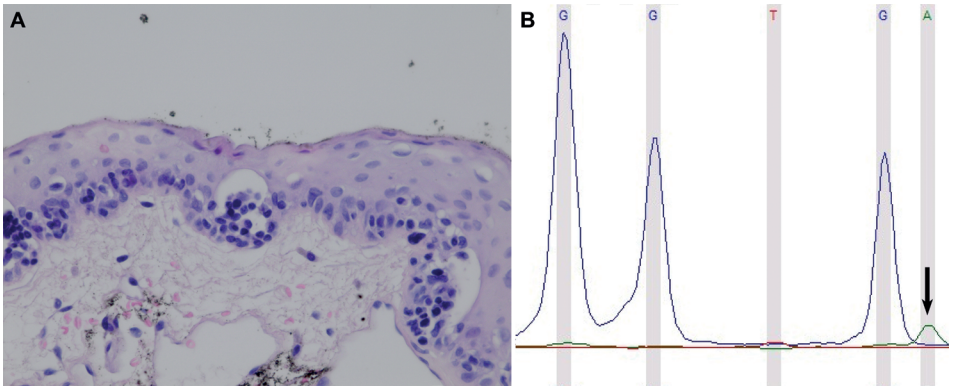


Figure 2. Histology and SnaPShot analysis results of case 72, primary acquired melanosis with moderate atypia (**A**), 250C>T mutation (**B**) indicated by arrow.

Table 3. Overview of the *TERT* promotor mutations found in different types of melanocytic conjunctival and uveal lesions.

	Conjunctival naevi	PAM without atypia	PAM with atypia	Conjunctival melanoma	Uveal melanoma
	***n* (%)**	***n* (%)**	***n* (%)**	***n* (%)**	***n* (%)**
TERT mutation	0/56	0/13	2/25 (8.0%)	16/39 (41.0%)	1/102 (1.0%)
g.1295228C>T	-	-	1	3	0
g.1295242CC>TT	-	-	0	2	0
g.1295250C>T	-	-	1	11	1

Abbreviations: PAM = primary acquired melanosis.

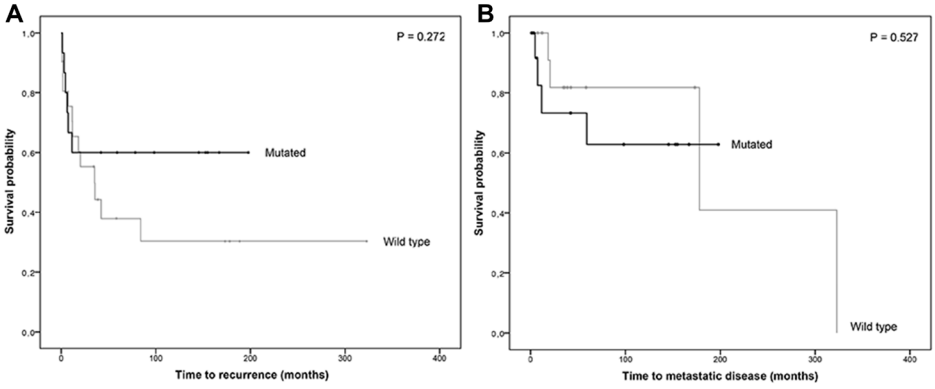


Figure 3. Kaplan-Meier survival estimate for the time to recurrence of conjunctival melanoma (**A**) and the time to metastasis of conjunctival melanoma (**B**), showing no significant differences among survival between the *TERT* mutated and wild-type cases.

atypia and conjunctival melanoma were significantly older than patients with PAM without atypia (64.0 versus 48.0, P = 0.025; 62.5 versus 48.0 years, P = 0.017). No significant difference in mean age was observed between PAM with atypia and conjunctival melanoma (64.0 versus 62.5 years, P = 0.708). Overall, *TERT* mutations did not significantly correlate with the development metastatic disease (P = 1.000). For subgroup analysis, no correlations could be calculated for UM. The conjunctival melanoma cases with *TERT* promoter mutation did not show a statistically significant correlation with adverse histologic prognostic factors when compared to nonmutated cases as to mean Breslow thickness (2.7 versus 2.1 mm, respectively, P = 0.824), mitotic count (14.6 versus 7.3, respectively, P = 0.138), or necrosis (P = 0.502). There was also no significant association between conjunctival location and the presence of a *TERT* promoter mutation in PAM with atypia and conjunctival melanomas (P = 0.153). No significant differences in clinical outcome were observed. The mean survival of *TERT* promoter mutated cases when compared to nonmutated cases did not differ significantly (75.3 versus 60.4 months, respectively, P = 0.490). Six of 16 patients with a *TERT* mutated tumour developed a recurrence of the melanocytic lesion after excision compared to 13 of 23 patients with a wild-type tumour (P = 0.272, **Figure 3A**). In total, 8 of 39 patients with conjunctival melanoma developed metastatic disease; four of these tumours harboured a *TERT* mutation (P = 0.527, **Figure 3B**). Three out of 16 mutated conjunctival melanoma cases died from metastatic disease compared to none of the wild-type cases.

DISCUSSION

Melanomas are the most common lethal primary ocular cancers. In the ophthalmic practice, melanomas mainly occur in two types, UM and conjunctival melanoma, which differ in both genetic and molecular background. We detected *TERT* promoter mutations in a high percentage (41%) of conjunctival melanomas as compared to only 1% of UM. These results are in accordance with one other cohort in which *TERT* promoter mutations were detected in approximately one-third of conjunctival melanoma.[7] We present the second case of a *TERT* promoter mutation in an otherwise cytogenetically and molecularly unremarkable case of UM. Both the *TERT* promoter mutated UM described here and the *TERT* promoter mutation-positive UM described by Dono et al.[9] have a cytogenetic low risk signature. Until now, *TERT* promoter mutations have been detected in only 1 out of a total of 97 UM-derived samples from two different cohorts.[7, 9] Uveal melanoma, in contrast to conjunctival melanoma, lack *BRAF* or *NRAS* mutations but frequently have *GNAQ*, *GNA11*, *BAP1* or *SF3B1* mutations.[9, 11] These findings confirm that since genetic alterations are different,[14] the molecular pathogenesis of UM is distinct from that of conjunctival or cutaneous melanoma. In contrast to earlier reports, the mere presence of *TERT* promoter mutations cannot be used to distinguish primary UM from (metastatic) cutaneous or conjunctival lesions.[7]

The core promoter region of the *TERT* gene encodes the rate-limiting catalytic reverse transcriptase subunit of the telomerase ribonucleoprotein complex. The g.1295228C>T, g.1295250C>T, or less common g.1295242CC>TT mutations upstream of the transcription start site create de novo CCGGAA/T general binding motifs for E-twenty six/ternary complex factors (Ets/TCF) transcription factors, which differs from preexisting GGAA/T Ets binding sites within the *TERT* promoter. The Ets transcription factors are downstream targets of RAS-RAF-MAPK pathways, and *TERT* promoter mutations are suggested to have synergistic effects with activating *BRAF* or *NRAS* mutations to promoter tumour cell proliferation. *BRAF* and *NRAS* mutations have been proposed to be driver mutations in the development of cutaneous melanocytic neoplasms since they are present in both benign and malignant neoplastic lesions.[15, 16]

We are the first to show the presence of *TERT* promoter mutations in a premalignant melanocytic lesion, PAM with atypia, and its absence in PAM without atypia and conjunctival naevi. The mutation frequency of 33% reported in primary cutaneous melanomas[6, 17] is similar to the mutation rate in primary conjunctival melanomas. Conjunctival melanomas, like cutaneous melanomas, frequently harbour *BRAF* or *NRAS* mutations.[7] The high frequency of *TERT* promoter mutations and the higher than expected rate of concomitant mutations in the *TERT* promoter and *BRAF* in cutaneous melanoma[6] might suggest that *TERT* promoter mutations are driver mutations in the pathogenesis of melanoma. However, in contrast to

BRAF mutations, *TERT* promoter mutations were not identified in benign cutaneous naevi (*n* = 34),[6, 17] nor in conjunctival naevi[7] or PAM without atypia. The possible influence of *TERT* promoter mutations in molecular progression is demonstrated by the identification of these mutations in a low percentage of premalignant melanocytic lesions PAM with atypia and in a higher percentage in conjunctival melanoma. In the past, it has been observed that mild atypia does lead to a highly increased chance for malignant progression. Although our cohort contained only 14 cases of PAM without atypia, this would be in agreement with that observation. The acquisition of *TERT* promoter mutations can be hypothesised to facilitate stabilisation of the transformed genome through reversal of telomeric loss. Many conjunctival melanocytic naevi carry *BRAF* mutations,[18] whereas *TERT* promoter mutations are detected only in primary melanoma and, as we have shown, in PAM with atypia. In cutaneous squamous cell carcinoma lesions a similar role for *TERT* promoter mutations can be observed, with mutations in 50% of squamous cell carcinoma and 9% of Bowen's disease (*in situ* lesions). In contrast, the role for *TERT* promoter mutations as a driver mutation is more convincing in urothelial cell neoplasms, where *TERT* promoter mutations are present in a high percentage (74%) in both low-grade and high-grade *in situ* urothelial cell carcinoma[13, 19] as well as in invasive urothelial cell carcinoma (53%).[13, 20]

The increased telomerase expression associated with *TERT* promoter mutations[5] offers new therapeutic possibilities in *TERT* mutated conjunctival melanoma. General reverse transcriptase inhibitors such as AZT (azidothymidine), which acts on the reverse transcriptase activity of *TERT*, may warrant investigation for potential therapeutic potential in conjunctival melanoma with *TERT* promoter mutations.[21] Another way to inhibit telomerase function is to reduce levels of *TERT* transcripts. The telomerase nucleotide bases inhibitor, imetelstat (GRN163L), has shown effectiveness in melanoma cell lines[22] and entered Phase I[23] and II clinical trials for other tumour types. More specific chemical inhibitors of telomere extension, MST-312, BIBR1532, b-rubromycin, PIPER {N,N0-bis [2-(1-piperidino) ethyl]-3,4,9,10-tetracarboxylic diimide}, and TmPyP4 are in experimental phase at this moment.[24]

In conclusion, we present the first data on *TERT* promoter mutations in PAM with atypia and propose its possible role as a molecular tumour progression marker for conjunctival melanocytic lesions.

ACKNOWLEDGEMENTS

Supported by Grants SNOO2012-21 and SNOO2012-13 from the Stichting Nederlands Oogheelkundig Onderzoek.

REFERENCES

1. Bishop KD, Olszewski AJ. Epidemiology and survival outcomes of ocular and mucosal melanoma: a population-based analysis. *Int J Cancer*. 2014;134:2961-2971.
2. Missotten GS, Keijser S, De Keizer RJ, De Wolff-Rouendaal D. Conjunctival melanoma in the Netherlands: a nationwide study. *Invest Ophthalmol Vis Sci*. 2005;46:75-82.
3. Triay E, Bergman L, Nilsson B, All-Ericsson C, Seregard S. Time trends in the incidence of conjunctival melanoma in Sweden. *Br J Ophthalmol*. 2009;93:1524-1528.
4. Yu GP, Hu DN, McCormick S, Finger PT. Conjunctival melanoma: is it increasing in the United States? *Am J Ophthalmol*. 2003;135:800-806.
5. Huang FW, Hodis E, Xu MJ, et al. Highly recurrent TERT promoter mutations in human melanoma. *Science*. 2013;339:957-959.
6. Horn S, Figl A, Rachakonda PS, et al. TERT promoter mutations in familial and sporadic melanoma. *Science*. 2013;339:959-961.
7. Griewank KG, Murali R, Schilling B, et al. TERT promoter mutations in ocular melanoma distinguish between conjunctival and uveal tumours. *Br J Cancer*. 2013;109:497-501.
8. Liau JY, Tsai JH, Jeng YM, et al. TERT promoter mutation is uncommon in acral lentiginous melanoma. *J Cutan Pathol*. 2014;41:504-508.
9. Dono M, Angelini G, Cecconi M, et al. Mutation frequencies of GNAQ, GNA11, BAP1, SF3B1, EIF1AX and TERT in uveal melanoma: detection of an activating mutation in the TERT gene promoter in a single case of uveal melanoma. *Br J Cancer*. 2014;110:1058-1065.
10. van Beek JG, Koopmans AE, Vaarwater J, et al. The prognostic value of extraocular extension in relation to monosomy 3 and gain of chromosome 8q in uveal melanoma. *Invest Ophthalmol Vis Sci*. 2014;55:1284-1291.
11. Koopmans AE, Vaarwater J, Paridaens D, et al. Patient survival in uveal melanoma is not affected by oncogenic mutations in GNAQ and GNA11. *Br J Cancer*. 2013;109:493-496.
12. Koopmans AE, Verdijk RM, Brouwer RW, et al. Clinical significance of immunohistochemistry for detection of BAP1 mutations in uveal melanoma. *Mod Pathol*. 2014;27:1321-1330.
13. Allory Y, Beukers W, Sagrera A, et al. Telomerase Reverse Transcriptase Promoter Mutations in Bladder Cancer: High Frequency Across Stages, Detection in Urine, and Lack of Association with Outcome. *Eur Urol*. 2014;65:360-366.
14. van den Bosch T, Kilic E, Paridaens D, de Klein A. Genetics of uveal melanoma and cutaneous melanoma: two of a kind? *Dermatol Res Pract*. 2010;2010:360136.
15. Davies H, Bignell GR, Cox C, et al. Mutations of the BRAF gene in human cancer. *Nature*. 2002;417:949-954.
16. Dhomen N, Reis-Filho JS, da Rocha Dias S, et al. Oncogenic Braf induces melanocyte senescence and melanoma in mice. *Cancer Cell*. 2009;15:294-303.
17. Vinagre J, Almeida A, Populo H, et al. Frequency of TERT promoter mutations in human cancers. *Nat Commun*. 2013;4:2185.
18. Goldenberg-Cohen N, Cohen Y, Rosenbaum E, et al. T1799A BRAF mutations in conjunctival melanocytic lesions. *Invest Ophthalmol Vis Sci*. 2005;46:3027-3030.
19. Kinde I, Munari E, Faraj SF, et al. TERT promoter mutations occur early in urothelial neoplasia and are biomarkers of early disease and disease recurrence in urine. *Cancer Res*. 2013;73:7162-7167.

20. Killela PJ, Reitman ZJ, Jiao Y, et al. TERT promoter mutations occur frequently in gliomas and a subset of tumours derived from cells with low rates of self-renewal. *Proc Natl Acad Sci U S A*. 2013;110:6021-6026.

21. Humer J, Ferko B, Waltenberger A, et al. Azidothymidine inhibits melanoma cell growth in vitro and in vivo. *Melanoma Res*. 2008;18:314-321.

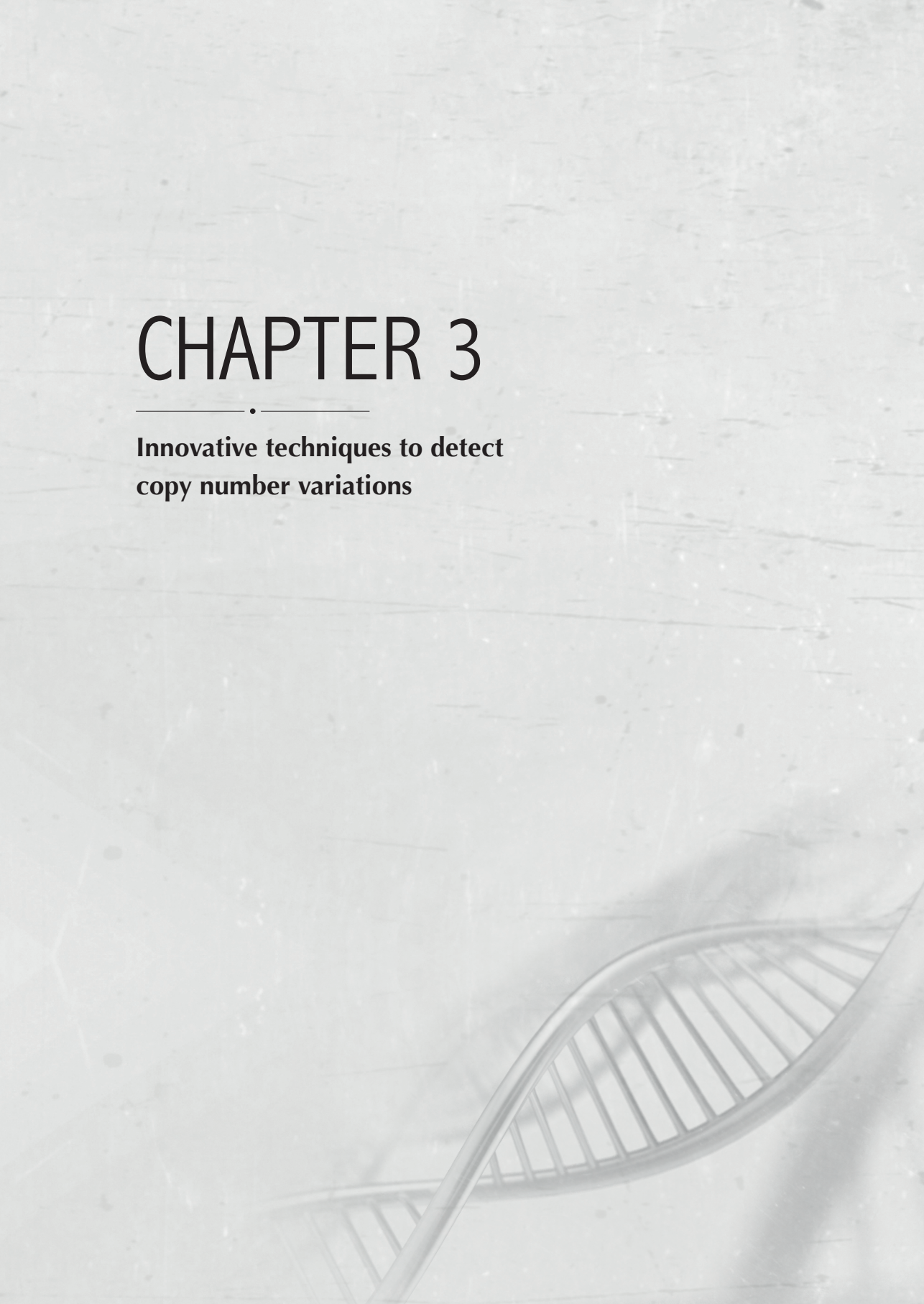
22. Go NF, Tressler RL, Harley CB, et al. Enhanced efficacy of GRN163L combined with melphalan in a human melanoma xenograft model. *AACR Meeting Abstracts*. 2005;2005:137-c-138.

23. Thompson PA, Drissi R, Muscal JA, et al. A phase I trial of imetelstat in children with refractory or recurrent solid tumours: a Children's Oncology Group Phase I Consortium Study (ADVL1112). *Clin Cancer Res*. 2013;19:6578-6584.

24. Low KC, Tergaonkar V. Telomerase: central regulator of all of the hallmarks of cancer. *Trends Biochem Sci*. 2013;38:426-434.

CHAPTER 3

Innovative techniques to detect copy number variations



CHAPTER 3.1

HaloPlex: a targeted approach for detecting gene mutations and copy number variations designed for uveal melanoma

Anna E. Koopmans, Mike M.P. van den Berg, Rutger W.W. Brouwer, Christel E.M. Kockx, Jolanda Vaarwater, Robert M. Verdijk, Mark Nellist, Wilfred F.J. van IJcken, Emine Kiliç and Annelies de Klein

Manuscript in preparation

CHAPTER 4

Histopathological and chromosomal studies in uveal melanoma

CHAPTER 4.1

Chemokine receptor CCR7 expression predicts poor outcome in uveal melanoma and relates to liver metastasis whereas expression of CXCR4 is not of clinical relevance

Thierry P.P. van den Bosch, Anna E. Koopmans, Jolanda Vaarwater, Mike M.P. van den Berg, Annelies de Klein and Robert M. Verdijk

Invest Ophthalmol Vis Sci. 2013 Sep;54:7354-7361

ABSTRACT

Purpose. To examine the prognostic relevance of expression of the chemokine receptors CCR7 and CXCR4 and its ligand CXCL12 in uveal melanoma in non-metastatic and metastatic patients with correlation to liver metastasis and overall survival.

Methods. Primary uveal melanoma specimens from 19 patients with correlating liver metastasis specimens and 30 primary uveal melanoma specimens of patients without metastasis were collected between the years 1988 and 2008. Expression of CCR7, CXCR4 and CXCL12 were studied using immunohistochemistry. Single nucleotide polymorphism arrays were used to examine gains or losses of chromosomes 1, 3, 6 and 8 and the regions of *CCR7* (17q12-q21.2), *CXCR4* (2q21) and *CXCL12* (10q11.1) genes.

Results. Strong cytoplasmic staining for CCR7 correlated with the presence of epithelioid cells (P = 0.037), tumour thickness (P = 0.011), lymphocytic infiltration (P = 0.041) and necrosis (P = 0.045). Nuclear staining for CXCR4 correlated with lymphocytic infiltration (P = 0.017). CXCL12 showed no correlation to histological parameters. Single nucleotide polymorphism analyses showed no copy number variations in the regions of *CCR7*, *CXCR4*, or *CXCL12*. Strong expression of CCR7 was observed in 76% of the metastatic patients and 0% of non-metastatic patients. In multivariate analysis, CCR7 staining was inversely correlated to overall survival and disease-free survival whereas CXCR4 nuclear staining was not.

Conclusions. Our data suggest that CCR7 plays a role in uveal melanoma metastasis and is associated with poor survival. CCR7 and its involved related pathways are of prognostic value in uveal melanoma and may prove to be a target for therapeutic intervention.

INTRODUCTION

Uveal melanoma (UM) is the most common type of primary eye cancer in adults. Approximately 0.7/100,000 of the Western population is affected yearly.[1] The age-adjusted incidence of UM (5.1 per million) has remained unchanged from 1973 to 2008. Despite new and more conservative treatments, survival has not improved during this time period.[2] Well-known clinical prognostic factors are age and location of the tumour. UM patients that are of older age tend to have a worse prognosis. The most important clinical prognostic factor is the tumour size, which is often used for selection of treatment. A large study showed that each increase in millimetre of tumour thickness increased the risk of metastasis by 5%.[3] The most common location for the initial metastasis is the liver. Median survival with initial liver metastasis is 4 to 12 months.[4] UM containing fast growing epithelioid cells, have aggressive behaviour, and is therefore are associated with a poor prognosis. Other histologic features that have been associated with mortality and metastasis are extrascleral extension, extracellular matrix patterns, mitotic count, tumour necrosis, and presence of lymphocytic infiltration.[5]

Chemokine receptor CCR7 is mainly expressed in lymphoid cells, its function is mainly mediating cell migration of naive lymphocytes and mature dendritic cells to secondary lymphoid organs towards chemokines CCL19 and CCL21. CCL19 and CCL21 are the ligands of CCR7 and are both mainly expressed by stroma cells in lymphoid tissues.[6] CCR7 has been shown to regulate integrins, which influence the transport of cancer cells and help them migrate through the extracellular matrix.[7] Wang et al.[8] found evidence that CCR7 mediates survival and invasion of metastatic squamous cell carcinoma cells through activation of PI3K. This indicates that CCR7 is involved in tumour progression and metastasis. It is known that chemokine receptors are expressed widely on different types of cancers such as non-small cell lung cancer, gastric cancer, head/neck cancer, and colon cancer.[9-12] CCR7 is also expressed on B-cell acute lymphatic leukaemia and chronic lymphatic leukaemia.[13] Strong expression of CCR7 has been associated with poor clinical outcome in patients with cutaneous melanoma.[14, 15] Recently, strong expression of CCR7 was found in UM cells.[16] The role of chemokine receptor CXCR4 has been described in multiple malignancies that metastasise to the liver, and therefore might provide a pathway for therapeutic intervention. In addition, CXCR4 also localises on vascular endothelial cells and mediates the angiogenic activity of the chemokine CXCL12.[17] A recent study shows that CXCR4 is commonly expressed in UM and correlates with epithelioid cell type, a well-established prognostic factor.[18] High levels of its ligand, CXCL12, in the liver offer an attractive explanation for the selective metastasis of UM to the liver.[19] It has been hypothesised that the CXCL12/CXCR4 pathway might mediate cancer cells to 'home' to specific secondary sites, thereby promoting organ-specific hepatic metastasis.[20]

In this study, we investigated the expression of CCR7 and CXCR4/CXCL12 in both primary and metastatic tumour samples. Furthermore, we examined whether expression levels are correlated with clinical, histopathologic, and chromosomal parameters as well as survival, to gain more insight into prognostic factors and possible opportunities for therapeutic intervention.

