GENETIC ABNORMALITIES IN UVEAL MELANOMA

Genetische afwijkingen in oogmelanomen

Nicole Corine Naus

ACKNOWLEDGEMENTS

This study was supported by grants from The Dutch Foundation Fighting against Blindness. The Rotterdam Blindness Foundation, The Hague Ophthalmic Foundation and the Revolving Fund of the University Hospital Rotterdam

Genetic abnormalities in uveal melanoma.
N.C. Naus
Thesis Rotterdam

Printed by: Print Partners Ipskamp, The Netherlands
Cover design: Michiel Postema

GENETIC ABNORMALITIES IN UVEAL MELANOMA

Genetische afwijkingen in oogmelanomen

Proefschrift

Ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de Rector Magnificus Prof.dr.ir. J.H. van Bemmel
en volgens besluit van het College voor Promoties

de openbare verdediging zal plaatsvinden op
woensdag 25 april 2001 om 11.45 uur

door

Nicole Corine Naus

geboren te Oss

PROMOTIECOMMISSIE

Promotor	Prof.dr. D. Bootsma
Overige leden	Prof.dr. G. van Rij Prof.dr. A. Geurts van Kessel Prof.dr. P.T.V.M. de Jong
Co-promotoren	Dr. A. de Klein Dr. G.P.M. Luyten

Celui qui disperse ses regards sur tout
ne voit rien ou voit mal

Denis Diderot

Voor pa en ma
Voor Michiel

CONTENTS

ABBREVIATIONS

ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia related
BRCA2	hereditary breast and ovarian cancer gene type 2
CDK	cyclin dependent kinase
CDKN2A	cyclin dependent kinase inhibitor 2a
CGH	comparative genomic hybridisation
Chk2	checkpoint kinase 2
DNS	dysplastic naevus syndrome
EORTC	European Organisation of Research and Treatment of Cancer
FAK	focal adhesion kinase
FAMMM	familial atypical multiple malignant mole syndrome
FISH	fluorescent *in situ* hybridisation
FNAB	fine needle aspiration biopsy
ICAM	intercellular cell adhesion molecule
kD	kilo Dalton
LOH	loss of heterozygosity
MDM2	murine double minute 2
MMAC	mutated in multiple advanced cancers
MTS1	multiple tumour suppressor gene
NCAM	neural cell adhesion molecule
$p14^{ARF}$	protein of 14kD, alternative reading frame of INK4A
$p16^{INK4a}$	protein of 16 kD, inhibitor of the cyclin dependent kinase 4A
PDK1	phosphonositide-dependent kinase 1
PI3-kinase	phosphatidylinositol 3-OH kinase
PtdIns(*x*,*y*)P	phosphatilyd insolitol *x*,*y* phospohate
PTEN	phosphatase and tensin homolog
RB	retinoblastoma gene
SKY	spectral karyotyping
TEP1	TGFβ-regulated and epithelial cell-enriched phosphatase 1
TNF	tumour necrosis factor
UV	ultraviolet light

CHAPTER

GENERAL INTRODUCTION

1.1 CLINICAL ASPECTS OF UVEAL MELANOMA

1.1.1 Epidemiology

Melanocytic tumours are believed to arise from the neural crest-derived melanocytes. Five to twelve percent of all melanomas are located in the eye, making it, after the skin, the second most common site of melanomas (Egan et al., 1988; Chang et al., 1998). Uveal melanoma is the most common form of primary eye cancer in adults, affecting approximately 7 per million people in the Western world each year (Egan et al., 1988). In persons over 20 years of age, melanoma is the reported diagnosis in 80% of all primary intraocular cancers. Most uveal melanomas are located in the ciliary body and/or the choroid (23% and 72%, respectively), whereas iris melanomas account for only a small percentage of these tumours (5%) (Yanoff and Fine, 1989). The majority of iris melanomas consist of non-metastasising lesions and will not be discussed in this thesis.

Although less than 2% of uveal melanoma patients have clinically detectable metastases at presentation, 50% of all patients ultimately die of metastases, predominantly located in the liver. The median survival time after discovery of the primary tumour is 6.5 years (McLean, 1993), and peaks between the second and the fourth year (Zimmerman et al., 1978). The 5, 10 and 15 year survival rates based on tumour related mortality are 72%, 59% and 53% respectively (Gamel et al., 1993; McLean and Gamel, 1998). As of yet, no effective treatment is available once metastatic disease has developed and the survival time after its diagnosis is limited to two to seven months (Seddon et al., 1983; Kath et al., 1993).

1.1.2 Therapy

Until recently, the standard therapy for uveal melanomas was enucleation of the tumour-containing eye. In the last three decades, a trend towards conservative treatment modalities has evolved. It has now become the first choice of treatment. The treatment the patient receives is based on specific findings with regard to tumour characteristics, visual acuity of the contralateral eye, the wishes and general health of the patient and the professional opinion of the ophthalmologist. Melanomas, greater than 12 mm in thickness and 16 mm in diameter, are generally treated with enucleation. Small and medium sized uveal melanomas (less than 12 mm thick and a largest basal diameter under 16 mm) can be treated with radiation therapy (proton beam, brachytherapy with or without transpupillary thermotherapy or stereotactic radiotherapy) and enucleation, or it can be decided to simply observe the tumour for further growth. The most effective treatment for these small and medium sized melanomas remains under discussion.

Currently, metastatic uveal melanoma is considered to be an incurable disease. As is reviewed by Woll (Woll et al., 1999), most clinical trials studying the impact of systemic therapy on metastatic uveal melanoma used only a limited number of patients and

therefore, a clear-cut statement of drug efficacy is difficult to make. However, patients with metastatic disease limited to the liver may benefit from chemo-embolisation with cisplatin, intra-arterial administration of carboplatin and fotemustine or hyperthermic perfusion with tumour necrosis factor (TNF) plus melphalan (Cantore et al., 1994; Bedikian et al., 1995; Leyvraz et al., 1997; Alexander et al., 1998). The ocular oncology groups of the European Organisation of Research and Treatment of Cancer (EORTC) are currently studying the effects of adjuvant therapies and new treatment protocols for metastatic uveal melanoma. In selected patients with solitary metastases, surgical excision has been reported to yield long-lasting control of local disease (Aoyama et al., 2000).

1.1.3 Predisposing factors

Certain conditions may predispose patients to uveal melanoma. Individuals with light coloured eyes, for example, have been found to be at greater risk for uveal melanoma than those with brown eyes (Gallagher et al., 1985; Tucker et al., 1985). Ultraviolet light (UV) exposure has also been suggested to increase the risk of uveal melanoma (Tucker et al., 1985; Holly et al., 1990). However, some investigators have found no increase of the incidence of uveal melanoma in the last 40 years. This is in contrast to cutaneous melanoma whose occurrence increased and with which a clear association between UV has been demonstrated. Also, no higher incidence in the most light-exposed areas of the eye was observed (Dolin et al., 1994; Dolin and Johnson, 1994; Schwartz et al., 1997) although a recent publication of Li et al. suggests a correlation between patterns of tumour initiation and solar light on the retinal sphere (Li et al., 2000). On a molecular level, no evidence has been found which supports the role of UV exposure in the development of uveal melanoma, i.e. no N-ras mutations have been discovered in uveal melanoma, in contrast to cutaneous melanoma (Mooy et al., 1991). Therefore, the association of UV and uveal melanoma remains controversial. Other predisposing factors may be ethnic background, as white individuals have an 8 times higher risk of developing uveal melanoma than individuals of African descent, and a three times higher risk than Asians (Woll et al., 1999). However, since ethnic background is correlated to eye colour, it remains unclear if both parameters are independent markers. Furthermore, factors such as socio-economic status, smoking and occupation have been postulated as to having an effect, but a causative agent has yet to be recognised.

A condition, which is known to increase the risk of uveal melanoma, is the oculodermal melanocytosis, also known as the nevus of Ota. Dysplastic nevus syndrome (DNS), neurofibromatosis, Li-Fraumeni syndrome and the BRCA2 linked breast and ovarian cancer syndrome have also been suggested to be associated with uveal melanoma.

Oculodermal melanocytosis is a congenital condition characterised by hyperpigmentation of the episclera, uvea and ipsilateral eyelid or the scalp. Although only 2% or less of all uveal melanomas are linked to oculodermal melanocytosis, the correlation between the two is well recognised (Gonder et al., 1982). It is estimated that oculodermal melanocytosis occurs approximately 35 times more often in uveal melanoma patients than in the normal population. In our study population of 80 uveal melanoma patients we found one patient with concurrent oculodermal melanocytosis

and uveal melanoma. This individual had a chromosome 9p21 deletion, suggesting the involvement of the $p16^{INK4a}$ gene (see chapter 1.2.2 and 8).
Individuals with DNS are known to be at increased risk of developing cutaneous melanoma and are frequently observed in familial melanoma kindreds. Uveal and conjunctival nevi both occur more commonly in patients with DNS and in patients with uveal melanoma more dysplastic nevi and cutaneous melanoma are observed than expected in the general population (Bataille et al., 1995; van Hees et al., 1998).
Another condition possibly associated with uveal melanoma is neurofibromatosis type 1 (Specht and Smith, 1988; Antle et al., 1990). This autosomal dominant multisystem disorder affects neural crest derived cells and causes an excess of melanocytes. However, within in our database containing more than 700 uveal melanoma patients, no patient suffering from neurofibromatosis type 1 was observed.
BRCA2 (hereditary breast and ovarian cancer gene type 2) linked breast and ovarian cancer has been theorised to be associated with uveal melanoma (Easton et al., 1997). Sinilnikova et al. investigated a series of 62 ocular melanoma patients with a family history of ocular melanoma or a family or personal history of breast and ovarian cancer. In 3 of their cases germline mutations in BRCA2 were detected to be likely associated with the cancer syndrome (Sinilnikova et al., 1999): two of these patients had a personal history of breast cancer and one had multiple relatives affected with breast cancer and ocular melanoma.
Furthermore, the autosomal dominant Li-Fraumeni syndrome predisposes patients to soft tissue sarcoma, brain tumours, leukemia, breast and other cancers. The presence of breast carcinoma in a family with uveal melanoma suggests the association between the Li-Fraumeni and uveal melanoma (Jay and McCartney, 1993). However, it remains unestablished whether this family is a true Li-Fraumeni family since no mutation analyses were performed.

1.1.4 Prognostic factors

Research on uveal melanoma has mainly been concentrated on the identification of prognostic factors for development metastatic disease . These prognostic factors can be subdivided into clinical, histopathological and genetic parameters.
Clinical markers predicting tumour growth and metastases in small uveal melanoma are described by Shields et al (Shields et al., 1995; Shields et al., 2000). A mnemonic, To Find Small Ocular Melanoma, was constructed to assist clinicians in detecting early choroidal melanoma. These factors for growth include: Thickness larger than 2 mm, subretinal Fluid, Symptoms, Orange pigment and Margin at the disc. Risk factors for metastases were analogous to those of tumour growth: thickness larger than 2 mm, location at the optic disc, symptoms and demonstrated growth. Additional clinical prognostic parameters include tumour margins anterior to the equator of the eye (Seddon et al., 1983), older age, the male gender (Folberg et al., 1993; Luyten et al., 1995) and tumour-induced glaucoma (Coleman et al., 1993).
The most consistent of the histopathological markers is the largest tumour diameter, in particular, the diameter which is in contact with the sclera (reviewed by Mooy and De Jong, 1996). Furthermore, an association between scleral invasion (Seddon et al., 1983), the standard deviation of the nuclear area (Coleman et al., 1996) and the presence of

epithelioid cells (Seddon et al., 1983; Coleman et al., 1993) with the outcome of the patient has been described. In the early nineties, Folberg et al. (Folberg et al., 1993) suggested that the presence of vascular networks, defined as at least three back to back closed vascular loops, is strongly associated with fatal metastatic melanoma. The relevance of this prognostic marker was also confirmed by others (McLean et al., 1997; Makitie et al., 1999). Furthermore, it was demonstrated that patients had a better survival if their tumour did not express HLA class 1 (Blom et al., 1997). Other histopathological markers have also been suggested in isolated reports, such as the expression of epidermal growth factor receptors (Hurks et al., 2000), the expression of the cell adhesion factors ICAM (intercellular cell adhesion molecule) (Anastassiou et al., 2000) and NCAM (neural cell adhesion molecule) (Mooy et al., 1995) and cyclin D1 (Coupland et al., 2000). However, the independent value of these parameters has yet to be confirmed and evaluated in larger studies. These factors all seem to correlate with the outcome of uveal melanoma patients. Nevertheless, predicting the outcome for an individual patient still remains difficult.
In 1996, it was shown that loss of chromosome 3, in comparison to tumour location or tumour diameter, is a more reliable prognostic parameter for decreased relapse-free and overall survival (Prescher et al., 1996). The chromosomal abnormalities and the prognostic relevance will be further discussed in paragraph 1.2.1 and chapter 2.