MATERIALS AND METHODS

Patient samples

Forty-nine UM paraffin samples, fresh UM tissue for DNA isolation, and corresponding metastatic liver paraffin samples were collected at the Department of Pathology of the Erasmus University Medical Centre between 1988 and 2008. Classical histopathologic parameters such as cell type, mitotic count (per 8 mm^2 equal to 50 high-power fields (HPF)), necrosis, lymphocytic infiltration, extraocular extension, and optical nerve invasion were scored by haematoxylin and eosin (H&E) staining, and extracellular matrix patterns with periodic acid-Schiff (PAS) staining without haematoxylin staining. Cell type was assigned according to the Callender classification system. The research followed the tenets of the Declaration of Helsinki.

Construction of Tissue Microarray (TMA) samples

The TMA contained 19 cases of UM patients with histologic confirmed metastasis and 30 patients without metastasis. These were constructed from representative areas of formalin-fixed specimens. The TMA consisted of random located 2-mm core samples of each case. The 4-µm sections were cut from TMA and were stained with H&E to confirm the presence of the expected tissue histology within each tissue core. Additional sections were cut for immunohistochemistry analyses and fluorescence *in situ* hybridisation (FISH) analyses.

Immunohistochemistry CCR7, CXCR4 and CXCL12

In the evaluation set, we assessed expression of CCR7, CXCR4, and CXCL12 by immunohistochemistry to determine specificity and sensitivity. The samples were scored positive or negative by masked screening. Immunohistochemistry was performed with an automated immunohistochemistry staining system (Ventana BenchMark ULTRA; Ventana Medical Systems, Tucson, AZ, USA) using alkaline phosphatase method for all antibodies and a red chromogen. Briefly, following deparaffinisation and heat-induced antigen retrieval for 64 min, with exception of CXCR4 (protease treatment for 4 min at 36˚C), the tissue sections were

incubated with primary antibody CCR7 (clone 150503, 1:32000 dilution), CXCL12 (clone 79018, 1:50 dilution), and CXCR4 (clone 44716, 1:128000 dilution; all from R&D Systems, Minneapolis, MN, USA) for 1 h at 36˚C. A subsequent amplification step was followed by incubation with haematoxylin II counter stain for 8 min and then blueing reagent for 8 min according to the manufacturer's instructions (Ventana). Liver, tonsil and breast tissue were used as positive controls for CCR7, CXCR4 and CXCL12. Tumours were scored according to the intensity as negative (-), mildly positive (+), moderately positive (++), or strongly positive (+++) of cytoplasmic staining for CCR7 and CXCL12. Nuclear and cytoplasmic staining was scored in a similar way for CXCR4. The histopathologic characterisation of the tissue sections and the immunohistochemistry staining's were independently evaluated by an ophthalmic pathologist.

DNA isolation

DNA was isolated from fresh received primary tumour samples using the QIAamp DNA-mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA concentration was measured using the NanoDrop ND-1000 Spectrophotometer (NanoDrop technologies, Wilmington, DE, USA) and Picogreen assay (Molecular Probes, Eugene, OR, USA). DNA was stored at -20˚C.

Single Nucleotide Polymorphism (SNP) array analysis

Two hundred nanograms of fresh primary tumour DNA were used as input for whole genome analysis by SNP array for each UM sample, to examine gains or losses of chromosomes 1, 3, 6 and 8 and the regions of *CCR7* (17q12-q21.2), *CXCR4* (2q21) and *CXCL12* (10q11.1) genes (Illumina 610Q BeadChip, Illumina, San Diego, CA, USA). The SNP array data were analysed with the Nexus 6 software (BioDiscovery, El Segundo, CA, USA).

FISH analysis

To validate non-random chromosomal anomalies on either the short arm (p) and/or long arm (q) of chromosomes 1, 3, 6 and 8, FISH was performed on fresh tumour material (n = 31) or primary UM paraffin samples (n = 15) as described before.[21]

Statistical analysis

We used several tests to assess whether clinical, histopathologic and chromosomal parameters were associated with expression levels. The χ^2-test and the Fisher's exact test were used for categorical variables. The Mann-Whitney test and the Kruskal-Wallis test were used for two and more than two continuous variables, respectively. For survival analyses both the

overall survival and the disease-free survival were taken into account. The primary endpoint for overall survival was defined as the time to death due to metastasis, whereby death due to other causes were treated as censored. The disease-free survival was defined as the time to the development of metastatic disease. The influence of single prognostic factors on overall survival and disease-free survival were assessed using the Kaplan-Meier method (for categorical variables) or the Cox proportional hazard analysis (for continuous variables). To identify the independent value of the prognostic factors on overall survival and disease-free survival, we used a multivariate Cox proportional hazard analysis with a forward stepwise method based on likelihood ratios. An effect was considered significant if the P-value was less than or equal to 0.05. The statistical analyses were performed with the SPSS software version 20.0 (IBM, Chicago, IL, USA).

RESULTS

A total of 49 patients were included in the study. Twenty-three of the patients were male and 26 were female. The mean age was 57 years (range 27 – 84); the mean largest tumour diameter was 12.5 mm (range 5.0 – 19.0) and the mean tumour thickness was 6.9 mm (range 2.0 – 20.0). Twenty-three tumours were classified as spindle cell type, five as epithelioid cell type and 21 as mixed. The mean overall survival (follow-up) was 171 months for the non-metastatic group and 69 months for the patients who developed metastatic disease. Four patients died due to another cause. The clinical and histopathologic tumour characteristics for non-metastatic patients and metastatic patients are shown separately in **Table 1** and **Table 2**, respectively.

Table 1. Patient characteristics of the investigated study group stratified for the presence of metastatic disease

Patient characteristics	Patients without metastasis	Patients with metastasis
	No. of patients (*n*)	**No. of patients (*n*)**
Male	14/30	9/19
Female	16/30	10/19
	Mean (range)	**Mean (range)**
Age at time of diagnosis, years	57.7 (34.0 – 84.0)	56.2 (27.0 – 84.0)
Tumour thickness, mm	8.0 (2.0 – 20.0)	5.1 (2.0 – 11.0)
Largest tumour diameter, mm	12.2 (5.0 – 18.0)	12.9 (10.0 – 19.0)
Overall survival, months	171.24 (52.93 – 272.39)	69.02 (6.83 – 181.82)
Disease-free survival, months	171.24 (52.93 – 272.39)	59.78 (6.83 – 156.88)

Table 2. Histopathologic parameters of the investigated study group stratified for the presence of metastatic disease

Histopathologic parameters	Patients without metastasis	Patients with metastasis
	No. of patients (*n*)	No. of patients (*n*)
Localisation tumour		
Choroid	25/30	14/19
Ciliary body	5/30	5/19
Cell type		
Spindle	19/30	4/19
Mixed	10/30	11/19
Epithelioid	1/30	4/19
Epithelioid cells		
Absent	18/30	4/19
Present	12/30	15/19
Necrosis		
Absent	19/30	11/19
Present	11/30	8/19
Extracellular matrix patterns		
Absent	23/30	10/19
Present	7/30	9/19
Lymphocytic infiltration		
Absent	25/30	10/19
Present	5/30	9/19
Extraocular extension		
Absent	29/30	17/19
Present	1/30	2/19
Optic nerve invasion		
Absent	30/30	19/19
Present	0/30	0/19
	Mean (range)	Mean (range)
Mitotic figures (per 8 mm^2)	4.2 (1.0 – 22.0)	8.0 (1.0 – 40.0)

CCR7 staining

Variation in intensity of cytoplasmic expression was evaluated for CCR7 and was ranked ranging from complete lack of expression to strong cytoplasmic expression (**Figure 1**). No nuclear staining was observed for CCR7. As demonstrated in **Table 3**, strong CCR7 expression was observed in 76% (13/17) of metastatic patients and 0% (0/26) of non-metastatic patients. When lymphocytic infiltration was observed the lymphocytes showed CCR7 ex-

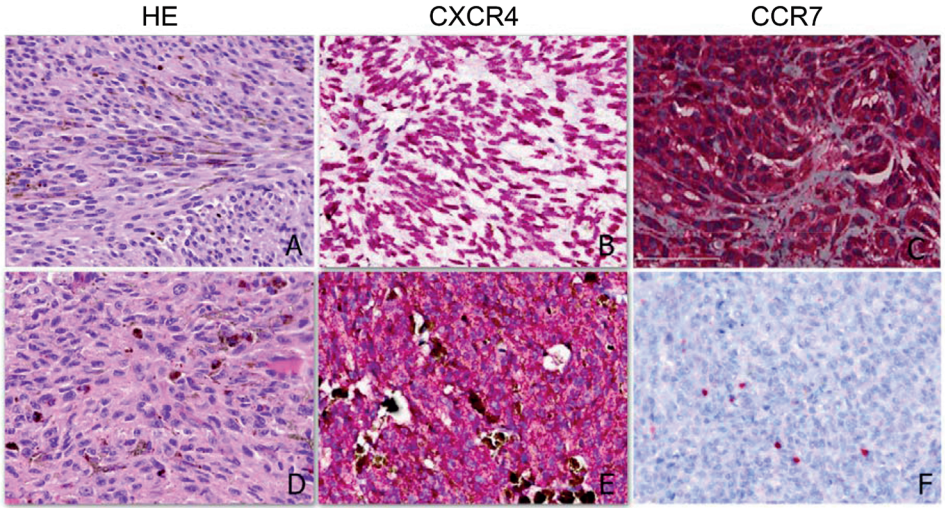


Figure 1. Histology and immunohistochemistry of chemokine receptor CXCR4 and CCR7 expression in uveal melanoma. Histologic parameters were scored using haematoxylin & eosin (H&E) staining (**A** and **D**). CXCR4 was scored with either strong nuclear staining (**B**) or cytoplasmic staining (**E**). CCR7 expression was scored with strong cytoplasmic staining (**C**, 40x) or negative staining (note positive lymphocytes and macrophages) (**F**). Original magnification for all panels x400.

Table 3. An overview of the numbers of cases with chemokine expression levels stratified for non-metastatic and metastatic uveal melanoma patients

	–	+	++	+++
Non-metastatic uveal melanoma, *n* = 30				
CCR7	12/30	14/30	4/30	0/30
CXCR4 (nuclear)	11/30	8/30	7/30	4/30
CXCR4 (cytoplasm)	5/30	5/30	13/30	7/30
CXCL12	24/30	6/30	0/30	0/30
Metastatic uveal melanoma, *n* = 19 (missing value, *n* = 2)				
CCR7	1/17	3/17	0/17	13/17
CXCR4 (nuclear)	6/17	2/17	1/17	8/17
CXCR4 (cytoplasm)	7/17	6/17	2/17	2/17
CXCL12	17/17	0/17	0/17	0/17

The expression levels are classified as: – = negative; + = mildly positive; ++ = moderately positive; +++ = strongly positive.

pression. Identical CCR7 expression patterns were found in the metastatic liver specimens. In a multivariate analysis, CCR7 expression was inversely correlated to overall survival ($P < 0.001$) (**Figure 2A**) and correlated with the adverse prognostic histologic parameters such as the presence of epithelioid cells ($P = 0.037$), tumour thickness ($P = 0.011$), lymphocytic

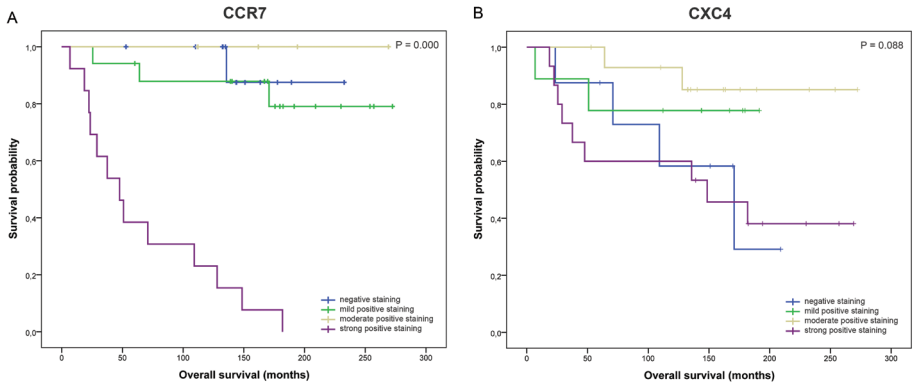


Figure 2. Overall survival analysis in relation to chemokine expression. Overall survival analysis showed a high significant inverse correlation with strong CCR7 expression (**A**), nuclear CXCR4 expression showed no significance with overall survival (**B**).

infiltration (P = 0.041) and necrosis (P = 0.045). The correlations are shown in **Table 4**. There was no correlation between CCR7 expression and chromosomal abnormalities or losses and gains in *CCR7* (17q12-q21.2) related regions using SNP analysis.

CXCR4/CXCL12 staining

Expression of CXCR4 showed both cytoplasmic and nuclear expression (**Figure 1**). The nuclear expression of CXCR 4 was correlated with lymphocytic infiltration (P = 0.017) (**Table 4**). Nuclear expression of CXCR4 was not significantly related to overall survival (P = 0.088) as shown in **Figure 2B**. When lymphocytic infiltration was observed the lymphocytes showed CXCR4 expression. There was an identical CXCR4 expression pattern in the metastatic liver specimens. There was no significant correlation between CCR7 expression and CXCR4 expression either cytoplasmic (6/47, P = 0.628) or nuclear (11/47, P = 0.838). CXCL12 was only mildly positive in the cytoplasm in a minority of the tumour samples (**Table 3**) and showed mild expression in the biliary epithelium of the liver samples. There were no correlations with chromosomal abnormalities or losses and gains in *CXCR4* (2q21) or *CXCL12* (10q11.1) related regions in SNP analysis.

Statistical analysis

Univariate analysis showed that the overall survival was significantly shorter in UM patients with tumours with the presence of epithelioid cells (P = 0.010), large tumour thickness (P = 0.026), presence of extracellular matrix patterns (P = 0.021), high mitotic count (8 mm^2 equal to 50 HPF) (P = 0.003), presence of lymphocytic infiltration (P = 0.011), chromosome

8p loss (P = 0.004), chromosome 8q gain (P = 0.005) and CCR7 expression (P < 0.001). Using multivariate analysis, only high mitotic count (hazard ratio (HR) 1.2, P = 0.007) and strong positive CCR7 expression (HR 479.1, P = 0.003) showed to be highly significant and independent adverse prognostic factors for overall survival.

For disease-free survival the univariate analysis showed that tumours with the presence of epithelioid cells (P = 0.006), tumour thickness (P = 0.018), presence of extracellular matrix patterns (P = 0.026), high mitotic count (P = 0.009), and presence of lymphocytic infiltration (P = 0.012), chromosome 8p loss (P = 0.008), chromosome 8q gain (P = 0.005) and CCR7 expression (P < 0.001) were significantly and inversely correlated with disease-free survival. In multivariate analysis, only high mitotic count (HR 1.1, P = 0.051), chromosome 8p loss (HR 8.4, P = 0.054) and a strong positive CCR7 expression (HR 435.3, P = 0.004) were independent predictors for disease-free survival.

DISCUSSION

In this study, we found a highly significant inverse correlation between both overall survival and disease-free survival, and CCR7 expression, which shows that CCR7 is associated with poor survival in uveal melanoma. In our immunohistochemistry experiments, we demonstrated that chemokine receptors CCR7 and CXCR4 are expressed on uveal melanoma samples. There were cases with negative CCR7 staining, but these cases showed positive internal controls in lymphocyte infiltration as shown in **Figure 1**. Negative staining for CCR7 did not significantly correlate with negative staining for CXCR4. Neither was there a significant correlation between CCR7 expression and CXCR4 expression in either cytoplasmic or nuclear pattern. The chemokine CXCL12 is rarely expressed in uveal melanoma at low levels. It is possible that immunohistochemistry might not be sufficiently sensitive to evaluate the differential expression of CXCL12 in formalin-fixated, paraffin-embedded tissues. Although CCR7 is linked mainly to lymphogenic metastasis,[11] our study strongly indicates that lymphatic dissemination is not the exclusive route for CCR7 associated metastasis. No lymphatic vessels are present in the choroid of the eye. Others have shown that CCR7 regulates migration and adhesion processes of metastatic squamous cell carcinoma cells via αvβ3 integrin,[10] these findings may be applicable to UM cells. In cutaneous melanoma αvβ3 has been correlated to metastatic behaviour,[22] and αvβ3 expression has been shown in primary UM and cell lines derived from the same tumours.[23-25] It can be hypothesised that CCR7 regulates migration and adhesion of UM cells through vascular endothelial cells, and thereby enable metastasis to secondary organs such as the liver. It is known that ligands of CCR7 (CCL19/CCL21) are expressed in the liver,[26, 27] thus it may be possible that the CCR7 pathway is activated in

Table 4. Correlations between CCR7, CXCR4 and CXCL12 expression and clinical, histopathologic, and chromosomal data

	CCR7				CXCR4 (nuclear)			CXCR4 (cytoplasm)				CXCL12		
	–	+	++/+++	P-value	–/+	++/+++	P-value	–	+	++/+++	P-value	–	+	P-value
	n = 13	*n* = 17	*n* = 17		*n* = 17	*n* = 30		*n* = 18	*n* = 11	*n* = 18		*n* = 23	*n* = 8	
Sex														
Male	6	7	9	0.788†	7	15	0.560†	9	8	5	0.059†	13	1	0.045†
Female	7	10	8		10	15		9	3	13		10	7	
Epithelioid cells														
Absent	9	9	4	0.037†	10	12	0.214†	7	5	10	0.602†	14	5	1.000*
Present	4	8	13		7	18		11	6	8		9	3	
Necrosis														
Absent	4	12	12	0.045†	13	15	0.076†	10	5	13	0.328†	12	7	0.108*
Present	9	5	5		4	15		8	6	5		11	1	
Extracellular matrix patterns														
Absent	11	11	10	0.302†	11	21	0.708†	13	7	12	0.879†	18	6	1.000*
Present	2	6	7		6	9		5	4	6		5	2	
Optic nerve invasion														
Absent	13	17	17	-	17	30	-	18	11	18	-	23	8	-
Present	0	0	0		0	0		0	0	0		0	0	
Mean age at time of diagnosis, years	54.2	56.9	59.8	0.206‡	58.0	56.7	0.674‡	58.1	54.0	58.2	0.680‡	56.4	58.9	0.603§
Mean tumour thickness, mm	9.0	6.9	5.5	0.011‡	7.3	6.8	0.463‡	6.5	7.6	7.1	0.624‡	8.1	7.6	0.285§
Mean largest tumour diameter, mm	13.2	11.8	12.5	0.602‡	12.7	12.3	0.867‡	11.4	13.4	12.9	0.182‡	12.6	11.4	0.601§
Mean mitotic figures (per 8 mm^2)	6.1	5.2	6.1	0.807‡	4.5	6.5	0.621‡	6.7	4.3	5.8	0.244‡	4.6	3.0	0.443§

Table 4. Correlations between CCR7, CXCR4 and CXCL12 expression and clinical, histopathologic, and chromosomal data (continued)

	CCR7			CXCR4 (nuclear)			CXCR4 (cytoplasm)			CXCL12		
	−/+ *n* = 30	++/+++ *n* = 17	P-value	−/+ *n* = 17	++/+++ *n* = 30	P-value	−/+ *n* = 29	++/+++ *n* = 18	P-value	− *n* = 23	+ *n* = 8	P-value
Localisation tumour												
Choroid	25	12	0.460*	12	25	0.460*	22	15	0.719*	19	7	1.000*
Ciliary body	5	5		5	5		7	3		4	1	
Lymphocytic infiltration												
Absent	25	9	0.041*	16	18	0.017*	18	16	0.091*	19	7	1.000*
Present	5	8		1	12		11	2		4	1	
Extraocular extension												
Absent	30	15	0.126*	17	28	0.528*	27	18	0.517*	23	7	0.258*
Present	0	2		0	2		2	0		0	1	
Chromosome 1p loss												
Yes	7	5	0.438*	6	6	0.784†	7	5	0.647†	5	2	1.000*
No	15	5		9	11		10	10		11	4	
Chromosome 3 loss												
Yes	10	5	1.000*	9	6	0.062†	6	9	0.126†	6	4	0.341*
No	13	5		5	13		12	6		11	2	
Chromosome 6p gain												
Yes	10	1	0.055*	7	4	0.208†	5	6	0.611†	7	3	0.624*
No	11	9		8	12		11	9		10	2	
Chromosome 6q loss												
Yes	5	2	1.000*	6	1	0.037*	2	5	0.220*	5	0	0.290*
No	16	8		9	15		14	10		12	5	

	CCR7			CXCR4 (nuclear)			CXCR4 (cytoplasm)			CXCL12		
	−/+ *n* = 30	++/+++ *n* = 17	P-value	−/+ *n* = 17	++/+++ *n* = 30	P-value	−/+ *n* = 29	++/+++ *n* = 18	P-value	− *n* = 23	+ *n* = 8	P-value
Chromosome 6q gain												
Yes	3	0	0.533*	2	1	0.600*	1	2	0.600*	2	1	1.000*
No	18	10		13	15		15	13		15	4	
Chromosome 8p loss												
Yes	2	2	0.567*	1	3	0.620*	1	3	0.288*	1	0	1,000*
No	21	8		13	16		18	11		17	5	
Chromosome 8p gain												
Yes	8	1	0.217*	4	5	1.000*	6	3	0.698*	5	2	0.621*
No	15	9		10	14		13	11		13	3	
Chromosome 8q gain												
Yes	11	7	0.283*	8	10	0.797†	10	8	0.797†	7	2	1.000*
No	12	3		9	6		11	6				

† The χ^2-test was used to calculate correlations between categorical variables.
‡ The Kruskal-Wallis test was used to calculate correlations between more than 2 continuous variables.
* The Fisher's exact test was used to calculate correlation between two categorical variables.
§ The Mann-Whitney test was used to calculate correlation between two continuous variables.

metastatic events in patients with UM. Expression of CCL19 and CCL21 was not investigated in this study since no significant correlation between these chemokines and metastasis was described by Dobner et al.[16] Moreover, Rubie et al.[28] showed no significant differences in CCL19 protein and CCL19 gene expression between hepatocellular carcinoma, colon cancer tumour tissue and normal liver tissue.The authors hypothesise that immunohistochemistry might not be sufficiently sensitive to evaluate the differential expression of CCL19/CCL21 in formalin-fixated, paraffin-embedded tissues. A mixed or epithelioid cell type was significantly correlated with CCR7 expression (P = 0.006), which is a known histologic parameter associated with poor survival.[29] We also explored CXCR4 and its ligand CXCL12. CXCR4 is expressed in breast, prostate, pancreatic, renal, gastric carcinoma, skin melanoma, glioma, and leukaemias.[30] We found CXCR4 expression in uveal melanoma both cytoplasmic and/or nuclear localised, and similar expression patterns in associated liver biopsy specimens (n = 19). In contrast to studies in breast carcinoma[31] and melanoma cell lines,[19] we did not find loss of CXCR4 expression or shifts from cytoplasmic to nuclear expression in liver metastases of CXCR4 positive primary tumours. Other studies also observed no significant difference between expression levels of CCR7 and/or CXCR4 in paired primary and metastatic breast cancer[32] or in gastric cancer.[33] In our analyses, strong nuclear CXCR4 expression was not correlated with epithelioid cell type in contrast to previous work by Scala et al.[18] This may be related to sample size. CXCR4 expression showed no correlation with overall survival or disease-free survival. These last findings do not contradict earlier immunohistochemical studies of CXCR4 expression in primary UM samples.[16, 34, 35] However, we provide the first study to compare selected groups of histologically confirmed metastasis and a large group of long-term progression-free survival cases. Our data suggest the more important role for metastatic disease to be with CCR7 expression as compared to CXCR4 expression in uveal melanoma. On the other hand, immunohistochemistry may not be a sensitive enough tool for the detection of differential chemokine and chemokine receptor expression in tumours for the identification of prognostically significant differences. Other molecular techniques, such as mRNA nor even epigenetic regulators such as microRNA expression may provide more insight into the importance of chemokines in metastatic spread of uveal melanoma.