1.1.5 Fine needle aspiration biopsy

Uveal melanoma is an exceptional tumour in which the type of treatment is based solely on clinical judgement, without requiring a histopathological diagnosis. In most cases, this diagnosis can be reliably made based on the clinical findings, but more atypical cases challenge an accurate diagnosis. In these cases confirmation of the diagnosis using cytology is advised.
The first needle biopsies on solid ophthalmic tumours were performed in the late 19th and early 20th century. In the late 1950s, occasional reports of uveal melanoma diagnosed with 20 to 24 gauge needle biopsies were published (Veasey, 1951; Kauffman, 1952; Long et al., 1953; Makley Jr., 1967). However, the use of these large needles resulted in unacceptable morbidity and local tumour seeding, leading the technique to be abandoned in most centres by the end of the decade (Jensen and Andersen, 1959). Jakobiec et al. reintroduced the use of needle biopsies for solid intraocular tumours in 1979, by reporting on six cases examined by fine needle aspiration biopsies (FNABs) (Jakobiec et al., 1979). The FNAB method using 25 gauge needles and, in particular, the transvitreal biopsies have now proven to be safe and useful in diagnosing uveal melanoma. No severe ocular morbidity or tumour spread has been demonstrated using this technique (Glasgow et al., 1988; Shields et al., 1993; Char et al., 1996).
In cases of irradiation, no tumour tissue is available for histopathological evaluation. Most clinical prognostic markers can, in these cases, still be used to select patients with a high risk of metastases. The tumour diameter can also be measured *in vivo* using ultrasound methods. However, prognostic markers such as genetic changes, vascular patterns and cell type cannot be assessed when irradiation takes place. We used fluorescent *in situ* hybridisation (FISH) analysis on FNABs of enucleated eyes to investigate whether it could detect chromosomal changes in radiotherapy cases. This

method proved itself to be very reliable in *ex vivo* biopsies (chapter 4). Since these biopsies were taken under ideal conditions, a larger prospective study on *in vivo* biopsies has to be performed to confirm these data and to enable a translation of these data for clinical situations.

1.2 GENETIC BACKGROUND OF UVEAL AND CUTANEOUS MELANOMA

1.2.1 Cytogenetic findings

Cytogenetic and molecular genetic studies of leukemias provide important prognostic information and are thereby becoming the major tool in determining the appropriate treatment protocol. Furthermore, they have made a significant contribution to our understanding of the different haematological malignancies (Sandberg and Chen, 1994; McKenna, 2000). In contrast, data from the cytogenetic analysis on solid tumours are severely restricted due to the technical difficulties associated with obtaining good quality metaphase spreads for the analysis and due to the complexity of the tumour.
However, uveal melanomas were found to be more amenable to cytogenetic analysis than other solid neoplasms. From the late eighties on, several reports on the cytogenetic analysis of uveal melanoma have been published. It was found that in these tumours, monosomy 3, abnormalities of chromosome 6 and gain of chromosome 8q were non-random (Table 1.1, Figure 1.1).

TABLE 1.1 OVERVIEW OF CHROMOSOMAL ABERRATIONS (%)

	NO.	METHOD	LOSS OF 1P36	LOSS OF 3	GAIN OF 6P	GAIN OF 8(Q)
Own data (chapter 2)	80	cytogenetics /FISH	34	45	30	56
Sisley et al., 1997	42	cytogenetics	29	50	19	54
Prescher et al., 1996	54	cytogenetics/CGH	nr	57	nr	nr
White et al., 1998	54	cytogenetics	nr	50	46*	48
Parella et al., 1996	50	microsatellite	33	59	28	60
Mc Namara, 1997	17	FISH	nr	12	nr	nr

*abnormalities of chromosome 6

abbreviations: nr, not reported; FISH, fluorescent *in situ* hybridisation; CGH, comparative genomic hybridisation

Furthermore, changes in chromosome 11 and the loss of chromosome 2, 21 and the sex chromosomes were reported (Mukai and Dryja, 1986; Griffin et al., 1988; Horsman et al., 1990; Prescher et al., 1990; Sisley et al., 1990; Horsthemke et al., 1992; Prescher et al., 1992; Sisley et al., 1992; Dahlenfors et al., 1993; Horsman and White, 1993; Wiltshire et al., 1993; Prescher et al., 1994; Singh et al., 1994; Prescher et al., 1995;

Prescher et al., 1996; White et al., 1996; Sisley et al., 1997; Sisley et al., 1998; White et al., 1998; Parrella et al., 1999; Sisley et al., 2000). The large amount of publications suggests that extensive evaluation on this subject has been done. However, excluding our 80 cases, only 166 cases in total have been studied with cytogenetics or FISH analysis and another 40 cases with CGH, since most of the studies referred to repeatedly used cohorts of patients.

Chromosome 3 loss was found to occur most commonly in those tumours with gain of chromosome 8. These genetic changes were found to occur in particular in tumours arising from the ciliary body (Prescher et al., 1992; Sisley et al., 1992; Dahlenfors et al., 1993; Horsman and White, 1993; Sisley et al., 2000). Moreover, Parrella et al. proposed a bifurcated tumour pathway with mutual exclusivity of the loss of chromosome 3 and gain of chromosome 6p (Parrella et al., 1999). However, in our own series, we observed several cases where both the gain of chromosome 6p and loss of 3 took place, arguing against this proposed pathway.
The changes of chromosomes 1, 3, 6 and 8 are thought to be correlated with patient survival, as is described in paragraph 1.1.4 and chapter 2 of this thesis. Prescher et al. found a strong correlation between loss of chromosome 3 and a poor prognosis of the patient (Prescher, 1996). It was found that no patient with tumours with normal copies of chromosome 3 (n=24) died within 3 years after diagnosis, whereas only 57% of the patients with tumours with loss of a chromosome 3 (n=30) survived the same interval. Sisley et al. confirmed the prognostic value of monosomy 3 and demonstrated a strong inverse correlation between the presence of extra copies of 8q and survival (Sisley et al., 1997). They showed a dosage effect relationship of additional copies of chromosome 8q and the survival of the patient. However, our own data of 80 uveal melanomas suggest that gain of chromosome 8q is not an independent prognostic marker, since it is also correlated with the largest tumour diameter, which may influence the correlation with survival. Furthermore, the tumours with gain of chromosome 8q copies appear to have a growth advantage in cell culture, since after culture an accumulation of chromosomes 8q copies can be found. A link between chromosomal changes was also studied by White et al. (White et al., 1998). They demonstrated a correlation between chromosome 3 loss and chromosome 8q gain but moreover, they also found a protective effect from chromosome 6 abnormalities over abnormalities of chromosome 3 and 8. However, the abnormalities of chromosome 6 were not further specified as gain or loss of chromosome 6p or 6q and it remains unclear which biological mechanisms could explain this finding.
As is shown in chapter 2 of this thesis, we confirmed the significance of monosomy 3 as a prognostic marker, but found no independent correlation between chromosome 6 abnormalities and gain of chromosome 8q and survival. Additionally, we found that the effect of chromosome 3 loss was determined by chromosome 1p loss: only those patients with concurrent chromosomes 1p36 and 3 losses showed a decreased disease-free survival when compared to patients with only loss of chromosome 3 or with no loss of 1p36 and 3.