Ocular and cutaneous melanoma show different preferential sites for metastatic spread. Skin melanoma spread to lymph nodes, distant skin sites, lung, liver, central nervous system and bone. Uveal melanoma spreads haematogenous, with a high tendency to metastasise to the liver in 90 to 95% of the patients. The absence of lymphatics in the eye is one of the most important factors for the difference in metastatic spread. The predominance of liver metastasis cannot be solely explained by circulation because the lungs provide the first capillary bed that these cells would encounter. Therefore, it will most probably be a reflection of both preferential homing of cells to the liver combined with preferential growth and survival of

disseminated uveal melanoma cells in the hepatic microenvironment. The metastatic disease occurs almost exclusively in patients whose tumour show chromosome 3 loss. Tumours with concurrent loss of chromosome 1p and 3 are at higher risk of metastasising than the tumours with other aberrations.[36] Partial deletions or translocations have rarely been described on these chromosomes making it difficult to map putative tumour suppressor genes. However, recently a mutation in the *BAP1* gene, located on chromosome 3, has been identified in UM and this gene seems to play an important role in the tumour progression,[37] but most likely not in metastatic potential.[38] Apart from the CXCR4/CXCL12 and CCR7/CCL19-CCL21 pathways that we have investigated in this report, other pathways have been implicated in the preferential homing of tumour cells to the liver, such as hepatocyte growth factor (HGF) and it's corresponding receptor c-Met, and insulin-like growth factor 1 (IGF-1).[39] Although these pathways are not uniquely restricted to melanoma in combination, they may offer an explanation for the preferential metastatic spread of uveal melanoma to the liver, and more importantly may offer potential for therapeutic interventions.

In summary, our study shows that CCR7 expression in uveal melanoma plays a role in uveal melanoma metastasis, is independently associated with poor patient survival and can be used as a prognostic marker. CCR7 expression is not exclusively related to lymphogenic metastasis. Further research to validate our findings could be directed to the evaluation of CCR7 and CXCR4 mRNA expression by RT-PCR. Further research on CCR7 and associated pathways in uveal melanoma is necessary for therapeutic targeting.

ACKNOWLEDGEMENTS

The authors thank Sharmiela Ramlal for her excellent technical support in immunohistochemistry and Frank van de Panne for his assistance with preparation of the figures. This study was carried out by the Rotterdam Ocular Melanoma Study group, which is supported by a grant of the Combined Ophthalmic Research Rotterdam (CORR) and Stichting Nederlands Oogheelkundig Onderzoek (SNOO).

REFERENCES

1. van den Bosch T, Kilic E, Paridaens D, de Klein A. Genetics of uveal melanoma and cutaneous melanoma: two of a kind? *Dermatol Res Pract*. 2010;2010:360136.
2. Singh AD, Turell ME, Topham AK. Uveal melanoma: trends in incidence, treatment, and survival. *Ophthalmology*. 2011;118:1881-1885.
3. Shields CL, Furuta M, Thangappan A, et al. Metastasis of uveal melanoma millimeter-by-millimeter in 8033 consecutive eyes. *Arch Ophthalmol*. 2009;127:989-998.
4. Eskelin S, Pyrhonen S, Hahka-Kemppinen M, Tuomaala S, Kivela T. A prognostic model and staging for metastatic uveal melanoma. *Cancer*. 2003;97:465-475.
5. Gill HS, Char DH. Uveal melanoma prognostication: from lesion size and cell type to molecular class. *Canadian journal of ophthalmology Journal canadien d'ophtalmologie*. 2012;47:246-253.
6. Schaeuble K, Hauser MA, Rippl AV, et al. Ubiquitylation of the chemokine receptor CCR7 enables efficient receptor recycling and cell migration. *J Cell Sci*. 2012;125:4463-4474.
7. Li P, Zhao ZJ, Liu FY, et al. The chemokine receptor 7 regulates cell adhesion and migration via beta1 integrin in metastatic squamous cell carcinoma of the head and neck. *Oncol Rep*. 2010;24:989-995.
8. Wang J, Zhang X, Thomas SM, et al. Chemokine receptor 7 activates phosphoinositide-3 kinase-mediated invasive and prosurvival pathways in head and neck cancer cells independent of EGFR. *Oncogene*. 2005;24:5897-5904.
9. Li J, Sun R, Tao K, Wang G. The CCL21/CCR7 pathway plays a key role in human colon cancer metastasis through regulation of matrix metalloproteinase-9. *Dig Liver Dis*. 2011;43:40-47.
10. Li P, Liu F, Sun L, et al. Chemokine receptor 7 promotes cell migration and adhesion in metastatic squamous cell carcinoma of the head and neck by activating integrin alphavbeta3. *Int J Mol Med*. 2011;27:679-687.
11. Mashino K, Sadanaga N, Yamaguchi H, et al. Expression of chemokine receptor CCR7 is associated with lymph node metastasis of gastric carcinoma. *Cancer Res*. 2002;62:2937-2941.
12. Takanami I. Overexpression of CCR7 mRNA in nonsmall cell lung cancer: correlation with lymph node metastasis. *Int J Cancer*. 2003;105:186-189.
13. Calpe E, Codony C, Baptista MJ, et al. ZAP-70 enhances migration of malignant B lymphocytes toward CCL21 by inducing CCR7 expression via IgM-ERK1/2 activation. *Blood*. 2011;118:4401-4410.
14. Kuhnelt-Leddihn L, Muller H, Eisendle K, Zelger B, Weinlich G. Overexpression of the chemokine receptors CXCR4, CCR7, CCR9, and CCR10 in human primary cutaneous melanoma: a potential prognostic value for CCR7 and CCR10? *Arch Dermatol Res*. 2012;304:185-193.
15. Wiley HE, Gonzalez EB, Maki W, Wu MT, Hwang ST. Expression of CC chemokine receptor-7 and regional lymph node metastasis of B16 murine melanoma. *J Natl Cancer Inst*. 2001;93:1638-1643.
16. Dobner BC, Riechardt AI, Joussen AM, Englert S, Bechrakis NE. Expression of haematogenous and lymphogenous chemokine receptors and their ligands on uveal melanoma in association with liver metastasis. *Acta Ophthalmol*. 2012;90:e638-644.
17. Zhuo W, Jia L, Song N, et al. The CXCL12-CXCR4 chemokine pathway: a novel axis regulates lymphangiogenesis. *Clin Cancer Res*. 2012;18:5387-5398.
18. Scala S, Ierano C, Ottaiano A, et al. CXC chemokine receptor 4 is expressed in uveal malignant melanoma and correlates with the epithelioid-mixed cell type. *Cancer Immunol Immunother*. 2007;56:1589-1595.
19. Li H, Alizadeh H, Niederkorn JY. Differential expression of chemokine receptors on uveal melanoma cells and their metastases. *Invest Ophthalmol Vis Sci*. 2008;49:636-643.
20. Kryczek I, Wei S, Keller E, Liu R, Zou W. Stroma-derived factor (SDF-1/CXCL12) and human tumor pathogenesis. *Am J Physiol Cell Physiol*. 2007;292:C987-995.

21. Vaarwater J, van den Bosch T, Mensink HW, et al. Multiplex ligation-dependent probe amplification equals fluorescence in-situ hybridization for the identification of patients at risk for metastatic disease in uveal melanoma. *Melanoma Res*. 2012;22:30-37.

22. Natali PG, Hamby CV, Felding-Habermann B, et al. Clinical significance of alpha(v)beta3 integrin and intercellular adhesion molecule-1 expression in cutaneous malignant melanoma lesions. *Cancer Res*. 1997;57:1554-1560.

23. Baker JK, Elshaw SR, Mathewman GE, et al. Expression of integrins, degradative enzymes and their inhibitors in uveal melanoma: differences between in vitro and in vivo expression. *Melanoma Res*. 2001;11:265-273.

24. Elshaw SR, Sisley K, Cross N, et al. A comparison of ocular melanocyte and uveal melanoma cell invasion and the implication of alpha1beta1, alpha4beta1 and alpha6beta1 integrins. *Br J Ophthalmol*. 2001;85:732-738.

25. Seftor RE, Seftor EA, Hendrix MJ. Molecular role(s) for integrins in human melanoma invasion. *Cancer Metastasis Rev*. 1999;18:359-375.

26. Bonacchi A, Petrai I, Defranco RM, et al. The chemokine CCL21 modulates lymphocyte recruitment and fibrosis in chronic hepatitis C. *Gastroenterology*. 2003;125:1060-1076.

27. Rubie C, Oliveira V, Kempf K, et al. Involvement of chemokine receptor CCR6 in colorectal cancer metastasis. *Tumour Biol*. 2006;27:166-174.

28. Rubie C, Frick VO, Wagner M, et al. Chemokine expression in hepatocellular carcinoma versus colorectal liver metastases. *World J Gastroenterol*. 2006;12:6627-6633.

29. Scholes AG, Damato BE, Nunn J, et al. Monosomy 3 in uveal melanoma: correlation with clinical and histologic predictors of survival. *Invest Ophthalmol Vis Sci*. 2003;44:1008-1011.

30. Slettenaar VI, Wilson JL. The chemokine network: a target in cancer biology? *Adv Drug Deliv Rev*. 2006;58:962-974.

31. Ramos EA, Grochoski M, Braun-Prado K, et al. Epigenetic changes of CXCR4 and its ligand CXCL12 as prognostic factors for sporadic breast cancer. *PLoS One*. 2011;6:e29461.

32. Liu Y, Ji R, Li J, et al. Correlation effect of EGFR and CXCR4 and CCR7 chemokine receptors in predicting breast cancer metastasis and prognosis. *J Exp Clin Cancer Res*. 2010;29:16.

33. Ying J, Xu Q, Zhang G, Liu B, Zhu L. The expression of CXCL12 and CXCR4 in gastric cancer and their correlation to lymph node metastasis. *Med Oncol*. 2012;29:1716-1722.

34. Franco R, Botti G, Mascolo M, et al. "CXCR4-CXCL12 and VEGF correlate to uveal melanoma progression". *Front Biosci (Elite Ed)*. 2010;2:13-21.

35. Scala S, Ottaiano A, Ascierto PA, et al. Expression of CXCR4 predicts poor prognosis in patients with malignant melanoma. *Clin Cancer Res*. 2005;11:1835-1841.

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

37. Harbour JW, Onken MD, Roberson ED, et al. Frequent mutation of BAP1 in metastasising uveal melanoma. *Science*. 2010;330:1410-1413.

38. Farmer H, McCabe N, Lord CJ, et al. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature*. 2005;434:917-921.

39. Bakalian S, Marshall JC, Logan P, et al. Molecular pathways mediating liver metastasis in patients with uveal melanoma. *Clin Cancer Res*. 2008;14:951-956.

CHAPTER 4.2

The prognostic value of extraocular extension in relation to monosomy 3 and gain of chromosome 8q in uveal melanoma

Jackelien G.M. van Beek, Anna E. Koopmans, Jolanda Vaarwater, Johan J. de Rooi, Dion Paridaens, Nicole C. Naus, Annelies de Klein, Robert M. Verdijk and Emine Kiliç

Inves Ophthalmol Vis Sci. 2014;55:1284-1291

ABSTRACT

Purpose. To identify the prognostic value of extraocular extension in enucleated uveal melanoma patients and to correlate extraocular extension to chromosomal aberrations, metastasis-free survival and clinico-histopathological risk factors.

Methods. Retrospective study of patients with uveal melanoma treated with enucleation between 1987 and 2011. Melanoma-related metastasis and death were recorded. Statistical analysis (Log-rank test or Cox regression analysis) was performed to correlate metastasis-free survival with tumour characteristics, extraocular extension, episcleral diameter of the extraocular extension, cell type, extracellular matrix patterns, inflammation, loss of chromosome 3 and gain of chromosome 8q.

Results. In 43 (12%) of 357 patients, extraocular extension was observed. In this subset of patients, we noted a reduced survival of 70 months (105.5 months, $P = 0.010$) compared with patients without extraocular extension (175.8 months). Patients with gain of chromosomal region 8q in uveal melanoma with extraocular extension had an increased risk of metastatic disease ($P < 0.001$). In multivariate Cox proportional hazard analysis, largest basal tumour diameter ($P = 0.001$), extracellular matrix patterns ($P = 0.009$), episcleral diameter of the extraocular extension ($P = 0.016$), loss of chromosome 3 ($P < 0.001$) and gain of 8q ($P < 0.001$) were independent predictors for metastasis-free survival.

Conclusions. Larger episcleral diameter of the extraocular extension and additional gain of chromosome 8q in extraocular extension uveal melanoma correlates to a worse prognosis. Metastasis-free survival is significantly reduced in uveal melanoma with a large basal tumour diameter, extracellular matrix patterns, loss of chromosome 3, and gain of chromosome 8q.

INTRODUCTION

The age-adjusted incidence of uveal melanoma (UM) is 5.1 per million since 1973.[1] During the past decades, several risk factors have been identified and related to survival. Clinical factors that correlate with poor survival are a large tumour thickness and tumour basal diameter, ciliary body localisation, mushroom configuration and older age.[2, 3] The tumour size is of great importance, since each millimetre increase in tumour thickness seems to increase the risk of metastases by approximately 5%.[4] Histopathological risk factors associated with decreased survival are epithelioid cell type, high mitotic activity, presence of extracellular matrix patterns, and extraocular extension.[5-7] UM with epithelioid cells tend to have a more aggressive behaviour and are therefore related to a poor clinical outcome. From the known prognostic parameters, the genetic alterations are by far the most strongly associated with metastatic disease. Loss of chromosome 3 or monosomy 3 is observed in approximately 50% of the UM and is not only associated with clinical but also with histopathological prognostic factors and metastatic death.[8-11] A higher percentage of monosomy 3 leads to a poorer disease-free survival.[12] The same is true for gain of chromosome 8q and when these abnormalities occur simultaneously, the prognosis is even worse.[13] Van den Bosch et al.[12] showed that gradual increase in copy number of chromosome 8q shortened survival. Extraocular extension occurs in 2% to 15% of the UM.[3, 5, 14-16] Tumours with extraocular extension are classified in a different subcategory of the TNM classification and are associated with a worse prognosis.[3, 14] Moreover, the larger the extension diameter, the shorter the survival will be. The 5-year survival of UM patients with an extraocular extension of 5.1 mm or more is between 18% and 22%.[14, 16] Extraocular extension has been correlated with monosomy 3; however, no associations have been found between extraocular extension and chromosome 8q alterations.[5, 13] Therefore, the aim of this study was to identify monosomy 3 and gain of 8q as additional risk factors, besides clinical and histopathological factors, in UM with extraocular extension and correlate these with metastasis-free survival.

METHODS

Patients and clinical characteristics

A retrospective study was carried out by the Rotterdam Ocular Melanoma Study group, in patients with a choroidal or ciliary body UM who underwent primary or secondary enucleation from 1987 until 2011. We excluded patients with iris melanoma and cases in which no sufficient tumour material was available to describe the histopathological characteristics

of the tumour. The following data were recorded: gender, location of the tumour, date of enucleation, age at time of enucleation, development of metastases, and date and cause of death. If measurements of the tumour thickness and largest basal diameter from B-scan ultrasonography (US) were not available, we used the tumour's measurements before histological preparation. Tumour measurements obtained prior to fractionated stereotactic radiotherapy (fSRT) were used if patients had received primary fSRT. Data on extraocular extension was registered during US, surgery, or histopathologically. Patients with extraocular extension were selected based on their pathology report.

Informed consent was obtained prior to treatment and the study was performed according to guidelines of the Declaration of Helsinki. Until 1999 all patients were enucleated; hereafter, enucleation was performed only if the tumour was too large for fSRT (basal tumour diameter > 16 mm and tumour thickness > 12 mm) or if the patient requested enucleation.[17]

Metastasis-free survival was defined as the time in months from enucleation until the development of metastasis. We obtained survival data up to April 2013 by reviewing patients' charts and contacting their primary physician. Patients were screened for the presence of metastasis by testing liver enzymes in peripheral blood every 6 months for the first 5 years and thereafter annually. If these were elevated, an abdominal US or computed tomography scan was carried out.

Histopathology

Fresh tumour material was obtained within 1 h of enucleation and processed for further histopathological and cytogenetic analysis. Conventional histopathological examination with haematoxylin and eosin (H&E) staining of formalin-fixed and paraffin-embedded eyes was performed on all tumours and confirmed the origin of the tumour. The intraocular part of the tumours were evaluated for the presence of inflammation and necrosis. Inflammation was defined as any obvious clusters of lymphoid inflammatory cells in the tumour assed by H&E staining. Microfoci of necrosis were accepted as positive. H&E staining was used to differentiate between an epithelioid, mixed, or spindle-cell type according to the modified Callender classification. Extracellular matrix patterns were visualised in tumour specimens stained with periodic acid-Schiff (PAS) reagent. The mitotic rate was determined only in tumours with extraocular extension by counting the mitosis in 8 mm^2 equal to 50 high-power fields. Extraocular extension was confirmed by revision of all histopathological sections by an ophthalmic pathologist (RV), and was defined as tumour growth through the sclera and beyond the outer scleral surface. Subsequently, the largest diameter of the extension of the tumour on the sclera surface was measured. The surgical margin was examined for infiltrating UM cells extending from the extraocular extension. We determined the route of extraocular spread and involvement of optic nerve, ciliary body, or choroid.

Cytogenetic analysis

We determined the copy number status of chromosome 3 and 8 of the intraocular part of the primary tumour with fluorescence *in situ* hybridisation (FISH) analysis by using centromeric and locus-specific probes on directly fixated tumour cells for chromosome 3 and 8. A deletion was scored if more than 15% of the nuclei showed one signal for centromere 3 (probe Pα3.5) and/or 3q24 (probe YAC 827D3). Amplification was scored if more than 10% of the nuclei had three or more signals for 8q22 (probe RP-11-88J22). For tumour samples collected from December 2000, we used a probe located on 3q25 (RP11-64F6). FISH analysis was performed in most tumours. In some tumours, the chromosome status was solely based on comparative genomic hybridisation (CGH) (n = 8), karyotyping (n = 18), or single nucleotide polymorphism (SNP) array (n = 21). In 78 tumours, both FISH and SNP array were used to determine monosomy 3 or gain of 8q. CGH and FISH analysis were performed according to the protocol described by Naus et al.[18] For whole genome analysis, we used an SNP array (Illumina HumanCytoSNP-12 v2.1 BeadChip and Illumina 610Q BeadChip, Illumina, San Diego, CA, USA). Two hundred nanograms of fresh tumour DNA was used as input. The data were analysed with version 6 of the Nexus software (BioDiscovery, Inc., El Segundo, CA, USA). BioDiscovery's SNP-Rank Segmentation Algorithm, an extension of the Rank Segmentation algorithm (a statistically based algorithm similar to the Circular Binary Segmentation algorithm[19]), was used to make copy number as well as loss of heterozygosity (LOH) calls. SNP-Rank Segmentation takes into account both the log-R as well as the B-allele frequency value at each probe location to create a segment. The significance threshold for segmentation was set at 5.0×10^{-7}, also requiring a minimum of three probes per segment and a maximum probe spacing of 1000 kilobase pairs (Kbp) between adjacent probes before breaking a segment. The log ratio thresholds for single copy gain and single copy loss were set at 0.15 and -0.15, respectively. The log ratio thresholds for two or more copy gain and homozygous loss were set at 0.41 and -1.1, respectively. The homozygous frequency threshold was set to 0.95. The homozygous value threshold was set to 0.8. The heterozygous imbalance threshold was set to 0.4. The minimum LOH length was set at 100 Kbp. Polyploid tumours with a relative loss of chromosome 3 were also considered as monosomy 3 UM. This is also applicable for relative gain of chromosome 8q.

Statistical analysis

Tumours with an epithelioid and mixed cell type were classified as tumours containing epithelioid cells for further statistical analysis. The primary end point for metastasis-free survival was the development of metastatic disease. Cases in which the cause of death was unknown or not related to their UM, were treated as censored. The importance of prognostic factors

Table 1. Tumor characteristics in uveal melanoma patients with and without extraocular extension (EXE)

	Correlations			Univariate survival analysis	
Variable	**Patients without EXE (*n* = 314)**	**Patients with EXE (*n* = 43)**	**P-value**		**P-value**
Age at enucleation, *n* = 357, mean, y (range)	60.2 (21 – 90)	63.9 (29 – 86)	0.085[*]	HR = 1.019	**0.002**[†]
Gender, *n* = 357					
Male, *n* (%)	161 (51.3)	29 (67.4)	0.051[‡]	164.7 mo	0.617[§]
Female, *n* (%)	153 (48.7)	14 (32.6)		172.5 mo	
Tumor location, *n* = 357					
Choroid, *n* (%)	289 (92.0)	35 (81.4)	**0.043**[‡]	170.8 mo	0.249[§]
Ciliary body, *n* (%)	25 (8.0)	8 (18.6)		135.4 mo	
Tumor size					
Largest basal tumor diameter, *n* = 356, mean, mm (range)	12.3 (2.0 – 21.0)	4.2 (6.0 – 22.0)	**0.002**[*]	HR = 1.138	**<0.001**[†]
Tumor thickness, *n* = 355, mean, mm (range)	7.2 (1.0 – 24.0)	7.7 (1.5 – 22.0)	0.778[*]	HR = 1.060	**0.003**[†]
Epithelioid cells, *n* = 356					
Absent, *n* (%)	113 (36.1)	16 (37.2)	0.868[‡]	188.2 mo	**0.002**[§]
Present, *n* (%)	200 (63.9)	27 (62.8)		154.6 mo	
Extracellular matrix patterns, *n* = 302					
Absent, *n* (%)	148 (57.1)	19 (44.2)	0.136[‡]	167.6 mo	**<0.001**[§]
Present, *n* (%)	111 (42.9)	24 (55.8)		87.1 mo	
Inflammation, *n* = 255					
Absent, *n* (%)	50 (23.6)	33 (76.7)	**<0.001**[‖]	162.0 mo	0.686[§]
Present, *n* (%)	162 (76.4)	10 (23.3)		163.0 mo	
Extraocular extension, *n* = 43					
Largest episcleral diameter of the EXE, mean, mm (range)	0	2.9 (0.1 – 40.0)	**<0.001**[*]	HR = 1.120	**<0.001**[†]
Loss of chromosome 3, *n* = 286					
Absent, *n* (%)	96 (38.9)	14 (35.9)	0.860[‡]	151.6 mo	**<0.001**[§]
Present, *n* (%)	151 (61.1)	25 (64.1)		96.8 mo	
Gain of chromosome 8q, *n* = 279					
Absent, *n* (%)	80 (33.2)	11 (28.9)	0.711[‡]	169.8 mo	**<0.001**[§]
Present, *n* (%)	161 (66.8)	27 (71.1)		97.1 mo	

Abbreviations: EXE = extraocular extension; y = years; HR = hazard ratio; mo = months; [*] = Mann-Whitney test; [†] = Cox regression analysis; [‡] = Fisher's exact test; [§] = Log-rank test; [‖] = χ^2-test.
The P-values that were significant (defined as a P-value less than or equal to 0.05) are shown in bold.

on metastasis-free survival was assessed using the Log-rank test (for categorical variables) or Cox regression analysis (for continuous variables). The significance of associations between clinico-histopathological, chromosomal variables and extraocular extension were calculated with the Pearson's χ^2-test or Fisher's exact test (for categorical variables) and the Mann-Whitney test (for continuous variables). Multivariate analysis using the forward stepwise method was conducted for the variables that were significant in univariate analysis. A two-tailed P-value less than or equal to 0.05 was considered significant. Statistical analyses were performed with SPSS version 20.0 software (SPSS, SPSS Inc., Chicago, IL, USA).

RESULTS

Patients

In total, 357 patients were included in this study. The mean age was 61 years at time of enucleation (range 21 – 90). The mean largest basal tumour diameter was 12.5 mm (range 2.0 – 22.0) and the mean tumour thickness was 7.3 mm (range 1.0 – 24.0). Overall, 20 patients received fSRT as initial treatment, of whom two patients had extraocular extension. Two patients received brachytherapy and one patient received proton beam radiation before enucleation. These patients did not have extraocular extension. Genetic testing of the UM patient who received proton beam radiation was conducted 20 months after the radiation and revealed a normal chromosome 3 and 8 status.

The tumour characteristics for the patients with extraocular extension versus patients without extraocular extension are shown in **Table 1**. Extraocular extension was identified in 43 (12%) out of 357 patients (**Figure 1A**). The mean age of the patients with extraocular extension was 64 years (range 29 – 86). The mean largest basal tumour diameter and mean tumour thickness for this group of patients with extraocular extension were 14.2 mm (range 6.0 – 22.0) and 7.7 mm (range 1.5 – 22.0), respectively. Tumour localisation (P = 0.043) and largest basal tumour diameter (P = 0.002) correlated with extraocular extension (**Table 1**).

Histopathology

Several histopathological features were determined for the extraocular extension (**Figure 1B**). For instance, the (largest) episcleral diameter of the extraocular extension ranged from 0.1 to 40.0 mm with a mean of 2.9 mm. Necrosis was found in 23 of 43 histopathological slides. The mean mitotic rate was 9.95/8 mm^2 (range 0.00 – 29.00). Absence of inflammation (P < 0.001) was associated with extraocular spread (**Table 1**). Eleven of the choroidal tumours invaded the ciliary body and all the ciliary body tumours invaded the choroid. The tumours

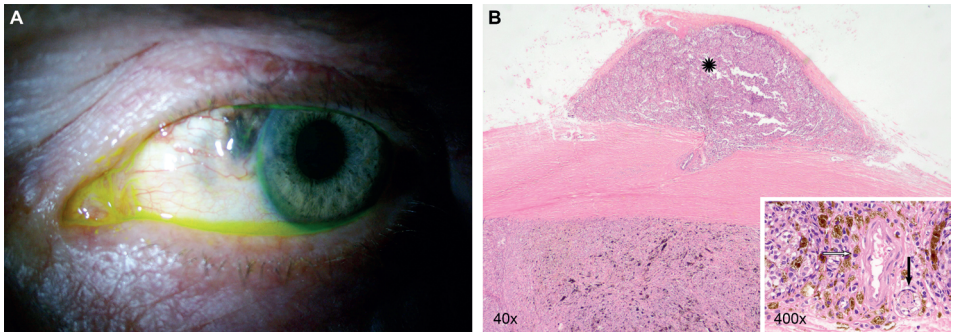


Figure 1. Slit lamp photograph of a ciliary body melanoma with extraocular extension (**A**). Extraocular extension of choroidal melanoma (indicated with asterisk) in hematoxylin and eosin (H&E) staining (40x) and an enlargement is shown with perineural (black arrow) and perivascular invasion (white arrow) of extraocular extension in H&E staining (400x) (**B**).

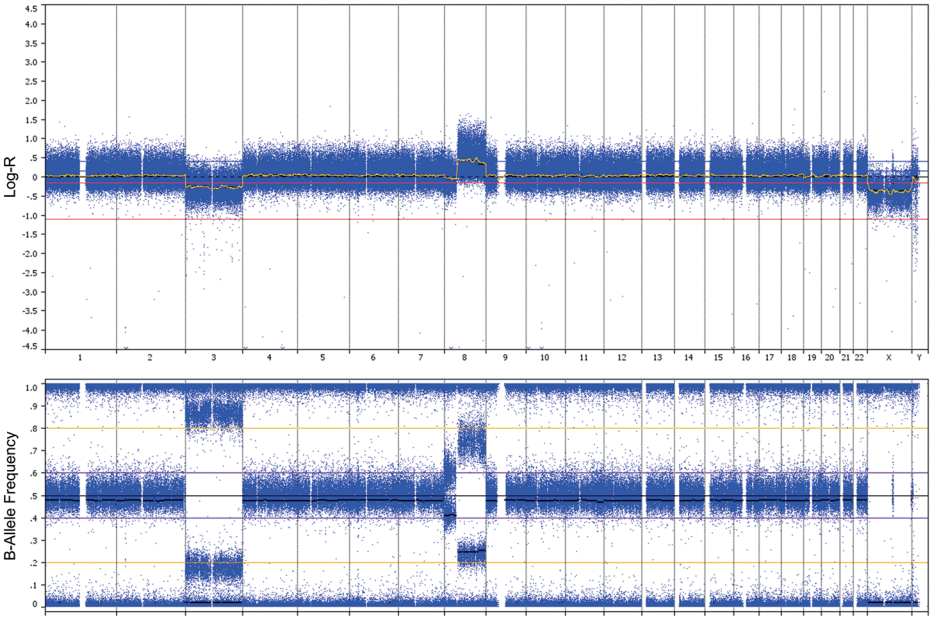


Figure 2. Single nucleotide polymorphism (SNP) array results of a male patient with uveal melanoma, showing loss of chromosome 3 and gain of chromsome 8q (Log-R upper panel). The lower panel represents the B-allele frequency showing loss of heterozygosity and allelic imbalance of chromosome 3 and 8, respectively.

did not show a significant difference in size of the mean largest basal tumour diameter and mean tumour thickness within the extraocular extension group (P = 0.615 and P = 0.517, respectively). Combinations of several routes of invasion of the extraocular extension were observed (**Figure 1B**). Seven UM invaded the perilimbal plexus, the anterior part of the eye. Five tumours invaded the equator of the eye through vortex veins and two UM invaded through the anterior ciliary arteries. Most of the tumours with extraocular extension were located posteriorly; 11 UM invaded through the long posterior ciliary nerve, 14 through the short posterior ciliary nerve, and 1 through both ciliary nerves. Besides these routes of invasion, 41 tumours also invaded perivascularly and 29 tumours invaded perineurally. In total, three UM with extraocular spread invaded the lamina cribrosa through three different routes: transscleral, and short and long ciliary nerves. However, the optic nerve resection margin was free of malignant cells.

In 20 patients, infiltrating UM cells extending from the extraocular extension were observed at the surgical margin. Of these patients, seven received post-operative irradiation and one patient with a 40-mm extraocular extension underwent an orbital exenteration. Thus far, orbital recurrence was noticed in one patient with a free surgical margin. Seven of the 20 patients with irradical enucleation were still alive at the last follow-up date. There was no significant difference in mean survival between patients with (72.7 months, 95% confidence interval (CI) 46.9 – 98.5, Log-rank test, P = 0.660) and without (120.6 months, 95% CI 67.7 – 173.6) a free surgical margin.

Cytogenetic analysis

Loss of chromosome 3 was present in 61.5% (176/286) of all UM and in 64.1% (25/39) of the tumours with extraocular extension (**Table 1**). This was not statistically different from cases without extraocular extension. Gain of chromosome 8q was present in 67.4% (188/279) of all UM and in 71.1% (27/38) of the UM with extraocular extension. Forty-seven patients had gain of 8q with disomy of chromosome 3, and 141 patients had gain of 8q combined with monosomy 3. An example of a case with loss of chromosome 3 and gain of chromosome 8q on SNP array is depicted in **Figure 2**. Twenty-two extraocular extension patients showed gain of chromosome 8q combined with monosomy 3. Due to lack of material, chromosome 3 and 8 status could not be examined in all extraocular extension patients.

Survival analysis

The mean metastasis-free survival of the overall group was 76.6 months (range 0 – 308.5). Irrespective of extraocular extension, 145 patients (40.6%) developed metastasis with a mean survival of 41.8 months (range 0 – 207.7) and 158 patients (44.3%) were alive at the end of

Table 2. Follow-up of patients stratified for the presence of extraocular extension (EXE)

	Patients without EXE (*n* = 314), *n* (%)	Patients with EXE (*n* = 43), *n* (%)
Alive	144 (45.9)	14 (32.6)
Melanoma-related death and metastases	121 (38.5)	24 (55.8)
Death due to other cause	38 (12.1)	5 (11.6)
Lost to follow-up	11 (3.5)	0 (0.0)

Abbreviations: EXE = extraocular extension.

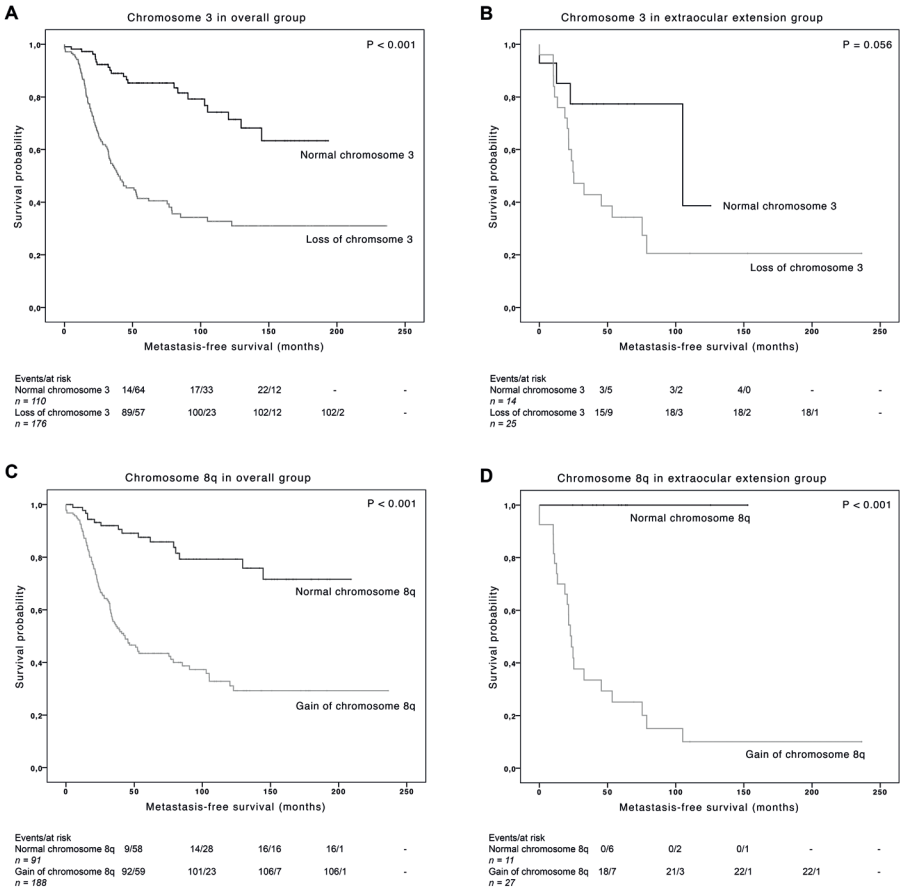


Figure 3. Survival probability plots for chromosome 3 and 8q in the overall group (**A** and **C**) and in the extraocular extension group (**B** and **D**).

Table 3. Cox multivariate regression analysis correlating with metastatic disease

	P-value	HR	95.0% CI
Largest basal tumour diameter	0.001	1.094	1.037 – 1.159
Extracellular matrix patterns	0.009	1.674	1.137 – 2.464
Largest episcleral diameter of the EXE	0.016	1.078	1.014 – 1.147
Loss of chromosome 3	< 0.001	2.634	1.570 – 4.420
Gain of chromosome 8q	< 0.001	2.874	1.651 – 5.005

Abbreviations: EXE = extraocular extension; HR = hazard ratio; CI = confidence interval.

the follow-up with a mean survival of 104.3 months (range 0.8 – 308.5). Forty-three patients (12.0%) died due to other disease causes, such as a ruptured aneurysm or a myocardial infarction. The mean survival of this group was 93.4 months (range 0.2 – 270.0). Eleven patients were lost to follow-up, of which five patients moved abroad and the other six patients moved to another city and did not provide their general practitioner with information or withdrew from ophthalmologic follow-up.

The follow-up of patients with extraocular extension is shown in **Table 2**. The survival was significantly reduced in patients with extraocular extension versus without extraocular extension (105.5 months vs. 175.8 months, respectively, Log-rank test, P = 0.010).

Univariate analyses of prognostic factors showed a significantly shorter metastasis-free survival in tumours with a larger episcleral diameter of the extraocular extension (hazard ratio (HR) 1.120, P < 0.001), epithelioid cells (154.6 vs. 188.2 months, P = 0.002), extracellular matrix patterns (87.1 vs. 167.6 months, P < 0.001), monosomy 3 (96.8 vs. 151.6 months, P < 0.001) and gain of 8q (97.1 vs. 169.8 months, P < 0.001) (**Table 1**). In addition, we conducted univariate survival analysis for extraocular extension UM patients only and the episcleral diameter of the extraocular extension remained significant (HR 1.079, P = 0.040).

The metastasis-free survival was significantly longer in the overall group without chromosomal aberrations compared with patients with these aberrations (**Figures 3A** and **3C**). UM with disomy 3 and normal 8q versus gain of 8q (171.6 vs. 123.2 months, P = 0.004) showed a prolonged survival compared with UM with monosomy 3 and normal 8q versus gain of 8q (143.5 vs. 78.1 months, P < 0.001).

In the subgroup of extraocular extension, patients with and without monosomy 3 had a survival of 3.5 months and 92.0 months, respectively (P = 0.056) (**Figure 3B**). Patients with extraocular extension and gain of 8q had a reduced survival compared with patients with normal chromosome 8q (P < 0.001) (**Figure 3D**). We validated the interaction between extraocular extension and gain of 8q and its effect on the metastasis-free survival in a separate multivariate model.

In multivariate analysis, the largest basal tumour diameter, extracellular matrix pat-

terns, episcleral diameter of the extraocular extension, chromosome 3 loss (HR 2.634, P < 0.001), and chromosome 8q gain (HR 2.874, P < 0.001) were independent prognostic factors on metastasis-free survival (**Table 3**). Prognostic factors, such as presence of epithelioid cells, extraocular spread in general, tumour thickness, and age, were rejected after multivariate analysis.

DISCUSSION

UM patients with extraocular extension are a clinically challenging group of patients, as there are only a few studies that have a large cohort of patients for analysis and often a limited duration of follow-up. In our study, we reviewed 357 ciliary body and choroidal UM of which 43 (12%) had extraocular extension with a mean follow-up of 6.4 years (range 0 – 25.7 years). As observed in previous studies, we also found that UM patients with loss of chromosome 3 and/or gain of chromosome 8q in their melanoma had a significantly reduced metastasis-free survival (P < 0.001). In addition, we observed that gain of chromosome 8q was associated with a worse prognosis in patients with extraocular extension. Besides that, patients with extraocular extension developed metastases or died due to metastases almost 6 years earlier, on average, compared with patients without extraocular extension (Log-rank test, P = 0.010).

Monosomy 3 and gain of chromosome 8q (or concurrent presence of abnormalities on chromosome 3 and 8) and extraocular extension have already separately been identified as risk factors in several other studies.[3, 11, 13, 14, 20] Nevertheless, gain of chromosome 8q in combination with extraocular extension has not been related to survival. A near significant trend (P = 0.056) was observed between monosomy 3 and extraocular extension regarding survival. With a larger patient group, a relation to patient survival could be noted. Histopathological factors have been described and related to survival in patients with extraocular extension. Coupland and associates[5] found that epithelioid cell type and high mitotic rate were related to extraocular spread and poor prognosis. In our series, UM with an epithelioid cell type were also related to a reduced survival, though this did not correlate with extraocular extension (P = 0.868). Because the percentages of epithelioid cells in the group of extraocular extension and without extension were similar, and although a difference in survival was measured, epithelioid cell type appeared not to be the most important prognostic factor in our population. In our multivariate analysis, the presence of epithelioid cells, extraocular spread in general, tumour thickness, and age were rejected. These prognostic factors were significant predictors of survival in the univariate analysis. In the multivariate analysis, age nearly reached statistical significance as an independent prognostic marker (P = 0.051). In

previous studies, older age and presence of epithelioid cells have proven to have a significant effect on survival.[3, 5]

In concordance with previous studies, we also found that clinical factors, such as a larger basal tumour diameter and the presence of extracellular matrix patterns, were associated with a decreased survival, whereas the size of the extraocular extension did not correlate significantly with metastatic death in all studies.[3, 5, 14] With an increasing size of extraocular extension diameter, the 5-year survival seems to decline: 81% in patients without extension, 49% in patients with a 0.1- to 5-mm extension diameter, and 18% in patients with 5.1-mm or more extension diameter.[14] In our analysis, we found that an increase of 1 mm in episcleral diameter led to a nearly 1.1 times increase in risk of developing metastatic disease (HR 1.078). We had only three patients in the subcategory of greater than or equal to 5.1 mm, and for this reason we could not perform statistical analyses for this group. In these three patients, one patient had metastasis at time of diagnosis (diameter extension of 40 mm) and another patient died due to a non-melanoma-related cause without metastasis (diameter extension of 9 mm) with a follow-up of 110.4 months at an age of 86 years. The third patient with a 6-mm extension was still alive at 41.9 months and had other favourable prognostic factors, such as the absence of genetic aberrations, absence of extracellular matrix patterns, absence of mitotic figures, and a free surgical margin. Interestingly, all three UM contained epithelioid cells.

Orbital recurrence has been reported in 3% to 23% of the patients undergoing enucleation for UM with extraocular extension.[16, 21-23] In our study, only one patient, with an initial tumour free surgical margin, had an orbital recurrence after 7 months and was exenterated. Nevertheless, orbital recurrence is described even 20 and 42 years after enucleation.[24] In 20 of 43 UM patients with extraocular spread, melanoma cells extending from the extraocular extension were found at the surgical margin. Of the irradiated patients, the mean survival was 71.2 months (range 10.4 – 257.1), and was almost similar to patients without additional treatment, 79.2 months (range 0 – 254.2). Nevertheless, incomplete surgical removal of the tumour, especially if the extraocular part of the tumour is nonencapsulated, remains one of the most important risk factors for orbital recurrences.[23] In our group of patients with incomplete resection, we found no cases with orbital recurrence. Nowadays most patients with extraocular extension will be treated with additional therapy or surgery.

In this study, we associated extraocular extension with chromosomal abnormalities of chromosome 3 and 8 in UM. Compared with other studies, our patient group has a long follow-up with a mean metastasis-free survival of 6.4 years, and only a few patients were lost to follow-up. Extraocular extension was histologically proven and reviewed by an ocular pathologist in a relatively large group of UM. Because this is a retrospective study, some data were missing. For example, we could not detect histopathological or chromosomal aberra-

tions in all patients due to necrosis or lack of material. Because we studied only enucleated eyes and not patients who have had eye-conserving treatments, our group contained relatively large UM. This selection bias could influence survival, because in general larger tumours have a worse prognosis. Still, in our multivariate analysis, other parameters remained significantly associated with a decreased survival. Chromosome 3 and 8q status was determined in almost all patients with FISH, and in some cases with additional SNP array analysis. Intratumour heterogeneity has been described in a small number of UM in our research group previously, although no structural difference in monosomy 3 distribution occurred between the base and the apex of the tumour.[25] On the other hand, genetic heterogeneity of chromosome 3 and 8 has been reported between the intraocular and extraocular part of the UM, and for monosomy 3 between the apex and base of the tumour.[26, 27] This variation of monosomy 3 in intra- and extraocular parts of UM was demonstrated by Lake et al.[26] with multiplex ligation-dependent amplification (MLPA) in only ten patients. Despite a certain heterogeneity, tumours can be classified correctly for monosomy 3 or gain of chromosomal region 8q, as is the case in our study, as we used either FISH and/or confirmed these results with SNP array in a large group of our patients. Moreover, from previous studies we know that the percentage of chromosomal aberrations does not influence the development of metastases, but can influence the time to development of metastatic disease.[12] In our series, we found that metastasis-free survival is significantly reduced in UM with a large basal tumour diameter, extracellular matrix patterns, loss of chromosome 3, and gain of chromosome 8q. Loss of chromosome 3 itself is not related with extraocular extension, but a gain of chromosomal region 8q in tumours with extraocular extension increases the risk of metastatic disease.

ACKNOWLEDGEMENTS

Supported by the Stichting Nederlands Oogheelkundig Onderzoek (SNOO). The authors alone are responsible for the content and writing of the paper.