In most cases, whole chromosomes or chromosome-arms are affected, hampering the identification of loci specific for uveal melanoma development or progression.

However, some chromosome regions, such as 3p13 (Blasi et al., 1999) and 8q23-24~qter, have been suggested as potential target regions (Speicher et al., 1994). Using spectral karyotyping (SKY) and comparative genomic hybridisation (CGH) analysis on uveal melanoma with complex chromosomal abnormalities, we were able to discover a small deletion of chromosome 3q13~21. Furthermore, we found two regions on chromosome 8q, i.e. 8q21.1~21.2 and 8q23~24 which could be the target sites of amplification (Naus et al., 2001).

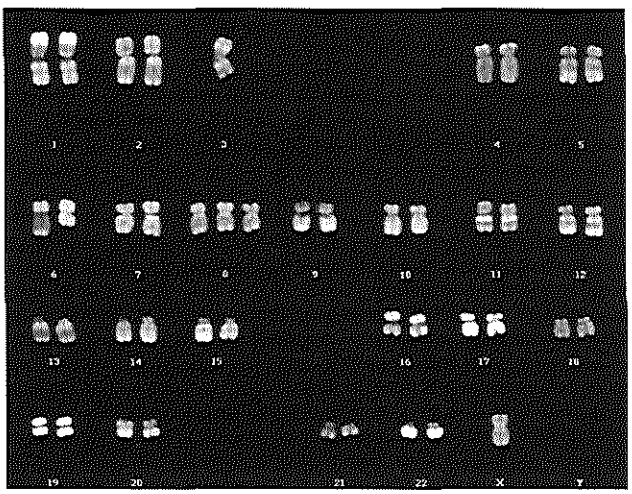


Fig. 1.1 Karyogram of tumour EOM207. This tumour showed a -3, i(6)(p) and +8, typical for uveal melanoma. Furthermore, there is a loss of the Y chromosome, a common feature in solid tumours.

In cutaneous melanoma, chromosomal abnormalities involving chromosomes 1 and 6 are also present. As is reviewed by Fountain et al. (Fountain et al., 1990), the most frequent abnormality consists of rearrangements of the distal part of the short arm of chromosome 1, occurring in about 80% of all cases. Chromosome 6 also appears to be altered frequently (66% of the cases) and the 6q11-q26 region shows the highest frequency of rearrangements as a result of deletion, translocation or due to the formation of an isochromosome of its short arm. Moreover, changes in chromosomes 7 and 9 were found to occur in 61% and 44% respectively of the cutaneous melanoma. Chromosomes 2, 3, 10 and 11 also show significant involvement, with alterations of these chromosomes being present in approximately 30% of all melanomas. In literature, only few cases of abnormal karyotypes in nevi or dysplastic nevi have been described. Changes of chromosomes 4, 8, 9 and 10 were, however, consistently found. Chromosome 9 was involved in three out of nine nevi, whereas chromosome 4, 8 and 10 changes were found in only two cases. The overall infrequent involvement of chromosome 4 and 8 in cutaneous melanoma suggests that these changes happened by chance or were actually in-vitro artefacts (Becher et al., 1983). Using microsatellite

analysis, no LOH for regions 9p21-22, 5q22, 11q23 and 17q21 could be demonstrated in 26 common nevi LOH (Birindelli et al., 2000). Based on the results of the karyotype and molecular genetic analyses of nevi and melanomas, a tumour progression model, from melanocyte to metastatic melanoma has been proposed (Figure 1.2).

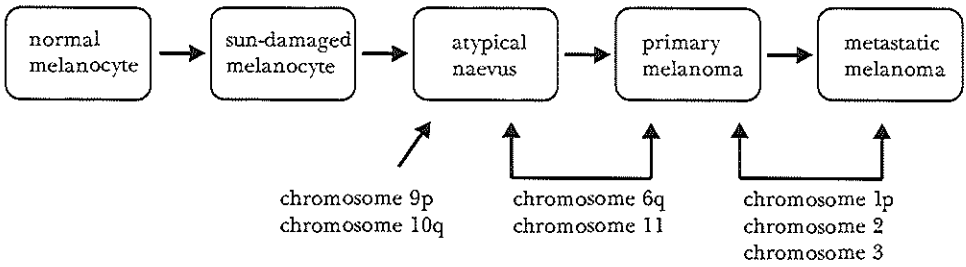


Fig 1.2 Tumour progression model for cutaneous melanoma. (Adapted from Fountain et al., 1990)

Although chromosome 1 and 6 changes are common in both cutaneous and uveal melanoma, their genetic tumour progression pathway seems to be different. Loss of chromosome 2, 9p, 10q and 11, frequently seen in cutaneous melanoma, are rarely found in uveal melanoma. On the other hand, monosomy 3 is rather specific for uveal melanoma. Furthermore, we were able to show that mutual loss of chromosomes 1p and 3 was associated with a more aggressive phenotype (chapter 2). Recent data also suggest that loss of chromosome 1p is only present in primary uveal melanoma that had metastasised and in its metastases (Aalto et al., 2001). These data point to putative tumour suppressor genes on chromosomes 1p and 3 involved in tumour progression. A tumour progression pathway is, however, difficult to propose, based on the genetic abnormalities found since no or few genetic information is available from normal uveal melanocytes, uveal nevi or suspected uveal melanoma. In three cases of suspected uveal melanoma we performed FISH analysis on FNABs (chapter 5). In one case gain of chromosome 8 was found, suggesting malignant transformation. In the other cases, no chromosomal abnormalities could be detected. Since this study was performed on only three tumours, no general conclusions can be drawn.

1.2.2 Genes

In 1971, Knudson proposed the two-hit hypothesis to explain cancer predisposition. This model still holds for most tumour suppressor genes: two successive mutations (hits), which inactivate both copies of the tumour suppressor gene, are needed for tumour formation (Knudson, 1971; Knudson, 1993).

Germline mutations of one allele of a tumour suppressor gene, such as the retinoblastoma (RB) gene, predispose one to tumour formation. A somatic mutation of the second allele subsequently results in the tumour initiation and/or progression (Figure 1.3).