REFERENCES

1. Singh AD, Turell ME, Topham AK. Uveal melanoma: trends in incidence, treatment, and survival. *Ophthalmology*. 2011;118:1881-1885.
2. Singh AD, Shields CL, Shields JA. Prognostic factors in uveal melanoma. *Melanoma Res*. 2001;11:255-263.
3. Shields CL, Kaliki S, Furuta M, et al. American Joint Committee on Cancer Classification of Posterior Uveal Melanoma (Tumor Size Category) Predicts Prognosis in 7731 Patients. *Ophthalmology*. 2013;120:2066-2071.
4. Shields CL, Furuta M, Thangappan A, et al. Metastasis of uveal melanoma millimeter-by-millimeter in 8033 consecutive eyes. *Arch Ophthalmol*. 2009;127:989-998.
5. Coupland SE, Campbell I, Damato B. Routes of extraocular extension of uveal melanoma: risk factors and influence on survival probability. *Ophthalmology*. 2008;115:1778-1785.
6. Folberg R, Rummelt V, Parys-Van Ginderdeuren R, et al. The prognostic value of tumor blood vessel morphology in primary uveal melanoma. *Ophthalmology*. 1993;100:1389-1398.
7. McLean IW, Keefe KS, Burnier MN. Uveal melanoma. Comparison of the prognostic value of fibrovascular loops, mean of the ten largest nucleoli, cell type, and tumor size. *Ophthalmology*. 1997;104:777-780.
8. Kilic E, van Gils W, Lodder E, et al. Clinical and cytogenetic analyses in uveal melanoma. *Invest Ophthalmol Vis Sci*. 2006;47:3703-3707.
9. Horsman DE, Sroka H, Rootman J, White VA. Monosomy 3 and isochromosome 8q in a uveal melanoma. *Cancer Genet Cytogenet*. 1990;45:249-253.
10. Prescher G, Bornfeld N, Becher R. Nonrandom chromosomal abnormalities in primary uveal melanoma. *J Natl Cancer Inst*. 1990;82:1765-1769.
11. Sisley K, Rennie IG, Parsons MA, et al. Abnormalities of chromosomes 3 and 8 in posterior uveal melanoma correlate with prognosis. *Genes Chromosomes Cancer*. 1997;19:22-28.
12. van den Bosch T, van Beek JG, Vaarwater J, et al. Higher percentage of FISH-determined monosomy 3 and 8q amplification in uveal melanoma cells relate to poor patient prognosis. *Invest Ophthalmol Vis Sci. 2012;53:2668-2674.*
13. White VA, Chambers JD, Courtright PD, Chang WY, Horsman DE. Correlation of cytogenetic abnormalities with the outcome of patients with uveal melanoma. *Cancer*. 1998;83:354-359.
14. Kujala E, Damato B, Coupland SE, et al. Staging of ciliary body and choroidal melanomas based on anatomic extent. *J Clin Oncol*. 2013;31:2825-2831.
15. Affeldt JC, Minckler DS, Azen SP, Yeh L. Prognosis in uveal melanoma with extrascleral extension. *Arch Ophthalmol*. 1980;98:1975-1979.
16. Pach JM, Robertson DM, Taney BS, et al. Prognostic factors in choroidal and ciliary body melanomas with extrascleral extension. *Am J Ophthalmol*. 1986;101:325-331.
17. Muller K, Nowak PJ, de Pan C, et al. Effectiveness of fractionated stereotactic radiotherapy for uveal melanoma. *International journal of radiation oncology, biology, physics*. 2005;63:116-122.
18. Naus NC, van Drunen E, de Klein A, et al. Characterization of complex chromosomal abnormalities in uveal melanoma by fluorescence in situ hybridization, spectral karyotyping, and comparative genomic hybridization. *Genes Chromosomes Cancer*. 2001;30:267-273.
19. Olshen AB, Venkatraman ES, Lucito R, Wigler M. Circular binary segmentation for the analysis of array-based DNA copy number data. *Biostatistics*. 2004;5:557-572.
20. Damato B, Duke C, Coupland SE, et al. Cytogenetics of uveal melanoma: a 7-year clinical experience. *Ophthalmology*. 2007;114:1925-1931.

21. Shammas HF, Blodi FC. Orbital extension of Choroidal and ciliary body melanomas. *Arch Ophthalmol*. 1977;95:2002-2005.

22. Hykin PG, McCartney AC, Plowman PN, Hungerford JL. Postenucleation orbital radiotherapy for the treatment of malignant melanoma of the choroid with extrascleral extension. *Br J Ophthalmol*. 1990;74:36-39.

23. Starr HJ, Zimmerman LE. Extrascleral extension and orbital recurrence of malignant melanomas of the choroid and ciliary body. *Int Ophthalmol Clin*. 1962;2:369-385.

24. Shields JA, Shields CL. Massive orbital extension of posterior uveal melanomas. *Ophthal Plast Reconstr Surg*. 1991;7:238-251.

25. Mensink HW, Vaarwater J, Kilic E, et al. Chromosome 3 intratumor heterogeneity in uveal melanoma. *Invest Ophthalmol Vis Sci*. 2009;50:500-504.

26. Lake SL, Damato BE, Dopierala J, et al. Multiplex ligation-dependent probe amplification analysis of uveal melanoma with extraocular extension demonstrates heterogeneity of gross chromosomal abnormalities. *Invest Ophthalmol Vis Sci*. 2011;52:5559-5564.

27. Schoenfield L, Pettay J, Tubbs RR, Singh AD. Variation of monosomy 3 status within uveal melanoma. *Arch Pathol Lab Med*. 2009;133:1219-1222.

CHAPTER 4.3

Histopathologic, immunohistochemical, and cytogenetic analysis of primary clear cell melanoma of the uvea

Anna E. Koopmans*, Robert M. Verdijk*, Emine Kiliç,
Dion Paridaens and Annelies de Klein

JAMA Ophthalmol. 2013 Jun; 131(6):814-816

* These authors contributed equally to this work

The histological findings of malignant melanoma may be highly variable, and the tumour can mimic many other neoplasms.[1] We describe a case of clear cell uveal melanoma. Cytoplasmic clear cell change of uveal melanoma cells can be observed as balloon cells,[1] clear cells,[2] and signet ring cells.[1] The histologic differential diagnosis has to be made with metastatic clear cell carcinomas, seminoma, and clear cell sarcoma as well as xanthomatous cells.

REPORT OF A CASE

A 38-year-old male visited the outpatient department of Ophthalmology with signs of blurred vision and metamorphopsia due to a foreign body in the right cornea. Best-corrected visual acuity was 20/125 OD and 20/20 OS. On dilated funduscopic and ultrasonographic (US) examination of the left eye, a dome-shaped pigmented subretinal mass was seen with a thickness of 5 to 6 mm, a diameter of 15.5 mm, and medium to low internal reflectivity (**Figure 1A**). No atypical cutaneous pigmented lesions were observed. Systemic radiologic evaluation revealed no metastatic lesions. The patient opted for enucleation. After a follow-up of 16 months, there were no signs of metastases.

Sections of the eye confirmed a dome-shaped tumour composed of both spindle-type and epithelioid-type cells with clear cytoplasm in more than 75% of the tumour cells (**Figure 1B**). The cytoplasm stained positive with periodic acid–Schiff stain sensitive to diastase treatment compatible with glycogen (**Figure 1C**). Mitotic figures were present at 5 per 8 mm^2 (equivalent to 50 high-power fields). A closed-loop extracellular matrix pattern was not present. The tumour showed extrascleral extension with a diameter of 0.5 mm and a free orbital resection margin. Tumour cells stained positive for Melan-A, HMB-45 and tyrosinase, confirming melanocytic lineage (**Figure 1D**).

Fluorescence *in situ* hybridisation (FISH) analysis of a tumour sample revealed no chromosomal rearrangements at 22q12 (Vysis). Single-nucleotide polymorphism (SNP) array analysis (HumanCytoSNP-12 v2 BeadChip; Illumina) showed gain of chromosome 6p, loss of chromosome 6q, gain of chromosome 8q and loss of the long arm of chromosome 16. Sequence analysis of *BAP1* revealed a new missense mutation located in exon 5 (resulting in p.Met115Val). Using the *in silico* tool PolyPhen-2, this transition was predicted to be benign. A second variant was located in an intron. Immunohistochemistry for BAP1 showed normal protein expression.

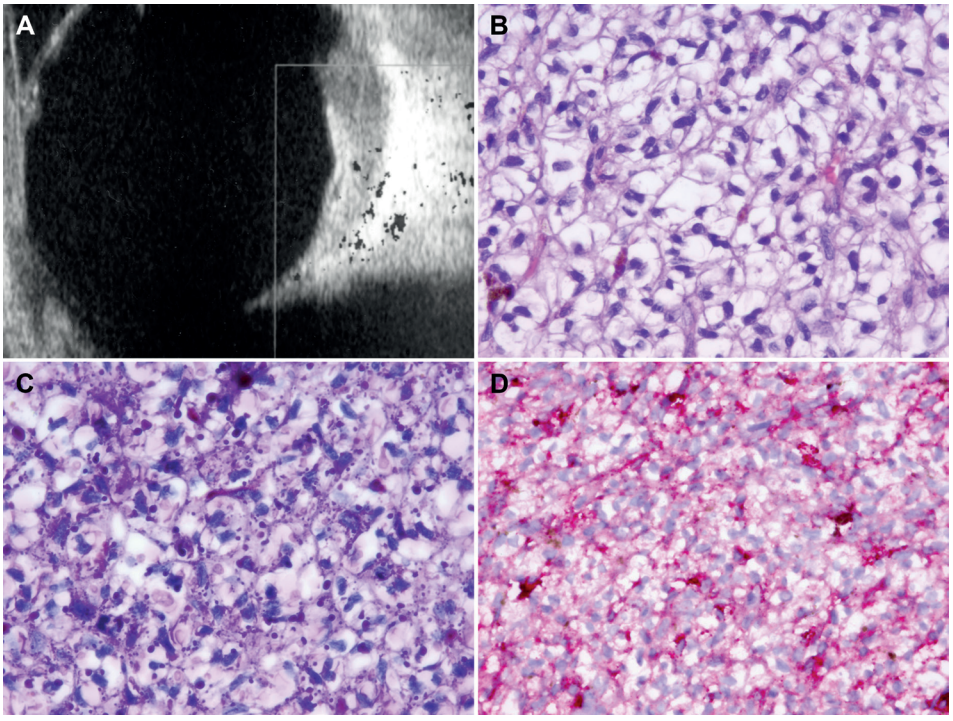


Figure 1. Ultrasonographic, histologic, and immunohistochemical appearance of the tumour. B-scan ultrasonography of the tumour shows a dome-shaped subretinal mass in the posterior pole (**A**). The tumour was mainly composed of spindle and irregular polygonal cells with distinct borders and a clear cytoplasm that sometimes contained brown pigment. The cells were interspersed in a delicate capillary network. Nuclei were enlarged with coarse open chromatin and prominent irregular nucleoli (haematoxylin-eosin, original magnification ×630) (**B**). The cytoplasm stained positive for periodic acid-Schiff sensitive to diastase treatment (original magnification ×630) (**C**). The cells stained positive for the melanocytic marker HMB-45 (original magnification ×400) (**D**).

COMMENT

Primary uveal melanoma is classified as spindle cell, mixed cell, or epithelioid cell type. The epithelioid cell type is associated with a significantly worse prognosis. Unusual cytomorphologic variants of malignant melanoma have been described as metaplastic, balloon cell, clear cell, signet ring cell, myxoid, small cell, oncocytic,[3] and rhabdoid melanoma.[1] Although the prognostic significance of these cytomorphologic variants is unknown, they should be recognized to avoid misdiagnosis with metastatic neoplasms. The clear cell change in melanoma cells can be due to accumulation of intracytoplasmic vimentin filaments as described in signet ring melanoma, to accumulation of fat as has been described in some cases of balloon cell melanoma,[4] or to accumulation of glycogen[5] as in our case. The histologic differential diagnosis has to be made with metastatic renal cell carcinoma, other metastatic clear cell

carcinomas, metastatic seminoma and metastatic clear cell sarcoma as well as benign inflammatory conditions with xanthomatous cells. For most of these differential diagnoses, immunohistochemical stains can be used to aid in diagnosis. In this case, we additionally were able to determine beyond doubt that clear cell uveal melanoma is distinct from clear cell sarcoma because the cytogenetic results showed typical changes of uveal melanoma and did not show the typical t(12;22)(q12q13) translocation that is associated with clear cell sarcoma and not with melanoma.[6] To our knowledge, this is the first report of clear cell uveal melanoma complete with cytogenetic investigations.

REFERENCES

1. Magro CM, Crowson AN, Mihm MC. Unusual variants of malignant melanoma. *Mod Pathol*. 2006;19 Suppl 2:S41-70.
2. Grossniklaus HE, Albert DM, Green WR, Conway BP, Hovland KR. Clear cell differentiation in choroidal melanoma. COMS report no. 8. Collaborative Ocular Melanoma Study Group. *Arch Ophthalmol*. 1997;115:894-898.
3. Verdijk RM, van den Bosch T, Naus NC, et al. Histopathologic, immunohistochemical, ultrastructural, and cytogenetic analysis of oncocytic uveal melanoma. *Arch Ophthalmol*. 2011;129:1501-1502.
4. Khalil MK. Balloon cell malignant melanoma of the choroid: ultrastructural studies. *Br J Ophthalmol*. 1983;67:579-584.
5. Nowak MA, Fatteh SM, Campbell TE. Glycogen-rich malignant melanomas and glycogen-rich balloon cell malignant melanomas: frequency and pattern of PAS positivity in primary and metastatic melanomas. *Arch Pathol Lab Med*. 1998;122:353-360.
6. Patel RM, Downs-Kelly E, Weiss SW, et al. Dual-color, break-apart fluorescence in situ hybridization for EWS gene rearrangement distinguishes clear cell sarcoma of soft tissue from malignant melanoma. *Mod Pathol*. 2005;18:1585-1590.

CHAPTER 5

Summary and general discussion

CHAPTER 5

Summary and general discussion

SUMMARY AND GENERAL DISCUSSION

In the last decade, genetic research in uveal melanoma (UM) has shifted from primarily cytogenetic workups to extensive molecular genetic analyses. Despite progress that has been made in understanding the genetic framework of UM, this disease still carries high mortality and patient survival rates have remained unchanged. Advances have been made in the primary treatment of UM with more eye-sparing treatment modalities; however, for liver metastases no standardised therapies are available. Research efforts are directed at the identification of prognostic parameters to select those patients who develop metastasis. The past years we have learned increasingly more about the genes involved in UM tumourigenesis. Only a few years ago prognostication of UM patients was primarily done only by examining the histopathological or chromosome status of the tumour. Nowadays, we can predict the patient prognosis more accurate based on chromosome and gene mutation status. The work presented in this thesis aimed at further enlightening the genetic background of UM and thereby providing possible new targets for therapy. We studied the prognostic value of several new genes in UM. In the previous chapters, the merits and limitations of each study have been described in detail. In the current chapter, a summary is presented and the main findings of this thesis are recapitulated and placed in a broader perspective. Special emphasis will be given on experimental considerations, the implications of our results for clinical practice, and finally, future prospects will be discussed.

Which genes are implicated in uveal melanoma carcinogenesis?

During the past decade, much effort has been made to identify genes involved in UM and recent advances in Next Generation Sequencing (NGS) have dramatically changed the process of gene identification. The human genome consists of approximately 21.000 genes. With exome or whole genome sequencing, scientists can detect both new and previously described sequence variants at gene level, and obtain information on depth coverage, heterogeneity, and accuracy of sequencing is provided.[1, 2] Although this technique has been available since 2004, only the past few years NGS has been widely applied in many hereditary and non-hereditary diseases as well as cancers. Target capture of custom designed regions and exome sequencing has allowed identification of causal variants in several Mendelian disorders,[3] variants associated with complex diseases,[4] and recurrently mutated cancer genes.[5] Interesting target genes in UM that harbour mutations, deletions and insertions have been discovered with NGS.[6, 7]

In the second chapter of this thesis, we performed targeted mutation analyses of several im-

plicating genes in UM to gain more insight in the tumourigenesis. Mutations in *GNAQ* and *GNA11* occur in roughly 5% of all tumours sequenced to date.[8] Several years ago, *GNAQ* was found to be frequently mutated in uveal melanocytic tumours, including benign nevi, primary melanomas of all stages and metastatic lesions.[9, 10] Mutations in *GNA11* (paralog of *GNAQ*, together referred to as Gα) were reported in blue naevi and UM shortly thereafter.[11] Other cancers with *GNAQ* mutations, although less studied, include a subset of cutaneous melanomas (6%),[9] leptomeningeal melanocytomas (50%) and melanomas arising from the meninges (25%).[12] In concordance with previous studies, we also found high mutation rates up to 93% in *GNAQ* as well as *GNA11* (codon Q209 and R183) in 92 primary UM (**Chapter 2.1**). *GNAQ* and *GNA11* encode the heterotrimeric guanine nucleotide-binding protein G subunit alpha q and 11, respectively, which play a role in G protein-coupled receptor (GPCR) signalling. Through mutations these subunits become activated and abrogate their intrinsic GTPase activity, which is required to return them to an inactive state. This oncogenic conversion is suggested to be the cause of constitutive mitogen-activated protein kinase (MAPK) pathway activation producing inappropriate proliferation,[13, 14] although the exact molecular events underlying these *GNAQ*/*GNA11*-driven malignancies are not yet defined. Two recent studies stated that Gα mutants found in UM promote tumourigenesis by activating YAP.[15 16] Nuclear localisation and dephosphorylation of YAP are triggered in Gα mutants. YAP is an oncoprotein and a key effector of the Hippo tumour suppressor pathway, which controls tissue growth and cell fate through the regulation of cell proliferation and apoptosis.[17] The major implication of these findings is that the classic GPCR signalling may not be the only mechanism for mutant *GNAQ*/*GNA11* activity. YAP is located on chromosome 11q22.2 that is generally not altered in UM. However, copy number variations in this region have been reported in the COSMIC database in other cancers, such as breast, lung, and ovary and pancreas cancer. More research is necessary to examine this chromosomal region and further unravel the role of YAP in UM. In our study, we found no association between *GNAQ*/*GNA11* mutations and metastasis or patient survival whereass in the literature several lines of evidence are presented suggesting that *GNA11* mutations might be more harmful than *GNAQ* mutations. In our cohort, patients with *GNAQ* and *GNA11* mutations had similar survival rates. Also, no associations were found with other prognostic factors, such monosomy 3, gain of chromosome 8q or *BAP1* mutations. This supports the hypothesis that these oncogenic mutations are early or perhaps initiating events in UM progression and they require additional mutations to acquire metastatic potential.

Loss of chromosome 3 or monosomy 3 is by far the most important chromosomal abnormality, and strongly associated with a decreased patient survival.[18-21] It is considered an early event in UM pathogenesis. In the past, research groups have delineated the minimal deleted

regions on chromosome 3 in attempt to find candidate genes on chromosome 3, but have been unsuccessful in identifying the specific mutations needed to establish pathogenetic relevance.[22-27] Just before we started on the studies in this thesis, Harbour et al. discovered an implicating tumour suppressor gene on chromosome 3, *BAP1*, in predominantly metastasising UM.[6] We evaluated the effect of *BAP1* mutations in 74 primary UM (**Chapter 2.2**), and confirmed that *BAP1* mutated tumours were present in nearly half of UM, mostly monosomy 3 tumours, and that *BAP1* mutations enhance the metastatic potential of UM. As we have shown in **Chapter 2.2**, lack of BAP1 expression highly associates with *BAP1* mutations and our data shows that *BAP1* acts as a classic tumour suppressor gene in UM whereby loss of one copy of chromosome 3 may unmask inactivating mutations in *BAP1* on the remaining chromosome 3, which is in concordance with current literature.[28, 29]

It is well known that tumours with disomy 3 (a normal chromosome 3 status with two different allelic copies) rarely metastasise. Two genes, *EIF1AX* and *SF3B1*, have recently been investigated and literature suggests that they would have a protective effect regarding UM metastases since they are associated with disomy 3 UM.[7, 30, 31] We also found that *EIF1AX* mutations associate with low-grade metastatic UM (**Chapter 2.3**). *EIF1AX* encodes the essential eukaryotic translation initiation factor 1A (eIF1A) and stimulates the transfer of methionyl initiator tRNA to the 40S ribosomal subunit,[32] mediating start codon recognition in mRNA by assembly of the eukaryotic translation preinitiation complex (PIC).[33] This process is essential for accurate gene expression. It can be hypothesised in *EIF1AX* mutated tumours the recognition of 5′ AUG codon of other specific tumour-related transcripts might be suppressed, which may lead to an increased survival in those patients. Although it is true that the chromosome 3 status is an incredible strong predictive factor, we have a small group of patients in our cohort that develop metastasis despite the disomy 3 status of their tumour. In fact, in **Chapter 2.3** we state that *SF3B1* mutations and disomy 3 predispose to late metastases that occur on average 7.5 years after enucleation. *SF3B1* encodes a component of the spliceosome, and a recent study revealed that recurrent *SF3B1* mutations in UM were associated with alternative splicing of protein coding genes (*ABCC5* and *UQCC*), and of the long noncoding RNA *CRNDE*.[34] This long RNA transcript has also shown to be up regulated in solid tumours (such as colorectal cancer) and leukaemias.[35, 36] *SF3B1* mutations have been observed in 15% of the chronic lymphocytic leukaemia (CLL) and associate with a worse prognosis,[37] whereas these mutations in patients with myelodysplastic syndrome (MDS) are associated with a better prognosis.[38] Thus, it seems that *SF3B1* mutations have a diverse effect on patient survival in different kinds of cancer. Interestingly, the *SF3B1* mutated CLL cases all had deletions in chromosome 11q which is already associated with a poor prognosis in patients with CLL.[37] One can speculate that the *SF3B1* mutations in UM in combination with a high level of chromosome abnormalities would increase metastatic risk compared to those with a

SF3B1 mutation and less chromosomal abnormalities. However, when comparing the copy number status of the *SF3B1* mutated UM with metastases with the *SF3B1* mutated UM without metastases, this was not the case. Also, in CLL and MDS a different hotspot is affected (K700 instead of the R625 mutations observed in UM). Whether both mutated hotspots have a similar effect on alternative splicing remains to be determined and investigations aimed at comparing the downstream effects are ongoing.

In the majority of patients liver metastases are detected within two years after diagnosis. Recently, Dimicheli et al.[39] analysed the mortality dynamics of 3672 UM patients from three different databases and concluded that there is a bimodal pattern of mortality with a peak at three and nine years after treatment. This indicates that UM metastatic development is not a continuous growth model and that it is complex. Often micrometastatic spread is already present at time of diagnosis. Besides genetic features, tumour dormancy and seeding of metastasis before primary tumour removal are likely to play a role in this process. Despite successful eradication of the ocular tumour, about 50% of all UM patients develop metastatic disease.[40] Studies on liver biopsies have shown that the genetic profile of the metastasis corresponds to the primary tumour. Not many studies have been performed on metastases themselves. One study on metastases showed that *GNA11* mutations within the metastases were associated with a worse survival.[41] A recent study examined the BAP1 expression in five primary UM tissue as well as matching UM metastases and found complete concordance between the BAP1 expression.[42] We investigated primary tumour and several metastases material from one patient who developed metastasis 8 years after initial diagnosis (**Chapter 2.4**), and both tumour and metastases tissue harboured an identical *GNA11* and *SF3B1* mutation. These findings support our hypothesis that *SF3B1* plays a role in late metastasising UM.

Based on all these results we propose the following UM progression model (**Figure 1**), in which *GNAQ* and *GNA11* mutations occur early and play a role in melanocytic transformation, followed by either a *BAP1* or *SF3B1* mutation, which determines the risk for growth of metastases, or an *EIF1AX* mutation that plays a relative protective role with a low risk of metastasising.

In **Chapter 2.5**, we present the second documented case of *TERT* promoter mutation in UM. In comparison with other types of melanoma such as skin and conjunctival melanoma, *TERT* promoter mutations are extremely rare in UM. Only one other study by Dono et al.[43] revealed a single mutated UM in a group of 50 examined UM. Although these mutations are rare in UM, *TERT* promoter mutations are frequent in conjunctival melanoma (41%) and occur at lower frequency in primary acquired melanosis with atypia (8%). Since *TERT* was not altered in benign conjunctival melanocytic lesions, we propose a role for *TERT* promotor

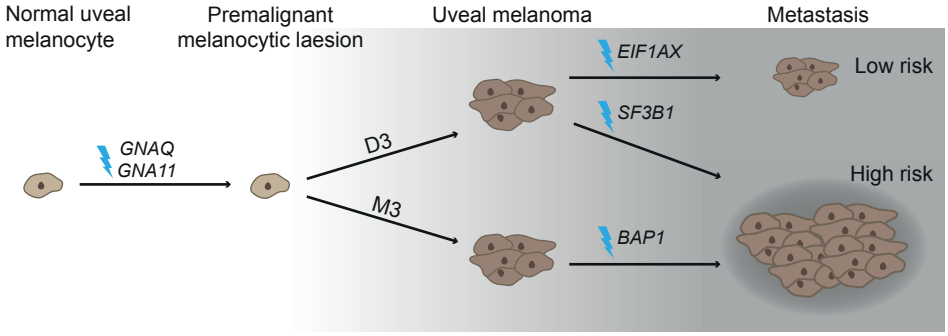


Figure 1. Simplistic tumour progression model with accumulation chromosome abnormalities and mutations in uveal melanoma. D3: disomy 3; M3: monosomy 3.

mutations as pathogenetic tumour progression marker in conjunctival melanocytic lesions. In addition, our findings confirm that the molecular pathogenesis of UM is distinct from conjunctival melanomas. Conjunctival melanomas are more similar to cutaneous melanomas that typically harbour *BRAF*, *NRAS*, and the recent discovered *TERT* mutations.[44-46]

Other frequent occurring chromosomal changes in UM, besides monosomy 3, include loss of 1p and 6q, and amplification of chromosome 6p and 8q. Previously, copy number variations in UM have been studied using low-resolution technologies such as karyotyping, fluorescence *in situ* hybridisation (FISH), comparative genomic hybridisation (CGH) and multiplex ligation-dependent probe amplification (MLPA). With specific NGS techniques not only variants can be detected in at base pair level but in some cases also copy number variations can be determined. One of such is the HaloPlex target enrichment NGS technique. Targeted capture followed by sequencing of selected genomic regions of interest provides an attractive, cost-effective alternative compared to whole genome sequencing. In **Chapter 3.1**, we validated the HaloPlex targeted capture method in 6 tumour samples and all the known mutations were validated. In addition, we concluded that the variant percentages were in general a good reflection of the copy number variation of chromosome 1p and 3. An advantage of NGS is that tumour heterogeneity, a frequent occurring phenomenon, can be detected. Tumour development is often associated with genomic instability and acquisition of genomic heterogeneity,[47] generating both clonal and non-clonal tumour cell populations.[48] In the HaloPlex pilot study, we observed one tumour with a clonal *BAP1* mutation, which was supported by the BAP1 expression data. Our group previously reported on intratumour heterogeneity of chromosome 3 and 8q in UM which is important to consider when taking biopsies for diagnostic purposes for instance.[49, 50]

Histopathological and chromosomal factors investigated

As mentioned previously, over the years many studies have been conducted finding prognostic factors in UM. Besides genetic parameters, there are a number of features that are predictive for metastasis, including large tumour diameter, ciliary body involvement, extraocular spread, a mixed or epithelioid melanoma cytomorphology, high mitotic count, vascular mimicry. Other potential prognostic features are the expression of chemokine receptors CCR7 and CXCR4, and increased expression of these chemokine receptors have been described in several types of cancer including UM.[51-53] We have investigated CCR7, CXCR4 and CXCL12 expression in 49 primary UM and corresponding liver metastasis (**Chapter 4.1**). A strong cytoplasmic staining for CCR7 correlated with bad prognostic features, such as epithelioid cell type and lymphocytic infiltration. In addition, strong CCR7 expression led to a significant worse prognosis in patients compared to those with CCR7 expression in a lesser grade or an absent CCR7 expression. These findings suggest the more important role for metastatic disease to be with CCR7 expression as compared with CXCR4 expression in UM. CCR7 could be used as prognostic marker in the future. Another well-established prognostic factor is extraocular extension of UM and these tumours are classified in a different subcategory of the TNM classification since they are associated with a worse prognosis.[54, 55] We revised 357 UM patients and 43 (12.0%) had extraocular extension of the tumour (**Chapter 4.2**). The risk of metastatic disease even increased when additional gain of chromosome 8q was present in tumours with extraocular extension. This study stresses the importance of both histopathological and chromosomal analyses in the work-up of UM.

In **Chapter 4.3**, an unusual case of UM with specific histopathological changes is demonstrated. Both spindle and epithelioid cells with a clear cytoplasm were found and the tumour had typical UM chromosomal alterations and not the translocation observed in clear cell sarcoma, concluding that this tumour was not associated with clear cell sarcoma but with UM. To date, no metastasis have been detected, but further follow-up in this patient is required to evaluate whether metastasis develop in this patient with a clear cell UM.

EXPERIMENTAL CONSIDERATIONS

Although well-established markers such as chromosome 3 loss and 8q gain are excellent for effective prognostication, combining the information on chromosomal aberrations with mutation analysis are important steps in a better prognostic prediction. The genetic landscape of this cancer is slowly coming into focus. In the past, genes involved in malignant melanoma development and progression have regularly been investigated, as these tumours are frequently compared. However, both types of melanoma have a different aetiology. Currently,

we are learning increasingly more about the genetics of UM. The investigated UM genomes display a mutational burden with abnormalities in the *GNAQ*, *GNA11*, *BAP1*, *SF3B1* and *EIF1AX* genes. Based on the studies described in this thesis, patients can now be prognostically categorised according to their mutational content: *BAP1* being responsible for early metastasis in monosomy 3 tumours, *SF3B1* for late metastasis in disomy 3 tumours and *EIF1AX* for a good prognosis. As mentioned previously, we know that *GNAQ* and *GNA11* mutations are early events in the pathogenesis. To this end, it would be interesting to perform mutation analysis on uveal naevi to gain more insight into the progression of a normal uveal melanocyte into an UM.

The experiments in this thesis have been performed on predominantly large tumours since these patients still get enucleated and tissue is available for genetic testing. Even though eye-conserving therapies are extensive used for small and medium-sized melanomas, they can still metastasise dependent on their chromosomal and mutational status. Biopsies are interesting for genetic prognostication purposes. Although, a risk of sampling error can occur if fine-needle aspiration biopsy (FNAB) or small incisional biopsy of the tumour is performed, misclassification due to tumour heterogeneity is reported in only 1% of tumours.[56] Tumour heterogeneity has been further investigated and it was concluded that analysis of tumour biopsies in UM gave an accurate prediction of the high-risk characteristics.[49] Still, when taking a FNAB or small incisional biopsy the risk of seeding and local metastasis remains a concern. Studies on the risk of local metastasis have been conducted and no increased risk was observed in FNABs[57] and the risk was even smaller if a transvitreal route was chosen for the FNAB.[58, 59] However, local recurrence or locoregional spread is still possible.[59, 60] Even though the risk for metastatic spread or local recurrence is minimal, the patient still needs surgery to obtain the tumour material. The surgery itself can have complications such as vitreous bleeding, retinal detachment, and endophthalmitis.[59, 61-63] Therefore, future experimental efforts need to be directed towards non-invasive detection of DNA abnormalities in blood or serum, which would be no extra burden to the patient since blood is routinely drawn to monitor basic liver functions of the patients, such as liver and kidney.

CLINICAL IMPLICATIONS

As metastatic disease occurrence has not changed in the past decade, it is likely that micrometastatic disease is present at time of diagnosis. For UM metastases, tumour-doubling times have been calculated with an median of 63 months, and it was assumed that with a constant growth rate, most metastases had initiated within 5 years before primary treatment.[64] Therefore, adjuvant treatment should be considered in an early stage of the disease,

and ideally at the time of diagnosis in order to prevent metastasis from growing. With the discovery of major key genes involved in UM development as *GNAQ*, *GNA11*, *BAP1*, *SF3B1* and *EIF1AX* mutations, further refinement of the classification of subclassification of UM is possible with mutation profiling in addition to chromosome 3 analysis. This will hopefully influence diagnostic concepts and therapeutic strategies based on the specific mutated gene. An accessible method for routinely prognostic testing BAP1 is immunohistochemistry since it allows clinicians to properly categorise UM patients, and is more practical and cheaper than NGS (**Chapter 2.2**). BAP1 immunohistochemistry can also be applied on biopsied tumour material (unpublished results), allowing also risk prediction of metastatic disease in patients with smaller melanomas. Another way to sequence multiple genes is with targeted NGS using special designed capture methods such as the HaloPlex (**Chapter 3.1**) or multiplex polymerase chain reaction (PCR).

While diagnostic methods for UM prognostication are rapidly improving, for the individual patient it is even more important to develop new therapeutic concepts. Although, the Gα-protein mutations themselves do not serve as a prognostic marker for the prediction of metastatic disease, due to the high frequency of occurrence they seem promising markers for tumour confirmation or therapeutic intervention in an early stage of the disease. The therapeutic goal is to inhibit downstream signalling molecules in the MAPK pathway that are activated. Preclinical studies show that inhibition of MAPK pathway in UM cell lines results in decreased cell proliferation.[9] There are several key molecules in the MAPK pathway, which have been explored as potential therapeutic targets. One of such is MEK, and Gα-protein mutant UM cells showed to be mildly sensitive to the MEK inhibitor AZD6244.[65] Another recent preclinical study proposed to target both the MAPK and PI3K/AKT pathway since both pathways are activated in UM. A combination of MEK and PI3K inhibition treatment resulted in induction of apoptosis in a Gα-mutant UM cells.[66] Another aspect to consider is that single-agent therapies for advanced cancers are rarely curative, due to the rapid development of resistance. A recent study showed that 6 out of 16 cutaneous melanoma tumours analysed in vitro acquired epidermal growth factor receptor (EGFR) expression after the development of resistance to BRAF or MEK inhibitors.[67] These tumours that developed vemurafenib resistance regained sensitivity to the drug after a so-called drug holiday. These findings highlight that not only combinations of certain drugs are important but also the timing of the therapy. Other potential targets in the MAPK pathway are currently being investigated, including protein kinase C, which is a component of signalling from GNAQ to ERK1/2.[68] Obstacles to these studies include the rarity of UM, and clinical trials are ongoing to evaluate the effect of these Gα-targeted regimens in UM patients. With the recent discovery of the involvement of the YAP-dependent pathway in Gα mutant UM (described above), YAP can also possible serve as a potential drug target for UM patients carrying mutations in *GNAQ* or *GNA11*. Verteporfin

has been identified as an inhibitor of YAP activity,[69] and recently, Feng et al.[16] and Yu et al.[15] demonstrated that verteporfin inhibits the growth of UM in xenograft mouse models. More studies are needed to explore the therapeutic potential of this in metastatic UM.

Therapeutically targeting UMs with a *BAP1* mutation works in a different manner than the Gα-protein mutations, since *BAP1* acts as a tumour suppressor gene. Regaining lost functions of suppressor genes are in general more challenging than inhibiting an overactive oncogene. Nevertheless, one preclinical study shows that histone deacetylase (HDAC) inhibitors can reverse the histone H2A hyperubiquitination that occurs in cultured UM cells depleted of BAP1, and it induces morphologic differentiation, cell-cycle exit, and shifts to a differentiated, melanocytic gene expression profile.[70] Examples of HDAC inhibitors are valproic acid, trichostatin A, LBH-589, and suberoylanilide hydroxamic acid.

Mutations in *SF3B1* occur in a variety of cancers and recently spliceosome inhibitor E7107 has been tested in patients with advanced solid tumours in a phase I clinical trial.[71] None of the patients were diagnosed with UM but since the same pathway is affected, perhaps this spliceosome inhibitor can be applicable for UM patients with a *SF3B1* mutation? Clinical trials are needed to evaluate the effect of these compounds in UM patients, and hopefully individualised patient care and targeted therapy based on mutational content will lead to improved patient survival.

FUTURE PROSPECTS

An important step has been made by identifying particular key genes in UM. A next step would be to non-invasively determine mutations in these genes. Exploring ways of non-invasive detection and screening of patients at high risk is a new field within UM research. Micro-RNAs (miRNAs) are used in many other types of cancer for diagnostic and classification purposes and have been reported as plasma or serum biomarkers for solid and haematological tumours.[72] In UM predictive plasma/serum biomarkers for metastasis are not available and biomarkers for early detection are important to identify patients at high risk for developing metastatic disease. It would be interesting to characterise miRNAs in UM, and especially the relation with development of metastases.

An autopsy study analysed the liver specimens from UM patients,[73] and this study indicated that UM latency was primarily due to the inability of cells in metastatic sites to grow. Single cells or a few cells in a single clump were found within the liver of otherwise asymptomatic patients. These were likely to be derived from circulating tumour cells.[73] Circulating tumour cells as well as circulating tumour DNA (ctDNA) can be assessed non-invasively in the peripheral blood circulation.[74, 75] Circulating cell-free tumour DNA, extracellular

DNA, has been suggested as a cancer biomarker for several types of solid tumours[76] next to circulating tumour cells.[74, 75] The ctDNAs are composed of small fragments of nucleic acid and not associated with viable cells or cell fragments.[77] Circulating tumour cells (CTCs) are cells shed form the primary tumour and circulate in the blood stream. In UM, CTCs have been explored before and the usefulness as an early prognostic marker has not been fully elucidated.[78, 79] Nevertheless, presence of CTCs was confirmed in patients with metastatic UM.[78, 79] For this purpose ctDNA seems to be more promising. With the current techniques, the ability to identify ctDNA has become easier as the protocols have been improved. For instance in solid tumours, such as colon carcinoma, a total amount of ctDNA has been related with the oncogenic load of the tumour and ability of the tumour to metastasise.[76] Detectable levels of ctDNA were present in 49% to 78% of the patients with localised tumours and in 86% to 100% of the metastatic colorectal, gastroesophageal, pancreatic and breast cancers. Within this study in many cases in which ctDNA was detected (81%), no circulating tumour cells were detectable. Specific mutations within the ctDNA were in concordance with the mutations detected in the primary tumour.[76] Also in UM the usefulness of ctDNA has been explored, and Madic et al.[80] have identified the presence of ctDNA in UM patients' serum. Studies with deep sequencing of the *GNAQ* and *GNA11* genes on ctDNA of metastasised UM have shown that this approach can be used for detection of disseminated disease.[81] Besides specific mutations, also copy number variations are detectable in circulating DNAs.[82] In concordance with prenatal testing for small amounts of foetal DNA in maternal blood also copy number variations, present in the tumour or metastases, can be identified.[74, 82]

Although progress has been made unravelling the genetic landscape of UM, more genes will probably get discovered with NGS in the future. In **Chapter 2.3** we showed that *BAP1*, *SF3B1* and *EIF1AX* mutations occur in a largely mutually exclusive pattern (**Figure 2, page 79**). Regardless of the Gα mutation status, nearly a quarter of UM had no mutations in either *BAP1*, *SF3B1* or *EIF1AX* suggesting that there are more genes involved in the UM tumour pathogenesis. Fundamental or basic research in the field of Ocular Oncology will still be necessary to gain more knowledge on current UM target genes as well as newly discovered genes, and to study their effect on the UM pathogenesis. It would be interesting to experiment with a zebra fish model. The zebra fish (Danio rerio) has been successfully applied as an organism to elucidate the aetiology of human genetic diseases and different types of cancer.[83, 84] Due to its transparent embryo that develops outside the mother's body the zebra fish represents an ideal vertebrate model system to study embryonic development. Gene expression in the zebra fish embryos can be manipulated with, for example, the morpholino antisense technology to achieve gene knockdown. It would be interesting to examine the effect of BAP1 depletion in zebra fish. Will these embryos develop different kind of tumours? In addition,

future experiments can focus on exposing zebra fish to different kinds of pharmacological compounds to examine the in vivo effect of newly developed medication.

In summary, UM is a genetic disease with a 50% risk of metastasis dependent on the tumours' chromosomal and mutational status, resulting in premature death. Genetic testing provides us with valuable prognostic information and will be critical to increase the understanding of UM progression. Targeting the pathways of *GNAQ/GNA11*, *BAP1* and *SF3B1* offer therapeutic opportunities, and clinical trials are ongoing. New non-invasive techniques such as screening of miRNAs and ctDNA will likely be crucial tools to improve diagnosis and therapy in the future by selecting patients for adjuvant treatment and monitoring for early metastatic disease.

REFERENCES

1. Metzker ML. Sequencing technologies - the next generation. *Nature reviews Genetics*. 2010;11:31-46.
2. Pareek CS, Smoczynski R, Tretyn A. Sequencing technologies and genome sequencing. *Journal of applied genetics*. 2011;52:413-435.
3. Gilissen C, Hoischen A, Brunner HG, Veltman JA. Unlocking Mendelian disease using exome sequencing. *Genome biology*. 2011;12:228.
4. Do R, Kathiresan S, Abecasis GR. Exome sequencing and complex disease: practical aspects of rare variant association studies. *Hum Mol Genet*. 2012;21:R1-9.
5. Myllykangas S, Ji HP. Targeted deep resequencing of the human cancer genome using next-generation technologies. *Biotechnology & genetic engineering reviews*. 2010;27:135-158.
6. Harbour JW, Onken MD, Roberson ED, et al. Frequent mutation of BAP1 in metastasizing uveal melanomas. *Science*. 2010;330:1410-1413.
7. Harbour JW, Roberson ED, Anbunathan H, et al. Recurrent mutations at codon 625 of the splicing factor SF3B1 in uveal melanoma. *Nat Genet*. 2013;45:133-135.
8. O'Hayre M, Vazquez-Prado J, Kufareva I, et al. The emerging mutational landscape of G proteins and G-protein-coupled receptors in cancer. *Nat Rev Cancer*. 2013;13:412-424.
9. Van Raamsdonk CD, Bezrookove V, Green G, et al. Frequent somatic mutations of GNAQ in uveal melanoma and blue naevi. *Nature*. 2009;457:599-602.
10. Bauer J, Kilic E, Vaarwater J, et al. Oncogenic GNAQ mutations are not correlated with disease-free survival in uveal melanoma. *Br J Cancer*. 2009;101:813-815.
11. Van Raamsdonk CD, Griewank KG, Crosby MB, et al. Mutations in GNA11 in uveal melanoma. *N Engl J Med*. 2010;363:2191-2199.
12. Kusters-Vandevelde HV, Klaasen A, Kusters B, et al. Activating mutations of the GNAQ gene: a frequent event in primary melanocytic neoplasms of the central nervous system. *Acta Neuropathol*. 2010;119:317-323.
13. Cohen C, Zavala-Pompa A, Sequeira JH, et al. Mitogen-actived protein kinase activation is an early event in melanoma progression. *Clin Cancer Res*. 2002;8:3728-3733.
14. Satyamoorthy K, Li G, Gerrero MR, et al. Constitutive mitogen-activated protein kinase activation in melanoma is mediated by both BRAF mutations and autocrine growth factor stimulation. *Cancer Res*. 2003;63:756-759.
15. Yu FX, Luo J, Mo JS, et al. Mutant Gq/11 Promote Uveal Melanoma Tumorigenesis by Activating YAP. *Cancer Cell*. 2014;25:822-830.
16. Feng X, Degese MS, Iglesias-Bartolome R, et al. Hippo-Independent Activation of YAP by the GNAQ Uveal Melanoma Oncogene through a Trio-Regulated Rho GTPase Signaling Circuitry. *Cancer Cell*. 2014;25:831-845.
17. Harvey KF, Zhang X, Thomas DM. The Hippo pathway and human cancer. *Nat Rev Cancer*. 2013;13:246-257.
18. Kilic E, van Gils W, Lodder E, et al. Clinical and cytogenetic analyses in uveal melanoma. *Invest Ophthalmol Vis Sci*. 2006;47:3703-3707.

19. Prescher G, Bornfeld N, Hirche H, et al. Prognostic implications of monosomy 3 in uveal melanoma. *Lancet*. 1996;347:1222-1225.

20. Sisley K, Parsons MA, Garnham J, et al. Association of specific chromosome alterations with tumour phenotype in posterior uveal melanoma. *Br J Cancer*. 2000;82:330-338.

21. White VA, Chambers JD, Courtright PD, Chang WY, Horsman DE. Correlation of cytogenetic abnormalities with the outcome of patients with uveal melanoma. *Cancer*. 1998;83:354-359.

22. Myatt N, Aristodemou P, Neale MH, et al. Abnormalities of the transforming growth factor-beta pathway in ocular melanoma. *J Pathol*. 2000;192:511-518.

23. Sisley K, Curtis D, Rennie IG, Rees RC. Loss of heterozygosity of the thyroid hormone receptor B in posterior uveal melanoma. *Melanoma Res*. 1993;3:457-461.

24. Zeschnigk M, Tschentscher F, Lich C, et al. Methylation analysis of several tumour suppressor genes shows a low frequency of methylation of CDKN2A and RARB in uveal melanomas. *Comparative and functional genomics*. 2003;4:329-336.

25. Cross NA, Ganesh A, Parpia M, et al. Multiple locations on chromosome 3 are the targets of specific deletions in uveal melanoma. *Eye (Lond)*. 2006;20:476-481.

26. Parrella P, Fazio VM, Gallo AP, Sidransky D, Merbs SL. Fine mapping of chromosome 3 in uveal melanoma: identification of a minimal region of deletion on chromosomal arm 3p25.1-p25.2. *Cancer Res*. 2003;63:8507-8510.

27. Tschentscher F, Prescher G, Horsman DE, et al. Partial deletions of the long and short arm of chromosome 3 point to two tumor suppressor genes in uveal melanoma. *Cancer Res*. 2001;61:3439-3442.

28. Yu H, Pak H, Hammond-Martel I, et al. Tumor suppressor and deubiquitinase BAP1 promotes DNA double-strand break repair. *Proc Natl Acad Sci U S A*. 2014;111:285-290.

29. Jensen DE, Proctor M, Marquis ST, et al. BAP1: a novel ubiquitin hydrolase which binds to the BRCA1 RING finger and enhances BRCA1-mediated cell growth suppression. *Oncogene*. 1998;16:1097-1112.