Our knowledge about tumour suppressor genes has expanded rapidly since that time and it became clear the first hit often consists of a small alteration such as a deletion or a point mutation, whereas the second hit mainly consists of gross chromosomal rearrangements. The latter can be seen as recurrent losses of chromosomal loci associated with specific human cancers.

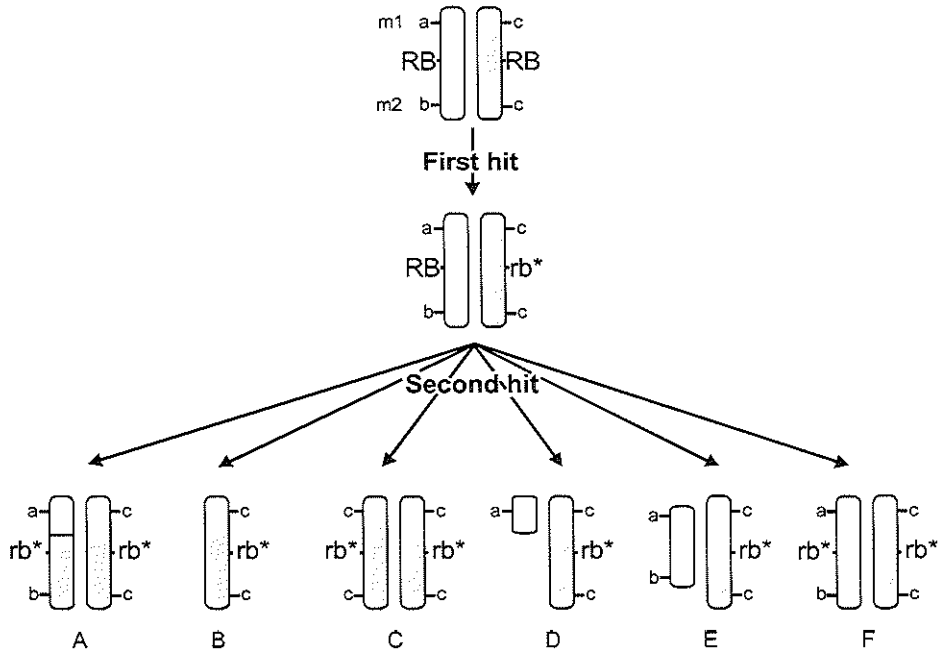


Fig. 1.3 Knudson's two hit model. RB, wildtype retinoblastoma allele; rb*, mutated allele; m1/m2, polymorphic markers at both sides of the gene; a/b/c, genotype at m1 or m2. In inherited cancers, the first hit is brought about by germline transmission of a mutated allele. The second allele can be inactivated by: A, mitotic recombination; B, loss of a chromosome; C, chromosome loss and consequent duplication; D, partial deletion; E, interstitial deletion or F, gene conversion. (adapted from (Pronk et al., 1994))

For several years now, these specific chromosomal abnormalities have been recognised in uveal melanoma (see paragraph 2.1: cytogenetic findings). However, the first hit, i.e. mutations or alterations within uveal melanoma specific genes are yet to be identified. In contrast to this, cutaneous melanomas display aberrations in specific tumour suppressor genes, such as $p16^{INK4a}$ (inhibitor of the cyclin dependent kinase 4a) and PTEN (phosphatase and tensin homologue). The role of p53 and its homologues, p63 and p73, remain to be established in both melanoma types. These genes and their biological functions will be discussed in the following paragraphs.

p16^{INK4a} and p14^{ARF} genes

The first report on the linkage of familial cutaneous melanoma to chromosome 9p21 was described in 1992 (Cannon-Albright et al., 1992). Subsequently, several studies have confirmed this result. It has been demonstrated that approximately 50% of all familial atypical multiple malignant mole (FAMMM) syndrome cases show linkage to this particular region (Goldstein et al., 1994; Gruis et al., 1995). Furthermore, cytogenetic and LOH studies revealed frequent heterozygous and homozygous losses in sporadic melanoma and melanoma cell lines (Fountain et al., 1992). This locus was found to harbour the p16^{INK4a} gene (Kamb et al., 1994), also known as MTS1 (multiple tumour suppressor gene 1) or CDKN2A (cyclin dependent kinase inhibitor 2A).

The p16^{INK4a} gene is composed of three exons and encodes a 156 aminoacid protein of 16kD. Its protein is a cell-cycle inhibitor, which can bind to complexes of cyclin-dependent kinases (CDK) 4 and 6 and cyclin D1. This binding inhibits the kinase activity and thus results in an arrest of the cell cycle in the G1 phase, thereby preventing abnormal cell growth and proliferation (Figure 1.4).

Homozygous loss, mutation or methylation of the p16^{INK4a} promotor region, results in an inefficient or absent binding to the CDKs. This enables damaged cells to progress throughout the cell cycle. The importance of the cell cycle regulatory function of the p16^{INK4a} protein is illustrated by a wide variety of tumours in which this gene is altered, such as gliomas, bile duct, oesophageal, pancreas and bladder carcinomas and some subtypes of leukemia (Ruas and Peters, 1998).

The 9p21 locus also harbours a *p16^{INK4a}*-related gene: the *p14^{ARF}* (alternative reading frame of *p16^{INK4a}*) gene. This gene uses an alternative exon 1 (exon 1β), located 10-20 kb upstream of exon 1α and different open reading frames for exons 2 and 3, resulting in a structurally different protein (132 amino acids). The gene has been designated the *p14^{ARF}* in human cells because of its predicted mass and *p19^{ARF}* in mouse cells.

The ARF protein binds to MDM2 (mouse double minute 2 gene), whose function is to bind p53 (see below) and target it for degradation via the ubiquitin pathway. The binding of ARF to MDM2, prevents p53 degradation (Figure 1.4) thereby can p53 exert its functions, such as initiation of apoptosis. Furthermore, p53 is also known to play a role in genome stability maintenance and it can target p21^{WAF1}. P21^{WAF1} is able to inhibit CDKs thereby interacting with the RB pathway and resulting in a G1/S arrest. P53 is considered to be the guardian of the genome and will be discussed below.

In contrast to the *p16^{INK4a}* gene, no mutations which selectively target exon 1β of *p14^{ARF}* are found in familial or sporadic cutaneous melanoma (FitzGerald et al., 1996; Fargnoli et al., 1998; Peris et al., 1999), although such mutations have been described in melanoma cell lines (Kumar et al., 1998). Recently, however, evidence was found that specific loss of the exon 1β was involved in familial cases of melanoma-neural system tumour syndrome (Randerson-Moor et al., 2001).