30. Martin M, Masshofer L, Temming P, et al. Exome sequencing identifies recurrent somatic mutations in EIF1AX and SF3B1 in uveal melanoma with disomy 3. *Nat Genet*. 2013;45:933-936.

31. Ewens KG, Kanetsky PA, Richards-Yutz JA, et al. Chromosome 3 Status Combined with BAP1 and EIF1AX Mutation Profiles are Associated with Metastasis in Uveal Melanoma. *Invest Ophthalmol Vis Sci*. 2014;55:5160-5167.

32. Chaudhuri J, Si K, Maitra U. Function of eukaryotic translation initiation factor 1A (eIF1A) (formerly called eIF-4C) in initiation of protein synthesis. *J Biol Chem*. 1997;272:7883-7891.

33. Jackson RJ, Hellen CU, Pestova TV. The mechanism of eukaryotic translation initiation and principles of its regulation. *Nat Rev Mol Cell Biol*. 2010;11:113-127.

34. Furney SJ, Pedersen M, Gentien D, et al. SF3B1 mutations are associated with alternative splicing in uveal melanoma. *Cancer Discov*. 2013;3:1122-1129.

35. Ellis BC, Molloy PL, Graham LD. CRNDE: A Long Non-Coding RNA Involved in CanceR, Neurobiology, and DEvelopment. *Frontiers in genetics*. 2012;3:270.

36. Graham LD, Pedersen SK, Brown GS, et al. Colorectal Neoplasia Differentially Expressed (CRNDE), a Novel Gene with Elevated Expression in Colorectal Adenomas and Adenocarcinomas. *Genes & cancer*. 2011;2:829-840.

37. Wang L, Lawrence MS, Wan Y, et al. SF3B1 and other novel cancer genes in chronic lymphocytic leukemia. *N Engl J Med*. 2011;365:2497-2506.

38. Papaemmanuil E, Cazzola M, Boultwood J, et al. Somatic SF3B1 mutation in myelodysplasia with ring sideroblasts. *N Engl J Med*. 2011;365:1384-1395.

39. Demicheli R, Fornili M, Biganzoli E. Bimodal mortality dynamics for uveal melanoma: a cue for metastasis development traits? *BMC Cancer*. 2014;14:392.

40. Singh AD, Turell ME, Topham AK. Uveal melanoma: trends in incidence, treatment, and survival. *Ophthalmology*. 2011;118:1881-1885.

41. Griewank KG, van de Nes J, Schilling B, et al. Genetic and clinico-pathologic analysis of metastatic uveal melanoma. *Mod Pathol*. 2014;27:175-183.

42. Kalirai H, Dodson A, Faqir S, Damato BE, Coupland SE. Lack of BAP1 protein expression in uveal melanoma is associated with increased metastatic risk and has utility in routine prognostic testing. *Br J Cancer*. 2014:111:1373-1380.

43. Dono M, Angelini G, Cecconi M, et al. Mutation frequencies of GNAQ, GNA11, BAP1, SF3B1, EIF1AX and TERT in uveal melanoma: detection of an activating mutation in the TERT gene promoter in a single case of uveal melanoma. *Br J Cancer*. 2014;110:1058-1065.

44. Heidenreich B, Rachakonda PS, Hemminki K, Kumar R. TERT promoter mutations in cancer development. *Curr Opin Genet Dev*. 2014;24:30-37.

45. Horn S, Figl A, Rachakonda PS, et al. TERT promoter mutations in familial and sporadic melanoma. *Science*. 2013;339:959-961.

46. Huang FW, Hodis E, Xu MJ, et al. Highly recurrent TERT promoter mutations in human melanoma. *Science*. 2013;339:957-959.

47. Bayani J, Selvarajah S, Maire G, et al. Genomic mechanisms and measurement of structural and numerical instability in cancer cells. *Semin Cancer Biol*. 2007;17:5-18.

48. Katona TM, Jones TD, Wang M, et al. Genetically heterogeneous and clonally unrelated metastases may arise in patients with cutaneous melanoma. *Am J Surg Pathol*. 2007;31:1029-1037.

49. Mensink HW, Vaarwater J, Kilic E, et al. Chromosome 3 intratumor heterogeneity in uveal melanoma. *Invest Ophthalmol Vis Sci*. 2009;50:500-504.

50. van den Bosch T, van Beek JG, Vaarwater J, et al. Higher percentage of FISH-determined monosomy 3 and 8q amplification in uveal melanoma cells relate to poor patient prognosis. *Invest Ophthalmol Vis Sci*. 2012;53:2668-2674.

51. Dobner BC, Riechardt AI, Joussen AM, Englert S, Bechrakis NE. Expression of haematogenous and lymphogenous chemokine receptors and their ligands on uveal melanoma in association with liver metastasis. *Acta ophthalmologica*. 2012;90:e638-644.

52. Li H, Alizadeh H, Niederkorn JY. Differential expression of chemokine receptors on uveal melanoma cells and their metastases. *Invest Ophthalmol Vis Sci*. 2008;49:636-643.

53. Scala S, Ierano C, Ottaiano A, et al. CXC chemokine receptor 4 is expressed in uveal malignant melanoma and correlates with the epithelioid-mixed cell type. *Cancer Immunol Immunother*. 2007;56:1589-1595.

54. Kujala E, Damato B, Coupland SE, et al. Staging of ciliary body and choroidal melanomas based on anatomic extent. *J Clin Oncol*. 2013;31:2825-2831.

55. Shields CL, Kaliki S, Furuta M, et al. American Joint Committee on Cancer classification of posterior uveal melanoma (tumor size category) predicts prognosis in 7731 patients. *Ophthalmology*. 2013;120:2066-2071.

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

57. Char DH, Kroll SM, Miller T, Castro J, Quivey J. Irradiated uveal melanomas: cytopathologic correlation with prognosis. *Am J Ophthalmol*. 1996;122:509-513.

58. Karcioglu ZA, Gordon RA, Karcioglu GL. Tumor seeding in ocular fine needle aspiration biopsy. *Ophthalmology*. 1985;92:1763-1767.

59. Glasgow BJ, Brown HH, Zargoza AM, Foos RY. Quantitation of tumor seeding from fine needle aspiration of ocular melanomas. *Am J Ophthalmol*. 1988;105:538-546.

60. Caminal JM, Sanz S, Carreras M, et al. Epibulbar seeding at the site of a transvitreal fine-needle aspiration biopsy. *Arch Ophthalmol*. 2006;124:587-589.

61. Scherfig E, Prause JU, Jensen OA. Transvitreal retinochoroidal biopsy. *Graefes Arch Clin Exp Ophthalmol*. 1989;227:369-373.

62. Cohen VM, Dinakaran S, Parsons MA, Rennie IG. Transvitreal fine needle aspiration biopsy: the influence of intraocular lesion size on diagnostic biopsy result. *Eye (Lond)*. 2001;15:143-147.

63. Faulkner-Jones BE, Foster WJ, Harbour JW, Smith ME, Davila RM. Fine needle aspiration biopsy with adjunct immunohistochemistry in intraocular tumor management. *Acta Cytol*. 2005;49:297-308.

64. Eskelin S, Pyrhonen S, Hahka-Kemppinen M, Tuomaala S, Kivela T. A prognostic model and staging for metastatic uveal melanoma. *Cancer*. 2003;97:465-475.

65. Mitsiades N, Chew SA, He B, et al. Genotype-dependent sensitivity of uveal melanoma cell lines to inhibition of B-Raf, MEK, and Akt kinases: rationale for personalized therapy. *Invest Ophthalmol Vis Sci*. 2011;52:7248-7255.

66. Khalili JS, Yu X, Wang J, et al. Combination Small Molecule MEK and PI3K Inhibition Enhances Uveal Melanoma Cell Death in a Mutant GNAQ- and GNA11-Dependent Manner. *Clin Cancer Res*. 2012;18:4345-4355.

67. Sun C, Wang L, Huang S, et al. Reversible and adaptive resistance to BRAF(V600E) inhibition in melanoma. *Nature*. 2014;508:118-122.

68. Wu X, Zhu M, Fletcher JA, Giobbie-Hurder A, Hodi FS. The protein kinase C inhibitor enzastaurin exhibits antitumor activity against uveal melanoma. *PLoS One*. 2012;7:e29622.

69. Liu-Chittenden Y, Huang B, Shim JS, et al. Genetic and pharmacological disruption of the TEAD-YAP complex suppresses the oncogenic activity of YAP. *Genes Dev*. 2012;26:1300-1305.

70. Landreville S, Agapova OA, Matatall KA, et al. Histone deacetylase inhibitors induce growth arrest and differentiation in uveal melanoma. *Clin Cancer Res*. 2012;18:408-416.

71. Eskens FA, Ramos FJ, Burger H, et al. Phase I pharmacokinetic and pharmacodynamic study of the first-in-class spliceosome inhibitor E7107 in patients with advanced solid tumors. *Clin Cancer Res*. 2013;19:6296-6304.

72. Allegra A, Alonci A, Campo S, et al. Circulating microRNAs: new biomarkers in diagnosis, prognosis and treatment of cancer (review). *Int J Oncol*. 2012;41:1897-1912.

73. Borthwick NJ, Thombs J, Polak M, et al. The biology of micrometastases from uveal melanoma. *J Clin Pathol*. 2011;64:666-671.

74. Jung K, Fleischhacker M, Rabien A. Cell-free DNA in the blood as a solid tumor biomarker--a critical appraisal of the literature. *Clin Chim Acta*. 2010;411:1611-1624.

75. Alix-Panabieres C, Schwarzenbach H, Pantel K. Circulating tumor cells and circulating tumor DNA. *Annu Rev Med*. 2012;63:199-215.

76. Bettegowda C, Sausen M, Leary RJ, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. *Sci Transl Med*. 2014;6:224ra24.

77. Stroun M, Lyautey J, Lederrey C, Olson-Sand A, Anker P. About the possible origin and mechanism of circulating DNA apoptosis and active DNA release. *Clin Chim Acta*. 2001;313:139-142.

78. Mazzini C, Pinzani P, Salvianti F, et al. Circulating tumor cells detection and counting in uveal melanomas by a filtration-based method. *Cancers (Basel)*. 2014;6:323-332.

79. Schuster R, Bechrakis NE, Stroux A, et al. Prognostic Relevance of Circulating Tumor Cells in Metastatic Uveal Melanoma. *Oncology*. 2011;80:57-62.

80. Madic J, Piperno-Neumann S, Servois V, et al. Pyrophosphorolysis-activated polymerization detects circulating tumor DNA in metastatic uveal melanoma. *Clin Cancer Res*. 2012;18:3934-3941.

81. Metz CH, Scheulen M, Bornfeld N, Lohmann D, Zeschnigk M. Ultradeep sequencing detects GNAQ and GNA11 mutations in cell-free DNA from plasma of patients with uveal melanoma. *Cancer Med*. 2013;2:208-215.

82. Norwitz ER, Levy B. Noninvasive Prenatal Testing: The Future Is Now. *Rev Obstet Gynecol*. 2013;6:48-62.

83. Faucherre A, Taylor GS, Overvoorde J, Dixon JE, Hertog J. Zebrafish pten genes have overlapping and non-redundant functions in tumorigenesis and embryonic development. *Oncogene*. 2008;27:1079-1086.

84. Feitsma H, Cuppen E. Zebrafish as a cancer model. *Mol Cancer Res*. 2008;6:685-694.

CHAPTER 6

Summary in Dutch

List of abbreviations

List of publications

About the author & PhD portfolio

Acknowledgements

SUMMARY IN DUTCH

NEDERLANDSE SAMENVATTING

NEDERLANDSE SAMENVATTING

Het uveamelanoom is een kwaadaardige vorm van kanker in het oog en ontstaat uit de gepigmenteerde cellen (melanocyten) in het vaatvlies (uvea) in het oog. Het is de meest voorkomende intra-oculaire maligniteit in Europa en de Verenigde Staten met een incidentie van zeven per miljoen mensen. Ongeveer de helft van de patiënten overlijdt uiteindelijk ten gevolge van levermetastasen. Er zijn bekende klinische, histopathologische en genetische factoren welke een invloed hebben op het al dan niet optreden van metastasen. De belangrijkste chromosoomverandering in de tumor is verlies van chromosoom 3 (monosomie 3). Ondanks de huidige sterk verbeterende oogsparende behandelingen van de primaire tumor (stereotactische bestraling, brachytherapie, protonenbestraling) en chirurgische verwijdering van het oog (enucleatie), is de prognose van patiënten met een uveamelanoom de afgelopen decennia niet veranderd. Wel zijn er in de laatste 5 jaar belangrijke inzichten verkregen door analyse van het tumor DNA met nieuwe moleculaire technieken. Zo kunnen we nu op basis van bepaalde genafwijkingen beter voorspellen welke patiënten metastasen zullen ontwikkelen. Daarnaast kunnen de onderzochte genen en pathways in de toekomst mogelijk als aangrijpingspunt dienen voor mutatiegerichte therapie in uveamelanomen. Het doel van dit proefschrift is om de genetische achtergrond van uveamelanomen verder op te helderen.

In hoofdstuk 2 van dit proefschrift worden verschillende kandidaat genen beschreven die betrokken zijn in de ontwikkeling van het uveamelanoom. Zo vonden we in **Hoofdstuk 2.1** dat het merendeel van de tumoren (93%) een specifieke hotspot mutatie in het *GNAQ* of *GNA11* gen hebben. Patiënten met *GNAQ* en *GNA11* mutaties hadden eenzelfde overlevingsduur en er werden geen associaties gevonden met andere prognostische factoren. Deze bevindingen ondersteunen de hypothese dat de oncogene *GNAQ/GNA11* mutaties vroeg in de tumorontwikkeling ontstaan en dat er additionele mutaties in andere genen nodig zijn voor het optreden van uitzaaiingen. Een gen wat wel een belangrijke rol speelt bij metastasering van uveamelanomen is het *BAP1* gen gelokaliseerd op chromosoom 3p21.1. Voorgaande studies hebben reeds laten zien dat verlies van chromosoom 3 sterk geassocieerd is met een verminderde overleving. **Hoofdstuk 2.2** laat zien dat afwijkingen in het *BAP1* gen voornamelijk voorkomen in agressieve tumoren met verlies van chromosoom 3. Patiënten met afwijkingen in dit gen hebben een zeven keer hoger risico op het ontwikkelen van metastasen vergeleken met patiënten zonder afwijkingen in dit gen. Daarnaast vonden wij dat *BAP1* mutaties sterk geassocieerd waren met een afwezige BAP1 eiwit expressie in de tumor. Op basis van deze bevindingen adviseren wij BAP1 immunohistochemische kleuringen standaard te verrichten in de histopathologische work-up van uveamelanomen.

Een andere groep van uveamelanomen zijn de tumoren met een normale chromosoom

3 status (disomie 3). Het merendeel van deze patiënten hebben een gunstige prognose en ontwikkelen zelden metastasen. Echter, in ons cohort is er een specifieke groep patiënten met disomie 3 die na enkele jaren toch metastasen ontwikkelen. In **Hoofdstuk 2.3** zien wij dat afwijkingen in het *SF3B1* gen hierbij een rol spelen. Patiënten met een disomie 3 tumor en een *SF3B1* mutatie hebben een hogere kans op het ontwikkelen van late metastasen. Daarnaast vonden we dat een ander gen, *EIF1AX*, een beschermend effect heeft op metastasering. Het belang van het *SF3B1* gen mutaties voor de voorspelling van late metastasering wordt nogmaals benadrukt in **Hoofdstuk 2.4** waarin een patiënt wordt beschreven die ongeveer 8 jaar na het stellen van de diagnose meerdere metastasen heeft ontwikkeld in de lever en alvleesklier. Wij vonden een *GNA11* en *SF3B1* mutatie in zowel de primaire tumor als ook in de metastasen.

Hoofdstuk 2.5 schets de rol van *TERT* promotor mutaties als tumor progressie marker in conjunctivale melanocytaire laesies. Deze mutaties zijn zeldzaam in uveamelanomen maar in conjunctiva melanomen (41%) en primary acquired melanosis (PAM) ofwel primaire verworven melanose (8%) komen deze *TERT* promotor mutaties frequent voor. Tevens bevestigen we in deze studie dat de moleculaire pathogenese van het uveamelanoom en conjunctiva melanoom wezenlijk verschillend is. Conjunctiva melanomen hebben meer overkomsten met huidmelanomen met mutaties in het *BRAF*, *NRAS* en *TERT* gen.

In het derde hoofdstuk van dit proefschrift evalueren we of Next Generation Sequencing met een speciaal ontworpen HaloPlex target enrichment kit een betrouwbare techniek is voor het opsporen van mutaties in de oogmelanoom kandidaat genen (**Hoofdstuk 3.1**). In deze pilotstudie werden zes tumoren met mutaties in *GNAQ*, *GNA11* en *BAP1* onderzocht en alle bekende mutaties werden met de HaloPlex techniek gevalideerd. Daarnaast waren de variantfrequenties over het algemeen een goede weerspiegeling van het aantal kopieën van chromosoom 1p en 3 in de desbetreffende tumoren. Gericht sequencing biedt een aantrekkelijk kosteneffectief alternatief voor genoom-breed sequencing indien slechts een gen panel getest dient te worden.

Naast genetische factoren zijn er ook andere klinische en histopathologische factoren welke van invloed zijn op de prognose van uveamelanoom patiënten, zoals de grootte van de tumordiameter, betrokkenheid corpus ciliare, extraoculaire extensie, een gemengd of puur epitheloïde cel morfologie, hogere mitosefrequentie en de aanwezigheid gesloten vaatpatronen. In **Hoofdstuk 4.1** hebben we de expressie van CCR7, CXCR4 en CXCL12 onderzocht in primaire uveamelanomen en bijbehorende levermetastasen. Een sterke cytoplasmatische CCR7 aankleuring was geassocieerd met prognostische kenmerken zoals een epitheloïde celtype en lymfocyten infiltratie in de tumor. Daarnaast hadden de patiënten met een sterke

CCR7 expressie in de tumor een significant slechtere overleving vergeleken met patiënten zonder CCR7 expressie. CCR7 zou daarom in de toekomst als prognostische marker kunnen dienen. Een ander bekende prognostische factor is extraoculaire extensie van de tumor waarbij de tumor door de sclera heen groeit. In **Hoofdstuk 4.2** reviseerden we 357 uveamelanomen waarvan 43 (12%) extraoculaire extensie hadden. Hieruit bleek dat het risico op metastasen verder toeneemt als er naast extraoculaire extensie ook winst van chromosoom 8q aanwezig is. Deze studie benadrukt het belang van zowel histopathologische als genetische analyses in de work-up van uveamelanomen. In **Hoofdstuk 4.3** wordt een zeldzaam geval van een clear cel uveamelanoom gepresenteerd met specifieke histopathologische veranderingen. De tumor had chromosomale afwijkingen (chromosoom 6p, 6q en 8q) passend bij een uveamelanoom en niet bij clear cel sarcoma. De typische t(12;22)(q12q13) translocatie welke gerelateerd is met clear cel sarcoma werd met behulp van fluorescentie *in situ* hybridisatie (FISH) niet gevonden. Tot op heden heeft de patiënt een gunstig beloop gehad, echter opvolging van het beloop is nodig om vast te stellen of er niet alsnog uitzaaiingen ontstaan.

Tenslotte worden in **Hoofdstuk 5** de belangrijkste bevindingen en implicaties van de resultaten in dit proefschrift bediscussieerd. Daarnaast worden in dit hoofdstuk overwegingen voor toekomstig onderzoek gegeven. De inhoud van dit proefschrift heeft ons meer inzicht gegeven tot het genetische profiel van uveamelanomen en is mogelijk bruikbaar voor het ontwikkelen van nieuwe gengerichte therapieën en non-invasieve detectiemethoden in uveamelanoom patiënten.

LIST OF ABBREVIATIONS

LIST OF ABBREVIATIONS

aa	amino acid
AKT3	v-akt murine thymoma viral ocogene homolog 3
ASAP1	arf-GAP with SH3 domain, ANK repeat and PH domain-containing protein 1
AZT	azidothymidine
BAP1	BRCA1-associated protein 1
BCL-2	B-cell CLL/lymphoma 2
bp	base pair
BARD1	BRCA1-associated RING domain protein 1
BCVA	best corrected visual acuity
BRAF	v-raf murine sarcoma viral oncogene homolog B1
BRCA1	breast cancer 1, early onset
BWA	Burrows-Wheeler Aligner
CCND1	G1/S-specific cyclin-D1
CDKN2A	cyclin-dependent kinase inhibitor 2A
cDNA	copy deoxyribonucleic acid
CD45	cluster of differentiation 45 (leukocyte common antigen, protein tyrosine phosphatase)
CI	confidence interval
CGH	comparative genomic hybridisation
CCL19	chemokine (C-C motif) ligand 19
CCL21	chemokine (C-C motif) ligand 21
CCR7	C-C chemokine receptor 7
CLL	chronic lymphocytic leukaemia
COSMIC	catalogue of somatic mutations in cancer
CT	computed tomography
CTCs	circulating tumour cells
ctDNA	circulating tumour deoxyribonucleic acid
CXCL12	C-X-C motif chemokine 12
CXCR4	C-X-C chemokine receptor 4
del	deletion
DNA	deoxyribonucleic acid
EGFR	epidermal growth factor receptor
eIF1A	eukaryotic translation initiation factor 1A
EIF1AX	eukaryotic translation initiation factor 1A, X-linked

Ets/TCF	E-twenty six/ternary complex factors
EXE	extraocular extension
FFPE	formalin-fixed paraffin-embedded
FISH	fluorescent *in situ* hybridisation
FNAB	fine-needle aspiration biopsy
fSRT	fractionated stereotactic radiotherapy
gamma-GT	gamma-glutamyl transpeptidase
GEP	gene expression profiling
GNAQ	guanine nucleotide-binding protein G subunit alpha q
GNA11	guanine nucleotide-binding protein G subunit alpha 11
GPCR	G-protein-coupled receptor
gp100	glycoprotein 100
GRN163L	imetelstat
Gα	*GNAQ* and *GNA11* genes
HBM	HCF1-binding domain-like motif
HCF1	host cell factor 1
HDAC	histone deacetylase
HGF	hepatocyte growth factor
hg19	human genome build 19
HMB-45	human melanoma black-45
HPF	high-power fields
HR	hazard ratio
H&E	haematoxylin and eosin
I-125	iodine-125
IGF-1	insulin-like growth factor 1
IHC	immunohistochemistry
InDel	insertion of deletion
ins	insertion
kb	kilobase
Kbp	kilo base pairs
LOH	loss of heterozygosity
LTD	largest tumour diameter
MAPK	mitogen-activated protein kinase
MAQ	multiplex amplicon quantification
Mb	megabase
MDS	myelodysplastic syndrome
MEK	mitogen-activated protein kinase kinase

Melan-A	melanoma antigen recognized by T cells (MART-1)
min	minutes
miRNA	micro ribonucleic acid
MLPA	multiplex ligation-dependent probe amplification
mo	months
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)
NBN	nibrin
NGS	Next Generation Sequencing
NLS	nuclear localisation signal
NRAS	neuroblastoma RAS viral (v-ras) oncogene homolog
N/A	not available
OCT	optical coherence tomography
OD	oculus dexter
OS	oculus sinister
PAM	primary acquired melanosis
PAS	periodic acid-Schiff
PCR	polymerase chain reaction
PIC	preinitiation complex
PI3K	phosphoinositide 3-kinase
PI3K/AKT	phosphoinositide 3-kinase/protein kinase B (PKB)
PTEN	phosphatase and tensin homolog
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
ROMS	Rotterdam Ocular Melanoma Study group
RT-PCR	real-time polymerase chain reaction
Ru-106	ruthenium-106
s	seconds
SER	secondary enucleation rate
SF3b	splicing factor 3B
SF3B1	splicing factor 3B subunit 1
SNP	single nucleotide polymorphism
S-100	acidic protein, 100% Soluble in ammonium sulphate at neutral pH
TERT	telomerase reverse transcriptase
TFSOM	to find small ocular melanoma
TMA	tissue microarray

TNM	tumour node metastasis
TP53	tumour protein p53
tRNA	transfer ribonucleic acid
TTT	transpupillary thermotherapy
UCH	ubiquitin carboxyl-terminal hydrolase
ULD	UCH37-like domain
UM	uveal melanoma
US	ultrasonography
UTR	untranslated region
VCF	variant calling file
y	years
YAP	Yes-associated protein
YY1	Yin Yang 1

LIST OF PUBLICATIONS

LIST OF PUBLICATIONS

Koopmans AE, van den Berg MMP, Brouwer RWW, Kockx CEM, Vaarwater J, Nellist M, van IJcken WFJ, Kiliç E, de Klein A. Haloplex: a targeted approach for detecting gene mutations and copy number variations designed for uveal melanoma. *Manuscript in preparation*.