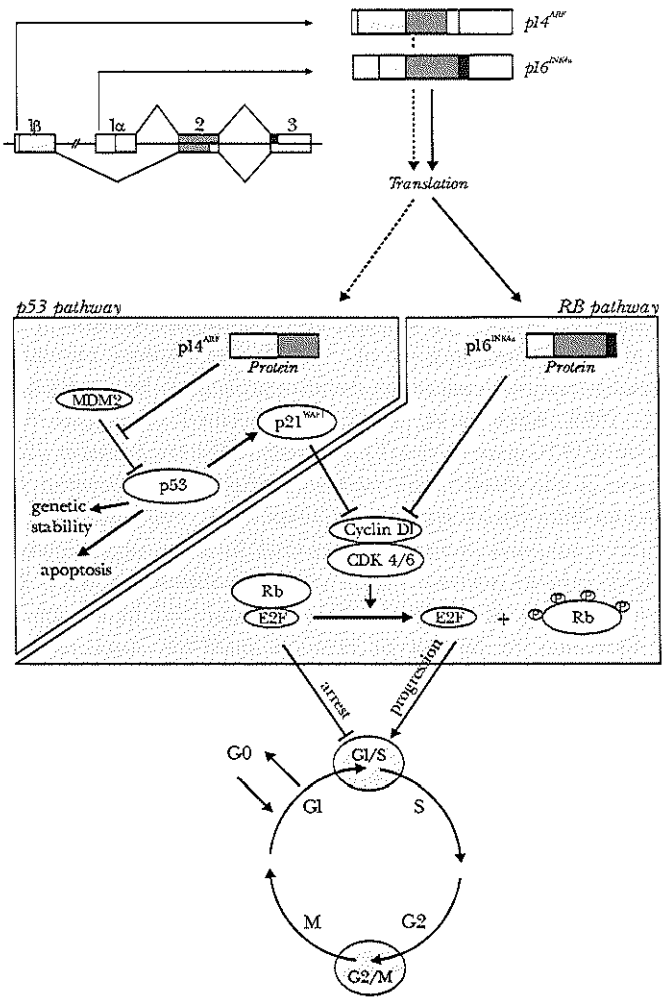


Fig. 1.4 Functions of $p16^{INK4a}$ and $p14^{ARF}$. Upper diagram: alternative transcripts are generated from the 9p21 locus. Exons are displayed as boxes and identified as exons 1β, 1α, 2 and 3. White boxes represent the untranslated region. Exon 1 is represented in light grey, exon 2 in dark grey and exon 3 in black. Middle diagram (grey): the p53 and RB pathways. Important key players are depicted in circles. Both pathways are linked via $p21^{WAF1}$, which inhibits cyclin dependent kinases. Lower diagram: cell cycle. S (synthesis), DNA replication; G1 and G2 (gap), cell growth and preparation for entering next phase; M (mitosis), cell division. G1/S and G2/M transition checkpoints are represented by grey circle.

We (chapter 7) and others (Ohta et al., 1994; Singh et al., 1996b) have shown that, in contrast to cutaneous melanoma, homozygous deletions and mutations of the $p16^{INK4a}$ gene rarely occur in uveal melanoma. However, whereas methylation of the promotor is

rare in cutaneous melanoma, we found methylation in four out of nine uveal melanoma cell lines. Normal expression of *p14ARF* was found in all cell lines tested.

PTEN gene

PTEN, also known as MMAC (mutated in multiple advanced cancers) or TEP-1, (TGFβ-regulated and epithelial cell-enriched phosphatase 1) is located on chromosome 10q23. It is a tumour suppressor gene which is often mutated or lost in several different tumour types, such as glioblastomas (Wang et al., 1997), prostate carcinomas (Cairns et al., 1997) breast carcinomas (Li et al., 1997) and endometrial carcinomas (Risinger et al., 1997). Furthermore, in 40% of the sporadic cutaneous malignant melanoma cell lines it is also mutated (Guldberg et al., 1997), but reports on the incidence of mutations in primary sporadic cutaneous melanoma seem to be conflicting (Boni et al., 1998; Tsao et al., 1998; Birck et al., 2000). As described in chapter 8, we found no abnormalities of the PTEN gene in uveal melanoma cell lines (Naus et al., 2000).
Germline mutations of the PTEN gene were identified in the Cowden and Bannayan-Zonana or Ruvalcaba-Riley-Smith syndrome (Nelen et al., 1997; Marsh et al., 1998). The phenotypes of these syndromes include hamartomas in many organ systems, such as the breast, thyroid, skin, central nervous system and gastrointestinal tract. Unlike Cowden syndrome no increased risk of malignancies is documented in the Bannayan-Zonana syndrome. This difference in phenotype remains puzzling, since identical mutations have been associated with both Cowden and Bannayan-Zonana syndromes. It might be that differences in other, as of yet unidentified, disease-modifying genes cause this variance in phenotype.
The PTEN protein is a dual-specific phosphatase which is able to dephosphorylate proteins (serine/threonine and thyrosine) and phospholipids such as phosphatidylinositol 3,4,5 triphosphate (PtdIns(3,4,5)P3) and phosphatidylinositol 3,4 biphosphate (PtdIns(3,4)P2) residues (Figure 1.5). PTEN counteracts phosphatidylinositol 3-OH kinases (PI3-kinases) functions, which are associated with cell growth and survival. PI3-kinases are activated by stimulation by several growth factors and phosphorylate PtdIns(4,5)P and PtdIns(4)P residues. Upon phosphorylation of these residues, other proteins, which associate with the phospholipids, such as AKT, are recruited. AKT, in turn, is phosphorylated by phosphonositide-dependent kinase 1 (PDK1) and is able to stimulate cell growth. By dephosphorylating PtdIns(3,4,5)P3 and PtdIns(3,4)P2, PTEN works in opposition to the PI3-kinase/AKT pathway, and thus functions as a growth suppressor (Di Cristofano and Pandolfi, 2000; Vazquez and Sellers, 2000). Additionally, PTEN plays a major role in inhibiting cell spreading and the formation of focal adhesions, by dephosphorylation of focal adhesion kinase (FAK)(Tamura et al., 1998).

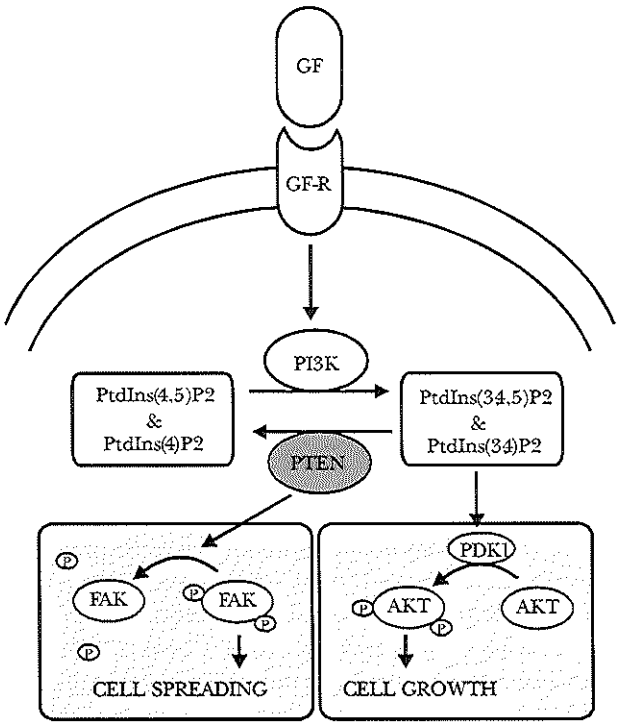


Fig. 1.5 PTEN signalling pathway. This diagram summarises the intracellular effects of PI3K activation by growth factors, which can be antagonised by PTEN. Phosphorylation of phosphatidyl inositol phosphates stimulates PDK1, resulting in phosphorylation of AKT and promoting cell growth. PTEN can also dephosphorylate FAK, hereby inhibiting cell spreading.

p53/p63/p73 genes

One of the most studied tumor-related genes is the *p53* gene, located on chromosome 17q13.1. This gene is mutated in over 50% of all human cancers. However, in cutaneous melanoma, the mutation rate seems to be low, although a recent report suggested that p53 mutation may be more common in certain types of melanomas, particularly those with a poorer prognosis (Akslen et al., 1998). In uveal melanoma, *p53* mutations do not appear to be the major cause of gene inactivation (Chana et al., 1999; Mouriaux et al., 2000), but it is possible that the *p53* pathway is functionally inactivated (Brantley Jr and Harbour, 2000).

P53 plays an important role in regulating the G1/S and G2/M transition of the cell cycle, in inducing apoptosis if the DNA damage is too severe to be repaired and in maintaining genome stability. These properties cause *p53* to be called the guardian of the genome (reviewed by Ko and Prives, 1996; Lohrum and Vousden, 2000). Normally, *p53* is not active, but it can be stimulated by different triggers:

Firstly, by DNA damage, such as that caused by ionising radiation. Double strand breaks activate ATM (ataxia telangiectasia mutated), which in turn activates Chk2 (checkpoint kinase 2).
Secondly, *p53* can be triggered by aberrant growth signals, such as those resulting from expression of RAS or MYC. In this case p53 depends on the activation via the $p14^{ARF}$ pathway.
Thirdly, by a wide range of stimuli, such as drugs and UV light. This pathway, which is independent from intact ATM or $p14^{ARF}$, may involve ATR (ataxia telangiectasia related), an ATM-related cell cycle checkpoint gene.
All pathways inhibit degradation of p53, thereby creating excess of p53. Mutations in *p53* or binding of viral proteins to p53 (in cervix carcinoma and lymphomas) can cause malfunction of p53. Alterations in genes whose products directly or indirectly interact with p53 (such as MDM2 and $p14^{ARF}$) or mislocalisation of p53 in the cytoplasm (Vogelstein et al., 2000) can also prevent a proper p53 response.
Much less is known about the *p53* homologues, *p63* and *p73*, whose structure is similar, but not identical, to that of p53. Each contains a transactivation domain (TAD), a DNA binding domain (DBD) and an oligomerisation domain. Both homologues also contain a steric alpha motif (SAM). SAM domains are known to be present in proteins involving regulation of development (Levrero et al., 2000). Unlike *p53*, both *p73* and *p63* give rise to differentially spliced mRNAs. At least 6 isoforms of *p73* and of *p63* have been identified. The isoforms of *p63* lacking exons 1 and 2 (ΔN forms) do not activate transcription, but can act as a dominant negative regulator of full length (TA)p63 and p53 proteins. *P73* can be stabilised by phosphorylation via c-Abl after treatment of cells with DNA damaging agents, such as cisplatin and taxol. This results in p73 mediated apoptosis.
P73 is located on chromosome 1p36, a region which is frequently deleted in uveal melanoma (Chapter 2) and other tumour types such as neuroblastoma, colorectal and breast carcinoma. In neuroblastoma, it was suggested that p73 is an imprinted gene, expressing only the maternal allele (Kaghad et al., 1997). However, recent studies seem to indicate that monoallelic expression of this gene is rare (Nomoto et al., 1998). Mutations in p73 are rarely found in human tumours. In the almost 900 tumours studied for mutations, including cutaneous melanoma, only three missense mutations have been found (Levrero et al., 2000).

P63 is the second *p53* related gene (also known as KET, p51 p40 and p73L) and is located on chromosome 3q27 (Yang et al., 1998). *P63* is predominantly expressed in the epidermis, cervix, urothelium and prostate and no mutations have been detected in human malignancies. However, *p63* mutations have been found in patients with ectodactyly, ectodermal dysplasia and facial clefts (EEC) syndrome. Knockout mice for *p63* also demonstrate major defects in limb, craniofacial and epithelial development (Mills et al., 1999; Yang et al., 1999), suggesting that *p63* predominantly plays a role in development.
Although it is assumed that the role of *p63* in tumorigenesis is limited, other data suggest that, in cancers, the balance between the TA and ΔN isoforms is disrupted. The ΔN isoforms of *p63* are known to have a dominant negative effect on both the TA-

isoforms of *p63* and on *p53*. As stated above, *p53* plays an important role as a controller of the G2/M and G1/S transitions and in genome stability.

No data on the involvement of the *p63* and *p73* gene in uveal melanoma are available yet. However, since these genes are both located on chromosomes frequently involved in uveal melanoma, i.e. chromosome 1p36 and 3, it will be interesting to investigate the role of these genes in uveal melanoma tumorigenesis.

1.3 AIMS AND OUTLINE OF THE THESIS

Research on uveal melanoma has largely been concentrated on the identification of prognostic markers, since it allows clinicians to select patients with a high risk of metastatic disease and to adjust the treatment protocols depending on the prognosis. I started the research on the effects of genetic abnormalities in uveal melanoma in 1996. At that time it was already known that recurrent chromosomal abnormalities, such as loss of chromosome 1p and 3 and gain of chromosome 6p and 8q, could be detected in these cancers. Furthermore, it was suggested that loss of chromosome 3 and gain of chromosome 8 were important prognostic markers for decreased survival in uveal melanoma patients.