Koopmans AE, Yavuzyigitoglu S, van Bodegom AJE, Vaarwater J, Verdijk RM, Brouwer RWW, van IJcken WFJ, Paridaens D, Kiliç E, de Klein A. *SF3B1* mutations predispose to late metastases whereas *EIF1AX* mutations prevent metastases in disomy 3 uveal melanoma. *Submitted.*

van Beek JGM, **Koopmans AE**, Vaarwater J, Verdijk RM, de Klein A, Naus NC, Kiliç E. Metastatic disease in uveal melanoma: importance of a genetic profile. *Submitted.*

Koopmans AE, Ober K, Dubbink HJ, Paridaens D, Naus NC, Belunek S, Krist B, Post E, Zwarthoff EC, de Klein A, Verdijk RM. Prevalence and implications of *TERT* promoter mutation in uveal and conjunctival melanoma and in benign and premalignant conjunctival melanocytic lesions. *Invest Ophthalmol Vis Sci.* 2014 Aug;26;55(9):6024-6030.

Brosens E, Ploeg M, van Bever Y, **Koopmans AE**, IJsselstijn H, Rottier RJ, Wijnen R, Tibboel D, de Klein A. Clinical and etiological heterogeneity in patients with tracheo-esophageal malformations and associated anomalies. *Eur J Med Genet*. 2014 Aug;57(8):440-452.

Koopmans AE, Verdijk RM, Brouwer RWW, van den Bosch TPP, van den Berg MMP, Vaarwater J, Kockx CEM, Paridaens D, Naus NC, Nellist M, van IJcken WFJ, Kiliç E, de Klein A. Clinical significance of immunohistochemistry for detection of BAP1 mutations in uveal melanoma. *Modern Pathol.* 2014 Oct;27(10):1321-1330.

van Beek JGM, **Koopmans AE**, Vaarwater J, de Rooi JJ, Paridaens D, Naus NC, de Klein A, Kiliç E. The prognostic value of extraocular extension in relation to monosomy 3 and gain of chromosome 8q in uveal melanoma. *Invest Ophthalmol Vis Sci.* 2014 Mar 4;55(3):1284-91.

van den Bosch TPP, **Koopmans AE**, Vaarwater J, van den Berg M, de Klein A, Verdijk RM. Chemokine receptor CCR7 expression predicts poor outcome in uveal melanoma and relates to liver metastasis whereas expression of CXCR4 is not of clinical relevance. *Invest Ophthalmol Vis Sci*. 2013 Sep;54(12):7354-7361.

Koopmans AE, Vaarwater J, Paridaens D, Naus NC, Kiliç E, de Klein A. Patient survival in uveal melanoma is not affected by oncogenic mutations in GNAQ and GNA11. *Br J Cancer*. 2013 Jul 23;109(2):493-496.

Koopmans AE, de Klein A, Naus NC, Kiliç E. Diagnosis and management of uveal melanoma. *European Ophthalmic Review*. 2013;7(1):56-60.

Verdijk RM, **Koopmans AE**, Kiliç E, Paridaens, de Klein A. Histopathologic, immunohistochemical, and cytogenetic analysis of primary clear cell melanoma of the uvea. *JAMA Ophthalmol*. 2013 Jun;131(6):814-816.

van Beek JGM, **Koopmans AE**, Verdijk RM, Naus NC, de Klein A, Kiliç E. Diagnosis, histopathologic and genetic classification of uveal melanoma. In: G. Huyn Thien Duc. *Melanoma – From Early Detection to Treatment*. 1st edn. Publisher: InTech, 2013:137-173.

de Klein A, **Koopmans AE**, Kiliç E. Multicolor FISH with improved sensitivity and specificity in the diagnosis of malignant melanoma. *Expert Rev Mol Diagn*. 2012 Sep;12(7):683-685.

Veenemans J, Milligan P, Prentice AM, Schouten LR, Inja N, van der Heijden AC, de Boer LC, Jansen EJ, **Koopmans AE**, Enthoven WT, Kraaijenhagen RJ, Demir AY, Uges DR, Mbugi EV, Savelkoul HF, Verhoef H. Effect of supplementation with zinc and other micronutrients on malaria in Tanzanian children: a randomized trial. *PLoS Med.* 2011 Nov;8(11).

ABOUT THE AUTHOR & PhD PORTFOLIO

ABOUT THE AUTHOR

Anna Elizabeth Koopmans was born on the 4th of July 1985 in Stockport, England. When she was five years old she moved with her family to the Netherlands. She graduated from bilingual VWO at the RSG 't Slingerbos, Harderwijk in 2003. After studying one year Biomedical Science at the University of Utrecht, she transferred to the Erasmus University Rotterdam to study Medicine. During her study, she worked as a medical student at the Department of Gastroenterology, Dermatology and Trauma Surgery at the Erasmus Medical Centre. She also worked as an administrative assistant at the Medical Services for asylum seekers (MOA) in a reception centre in Luttelgeest. In 2008, she spend five months in a rural area in Bondo, Tanzania working on malaria research under supervision of Dr. H. Verhoef and Dr. J. Veenemans (Wageningen University). After obtaining her medical degree, she began her PhD study in March 2011 described in this thesis under supervision of Dr. J.E.M.M. de Klein (Clinical Genetics) and Dr. E. Kiliç (Ophthalmology). She has presented her work at several national and international meetings. In March 2014, she started her residency in Ophthalmology at the Department of Ophthalmology at the Erasmus Medical Centre, headed by Prof.dr. J.R. Vingerling.

Erasmus MC
University Medical Center Rotterdam

PHD PORTFOLIO

Summary of PhD training and teaching

Name PhD student:	A.E. Koopmans
Erasmus MC Department:	Clinical Genetics & Ophthalmology
Research school:	Medisch Genetisch Centrum zuid-west Nederland (MGC)
PhD period:	March 2011 - February 2014
Promotor:	Prof.dr. J.R. Vingerling & Prof.dr. D. Paridaens
Supervisors:	Dr. J.E.M.M. de Klein & Dr. E. Kiliç

PhD training		
	Year	**Workload (hours/ECTS)**
General courses		
Biostatistical Methods I: Basic Principles part A (CC02), 24-28 September 2012, Rotterdam (Nihes)	2012	2.0 ECTS
English Biomedical Writing and Communication, December 2012 – March 2013, Erasmus MC, Rotterdam	2013	4.0 ECTS
Specific courses (e.g. Research school, Medical Training)		
Safe Laboratory Techniques, 9 June 2011, Leiden (MGC)	2011	8 hours
Biomedical Science containing the courses: Cell and Developmental Biology, Genetics, Biochemistry and Biophysics, Rotterdam (MGC)	2011 - 2012	6 ECTS
Next Generation Sequencing, 5-7 September 2011, Leiden (MGC)	2011	24 hours
Biomedical Research Techniques X, 10-14 October 2011, Rotterdam (Molmed)	2011	1.6 ECTS
SNP course VIII, 21-25 November 2011, Rotterdam (Molmed)	2011	2.0 ECTS
Nexus Training Course, 15-16 February 2012, Rotterdam (Molmed)	2012	0.8 ECTS
Bioinformatics Road show, 8-10 May 2012, Rotterdam (Molmed)	2012	0.9 ECTS
Literature course, December – February 2013, Rotterdam (MGC)	2013	56 hours
Seminars and workshops		
Bioinformatics workshop: Browsing Genes and Genomes with UCSC, 27 May 2011, Rotterdam (Molmed)	2011	0.4 ECTS
PhD student workshop, 14-17 June 2011, Maastricht (MGC)	2011	30 hours
Open Door Workshop: Working with the Human Genome Sequence, 14-16 May 2012, Hinxton, UK	2012	24 hours
The Follow-up Workshop on Photoshop and Illustrator CS5 for PhD-students and other researchers, 16 October 2012, Rotterdam (Molmed)	2012	0.3 ECTS
Workshop 'Effective Time Management: why is it so hard?', 26 March 2013, Rotterdam (Postdoc Network Erasmus MC)	2013	4 hours
Workshop Indesign CS5, 5 June 2013, Rotterdam (Molmed)	2013	0.1 ECTS

Presentations		
Weekly scientific seminars at the department of Clinical Genetics, Erasmus MC, Rotterdam (oral presentations)	2011 - 2013	15 hours
Weekly scientific and clinical seminars at the department of Ophthalmology, Erasmus MC, Rotterdam (oral presentations)	2012 - 2013	10 hours
(Inter)national conferences		
The International Conference: From DNA to Phenotype, 9-11 March 2011, Rotterdam (Molmed)	2011	1.4 ECTS
Nederlands Oogheelkundig Gezelschap (NOG) jaarvergadering 2011, 1 April 2011, Maastricht	2011	8 hours
European Association for Vision and Eye Research and the 40th Opthalmic Oncology Group (OOG) meeting, 5-8 October 2011, Crete, Greece; poster contribution '*BAP1* in uveal melanoma'	2011	40 hours
Ocular Oncology Course and the 41st Ophthalmic Oncology Group (OOG) meeting, 7-10 March 2012, Paris, France; oral presentation 'Mutation analyses in uveal melanoma'	2012	40 hours
Nederlands Oogheelkundig Gezelschap (NOG) jaarvergadering 2012, 28-30 March 2012, Groningen; oral presentation 'Oncogene *GNAQ* en *GNA11* mutaties in uvea melanomen'	2012	30 hours
Cambridge Ophthalmological Symposium, 'Cancer and the Eye', 13-14 September 2012, Cambridge, UK	2012	16 hours
Nederlandse Vereniging voor Humane Genetica (NVHG) – Najaarssymposium, 20- 21 September 2012, Arnhem	2012	16 hours
Dutch Ophthalmology PhD Students (DOPS), 25-26 January 2013, Nijmegen	2013	12 hours
The 43rd Ophthalmic Oncology Group (OOG) meeting, 7-10 February 2013, Ivalo, Finland; oral presentation '*BAP1* in uveal melanoma: mutations and gene expression'	2013	40 hours
Nederlands Oogheelkundig Gezelschap (NOG) jaarvergadering 2013, 20-22 March 2013, Groningen; oral presentation '*BAP1* mutaties en expressie in uvea melanomen'	2013	30 hours
The Association for Research in Vision and Opthalmology (ARVO) congress, 5-9 May 2013, Seattle, USA; poster presentation: '*BAP1* mutations in uveal melanoma'	2013	40 hours
Nederlandse Vereniging voor Humane Genetica (NHVG) – Najaarssymposium 'From Genome to Phenome', 3-4 October 2013, Arnhem; poster presentation '*BAP1* mutations in uveal melanoma'	2013	20 hours
The 45th Ophthalmic Oncology Group (OOG) meeting, 7-9 March 2014, Krakow, Poland; oral presentation '*SF3B1* and *EIF1AX* mutations in uveal melanoma protective or not?' (winner book prize for the best presentation)	2014	40 hours
Nederlands Oogheelkundig Gezelschap (NOG) jaarvergadering 2014, 26-28 March 2014, Maastricht; oral presentation '*SF3B1* en *EIF1AX* mutaties in oogmelanomen met een gunstig prognostisch profiel'	2014	30 hours

Teaching

	Year	Workload (Hours/ECTS)
Lecturing		
Analyst education Dept. of Clinical Genetics, 22 March 2012	2012	3 hours
Lecture 1st year Biology & Medical Laboratory Research students, 14 November 2013	2013	1 hour
Supervising practicals and excursions, Tutoring		
Junior Med School, lecturing high school students about FISH fluorescent microscope, 19 August 2011, Erasmus MC	2011	3 hours
Supervising Master's theses		
Mentor 4th year technical analyst student Mandy Mulder: 'Validatie Next Generation Sequencing met behulp van Sanger sequencing'	2012 - 2013	10 months
Mentor 4th year technical analyst student Mike van den Berg: 'HaloPlex as a method to sequence uveal melanoma-related genes in tumor DNA'	2012 - 2013	7 months
Supervising 4th year technical analyst student Thierry van den Bosch: 'Chemokine receptor CCR7 expression predicts poor outcome in uveal melanoma and relates to liver metastasis whereas expression of CXCR4 is not of clinical relevance'	2012 - 2013	7 months
Supervising 6th medical student Serdar Yavuzyigitoglu: '*SF3B1* mutations are associated with metastasised disomy 3 tumors in uveal melanoma'	2013	4 months

ACKNOWLEDGEMENTS

DANKWOORD

DANKWOORD

Met trots en voldoening schrijf ik dit dankwoord. Ik begon deze promotietraject om mijn horizon te verbreden en onderzoek in het laboratorium leek mij hier de uitgelezen kans voor. Het was een leerzame, leuke en soms ook minder leuke periode waarin ik mezelf beter heb leren kennen. Ik denk met veel plezier terug aan alle mooie ervaringen en contacten die ik heb opgedaan. Mijn dank gaat uit naar iedereen die direct of indirect betrokken was met het onderzoek.

Allereerst wil ik graag mijn co-promotoren dr. J.E.M.M. de Klein en dr. E. Kiliç bedanken. Beste Annelies, je hebt me veel geleerd over genetisch onderzoek. Dankzij jouw kennis en doeltreffendheid werd ik uitgedaagd en bijgestuurd waar nodig. Door te mogen werken in het lab, heb ik de kans gekregen om de moleculaire kant van de patiënt te ontdekken. Ik wil je van harte bedanken voor je begeleiding en betrokkenheid de afgelopen jaren.
Beste Emine, jij bent een voorbeeld voor mij en vele anderen. Je enorme veelzijdigheid aan kennis op zowel oogheelkundig, genetisch als basaal moleculair vlak en je *straight forward approach* heeft mij veel bijgebracht. Je liet me vrij waar nodig en stond altijd klaar als ik in de avonduurtjes of weekenden prangende vragen had. Mijn dank is groot.

Mijn promotoren, prof.dr. J.R. Vingerling en prof.dr. D. Paridaens. Beste Hans, ik waardeer het zeer dat je me het afgelopen jaar de kans gaf om de oogheelkunde opleiding te combineren met het afronden van mijn promotie. Beste Dion, hartelijk dank voor je betrokkenheid en advies gedurende het project en je klinische blik tijdens de ROMS vergaderingen. Het oogmelanoomonderzoek is een mooie samenwerking tussen het Erasmus MC en Het Oogziekenhuis Rotterdam.

De leden van de kleine commissie prof.dr. R.M.W. Hofstra, prof.dr. S. Sleijfer en prof.dr. S. Seregard dank ik allen dat u zitting hebt willen nemen in de leescommissie en dat u het manuscript binnen korte tijd heeft willen beoordelen. Prof.dr. S. Seregard, thank you for taking part in the inner doctoral committee and reviewing my manuscript in the short amount of time that you were given.

De hooggeleerde Prof.dr. E.C. Zwarthoff en Prof.dr. A.C. Moll wil ik bedanken voor het deelnemen in mijn grote commissie.

Dr. R.M. Verdijk, beste Rob, de pathologie is een essentieel onderdeel binnen het oogmelanoomonderzoek. Bedankt voor je enthousiasme, hulp en vooral voor het beoordelen van de

vele immunohistochemische coupes voor de BAP1, CCR7 en TERT projecten. Alle andere helpende handen bij de pathologie, betrokken bij de opvang en verwerking van de “ogen” bedankt. Daarnaast wil ik Ellen Zwarthoff en Irene Lurkin danken voor de samenwerking betreffende het TERT artikel. Voorgangers dr. N.C. Naus en dr. H.W. Mensink, beste Nicole en Hanneke, dank voor jullie betrokkenheid en interesse. Balie 2 van de poli Oogheelkunde, bedankt voor het prikken van alle bloedjes en natuurlijk bedank ik ook alle patiënten die mee hebben gewerkt aan het onderzoek.

Collega’s van de afdelingen Klinisch Genetica en Oogheelkunde: Bert, Cindy, Daphne, Elizabeth, Erwin, Hannie, Ilse, Marjan en Tom. Dank voor jullie behulpzaamheid en gezelligheid niet alleen op het lab of achter de pc (want tegenwoordig wordt steeds meer praktisch lab werk vervangen door computer data-analyse) maar ook tijdens de vele lunches en congressen. Bert, bedankt voor je enthousiasme en kritische feedback waar nodig en Tom, fijn dat je elke keer weer klaar stond om mij te helpen met m’n NGS data. Kamergenoot van het eerste uur, Jolanda, dank voor je gezelligheid, kennis, rust en het zijn van een stabiele factor binnen het oogmelanoomonderzoek. Andere (oud)kamergenootjes van 16.10: Esmay, Jackelien, Jan-Roelof, Magda, Serdar en Thomas. Het was een gezellige tijd op de 16e waar ik mij van het begin af aan thuis voelde. Magda, dank voor alle handige computertips! Jan-Roelof, jij kon het weekend altijd goed muzikaal inluiden op vrijdag, zouden ze ook moeten toepassen in de kliniek :-)… Jackelien, ik kon altijd goed met je lachen; succes met de laatste loodjes. Opvolgers Serdar en Kyra, heel veel succes met het voortzetten van het oogmelanoomonderzoek! Andere oogheelkunde onderzoekers: Frea, Gabriëlle, Henriët en Virginie. Ik heb het erg naar mijn zin gehad in Seattle!

Een andere afdeling waar ik heel wat uurtjes heb doorgebracht is het lab op de 9^{e} verdieping. Bianca, Herma, Jeanette, Josha, Leontine, Liesanne, Marialuisa, Marian, Marianne, Rachel, Renate en Ronald, dank voor jullie interesse en hulp waar nodig. Guido Breedveld, bedankt voor alle nuttige uitleg over PCR en Sanger sequencing vooral in het begin van mijn onderzoek. Mark Nellist, ik waardeer je inbreng in het BAP1 en HaloPlex artikel. Prof.dr. R. Willemsen, beste Rob, dank voor het nakijken van de zebravissen subsidieaanvraag.

Ook de hulp van de diagnostiek afdelingen op de 20^{e} en 24^{e} waardeer ik erg. Robert van der Helm en Guido Breedveld, bedankt voor het arraywerk. Joan Kromosoeto en Marjon Slegtenhorst, dank voor jullie hulp met het programma SeqNext. Berna Beverloo en Marjan van Til, bedankt voor het beoordelen van de FISH resultaten in eerdere perioden van het onderzoek.

De afgelopen jaren heb ik voor het NGS gedeelte samengewerkt met de afdeling Biomics onder leiding van dr.ir. W.F.J. van IJcken. Ik wil het gehele team van Biomics bedanken voor alle hulp, inzet en geduld (als ik keer op keer weer binnenstormde om iets vragen) en met name Rutger Brouwer en Christel Kockx.

Er zijn meerdere studenten voorbijgekomen de afgelopen jaren. Alice, Farzia, Mandy, Mike en Thierry bedankt voor jullie inzet; het was ontzettend leerzaam om studenten te mogen begeleiden en geeft ook beter inzicht in je eigen werkwijze… Succes met jullie volgende stappen!

Ik wil graag alles assistenten, oud-assistenten en stafleden van de poli Oogheelkunde van het Erasmus MC enorm bedanken. Nieuwe collega AIOSen: Dominiek, Laurens, Niki, Wishal, Tim, King, Özlem, Ruben, Gijsbert, Aron en Gabriëlle. De overgang van het lab naar de kliniek was even wennen; dank voor jullie begrip en support. Nicole van Basten, bedankt voor alle regelzaken het afgelopen jaar.

Een speciaal woordje wil ik richten aan mijn paranimfen. Jolanda, dank voor je hulp en vertrouwen in een goede afloop van dit boekje en een luisterend oor. Je zorgde er altijd voor dat ik me nadien weer vol moed in het schrijfwerk kon storten. Ik hoop dat we elkaar nog vaak zullen spreken! Kim, alweer 12 jaar geleden ontmoetten wij elkaar tijdens de studie Biomedische Wetenschappen, vrij snel werden we ook huisgenootjes en kort daarop verhuisden we beiden naar Rotterdam voor de opleiding Geneeskunde. Ik waardeer je humor en eerlijkheid, en voel me vereerd dat je mij ter zijde wilt staan.

Naast alle collega's, oud-collega's en onderzoekers, ben ik dank verschuldigd aan mijn vrienden die ik de afgelopen jaren toch wel een beetje heb verwaarloosd…Mijn *medic chicks* vriendinnetjes: Jorie, Lalini, Margaux, Margreeth, Michelle, Sanne, Wendelien en Wendy. Ondanks dat ik het afgelopen jaar wat minder van me heb laten horen, werden er gelukkig altijd wel etentjes en weekendjes georganiseerd waarbij ik kon aansluiten. Hier geniet ik enorm van. Aster, Adinda en Maaike, wat ben ik blij dat ik jullie heb leren kennen! Jullie vriendschap en vertrouwen in mij, betekenen heel veel voor me.

Mijn familie – ooms, tantes, neven en nichten – en natuurlijk mijn schoonfamilie (in het bijzonder Dick en Wies) dank ik voor hun interesse in de voortgang van mijn onderzoek.

Helen en Debby, wat ben ik bevoorrecht met zulke geweldige “kleine” zusjes! Altijd zijn jullie er voor mij en ik zal er altijd voor jullie zijn. Lieve pap en mam, ik prijs mezelf gelukkig dat jullie mijn ouders zijn. Jullie hebben mij geleerd door te zetten, ook als het even niet gaat zoals je wilt. Jullie steunen mij altijd onvoorwaardelijk en ik ben jullie hier erg dankbaar voor.

Lieve Jens, hoe jij me de afgelopen jaren gesteund hebt maar tegelijk ook ruimte gegeven hebt bewijst maar weer eens waarom we zo goed bij elkaar passen. Ik heb je lief, en ik dank je voor dat je mijn leven op alle bedenkbare manieren zoveel leuker maakt.