In the first part of this thesis, the cytogenetic aberrations found in these uveal melanomas will be discussed. In our laboratory, cytogenetic analysis on uveal melanomas has been performed since 1992. Until 1995, most tumours were analysed by conventional cytogenetic analysis only. After that time, fluorescent *in situ* hybridisation and, when possible, cytogenetic analysis was performed on all tumours. We characterised a series of 80 uveal melanomas for chromosomal changes, with the aim to identify correlations between these changes and tumour characteristics, and to confirm the correlations between chromosomal changes and survival. These data might enable us to narrow down the regions of interest on chromosome 1, 3, 6 and 8. This study is described in **chapter 2.**

In some cases, however, detailed cytogenetic analysis was not possible due to the presence of complex abnormalities. In **chapter 3** we describe the application of comparative genomic hybridisation (CGH) and/or spectral karyotyping (SKY) on two uveal melanoma cell lines and five primary uveal melanomas, with partially defined and/or complex abnormalities to further characterise the chromosomal changes.

The cytogenetic studies were performed on tumours available from enucleated eyes. However, more and more patients are now treated with radiotherapy and, as a result, the lack of tumour material compromises the evaluation of these chromosomal changes. In **chapter 4**, we describe the validation of using fine needle aspiration biopsies and fluorescent *in situ* hybridisation for the analysis of genetic prognostic markers. Therefore, we analysed 40 *ex vivo* uveal melanoma biopsies and the corresponding main tumour with FISH. To investigate whether chromosome analysis was also applicable on *in vivo* fine needle aspiration biopsies, we applied FISH analysis on biopsies of three patients suspected of uveal melanoma. These data are described in **chapter 5.**

The second part of this thesis is dedicated to the detection of molecular genetic changes in uveal melanoma. As described above, non-random chromosomal changes have been described in uveal melanoma but the molecular pathogenesis remains largely unknown. In contrast to uveal melanoma, much more is known about cutaneous melanoma, where genes such as $p16^{INK4a}/p14^{ARF}$ and *PTEN* are known to play a role in tumorigenesis. Since both cutaneous and uveal melanomas originate from neural crest derived melanocytes, we decided to test these genes in uveal melanoma cell lines. To

allow experimental studies investigating the molecular changes in uveal melanoma, we established and characterised primary and metastatic uveal melanoma cell lines (**chapter 6**). We studied the involvement of the $p16^{INK4a}$, $p14^{ARF}$ and the *PTEN* gene, which are known to be involved in cutaneous melanoma, in these cell lines. These studies are described in **chapter 7 and 8** of this thesis.

PART 1

CHROMOSOMAL ABERRATIONS IN UVEAL MELANOMA

CHAPTER

2

CONCURRENT LOSS OF CHROMOSOMES 1P AND 3 PREDICTS A DECREASED DISEASE FREE SURVIVAL IN EYE MELANOMA PATIENTS

Nicole Naus[1,2], Gregorius Luyten[1], Caroline Klaver[1,3], Ellen van Drunen[2], Anette Verhoeven[2], Cornelia Mooy[4], Paul Mulder[3], Dion Paridaens[6], Rosalyn Slater[2,5], Annelies de Klein[1,5]

From the Departments of [1]Ophthalmology, [2]Cell Biology & Genetics, [3]Epidemiology and Biostatistics, [4]Ophthalmopathology, [5]Clinical Genetics, Erasmus University Rotterdam and [6]The Rotterdam Eye Hospital, The Netherlands

Submitted for publication

ABSTRACT

Background Melanoma of the eye is a highly malignant disease with a mortality of 50%. It is suggested that chromosomal changes associate with a decreased survival of the patient. However, these studies had limited samples size, restricting statistical analysis. We aimed to assess the independent value of numerical changes of chromosomes 1,3, 6 and 8 on the disease free survival in a large series of eye melanoma patients.

Methods 80 eye melanomas were analysed for numerical changes of chromosomes 1, 3, 6 and 8 with cytogenetic analysis and/or fluorescent *in situ* hybridisation. The genetic data were correlated with disease outcome in a univariate and multivariate model using Kaplan-Meyer and Cox regression analyses.

Results After a mean follow-up time of 27 months, 17 patients had died or were suffering from metastatic disease. In the univariate analysis, loss of chromosome 3, gain of 8q and the presence of epithelioid cells were associated with a decreased disease free survival. In the multivariate analysis, we found that the effect of monosomy 3 on survival was largely modified by changes in chromosome 1p. We found that, regarding all chromosomal changes, only concurrent loss of chromosome 1p and 3 was an independent prognostic parameter for disease free survival (p=0.007)

Conclusions Our findings suggest a synergistic effect of genes on chromosomes 1p and 3 involved in melanoma progression. These chromosomal changes are an independent prognostic marker for disease free survival and may have implications for the treatment of eye melanoma patients.

INTRODUCTION

Melanoma of the eye, the most common form of primary eye cancer in adults, is a highly malignant disease with a mortality rate of fifty percent (Egan et al., 1988). Identification of patients at high risk of mortality may enable early detection of metastases in a stage in which adjuvant therapy may be justified. Chromosomal abnormalities are known to have prognostic importance in certain cancers, e.g. in leukemia patients (Dohner et al., 2000). This helps clinicians to plan treatments and alerts them to patients with a high risk of relapse or (Sandberg and Chen, 1994; McKenna, 2000). Eye melanoma, also referred to as uveal melanoma, are highly amenable for cytogenetic analysis and show mostly simple karyotypes, in contrast to most other solid tumours. Specific chromosomal changes such as variation in chromosomes 1p, 3, 6 and 8 are often found in these tumours (Sisley et al., 1990; Prescher et al., 1995). Loss of chromosome 3 and gain of chromosome 8 or 8q are associated with a high mortality rate, whereas abnormalities of chromosome 6 are suggested to correlate with a good prognosis. However, these data are from relatively small studies and the associations with other chromosome abnormalities and tumour parameters were not analysed.
In the present study we investigated the associations between chromosomal changes and tumour parameters such as cell type, tumour diameter. We examined, in the largest series up until now, the independent effect of numerical changes of chromosomes 1, 3, 6 and 8 on disease free survival in a series of 80 uveal melanoma patients.

METHODS

Patients and tumour samples

From March 1992 to July 2000, we collected tumour material of 112 consecutive patients who underwent enucleation of the tumour-containing eye for ciliary body or choroidal melanoma. Informed consent was given prior to enucleation and the study was performed according to the tenets of the Declaration of Helsinki. Until the introduction of routine cytogenetic analysis for eye melanoma in May 1994, only very few cases were karyotyped (4 out of 31). From May 1994 until January 1995 cytogenetic analyses were performed (n=15) which was successful in 11 cases. From January 1995 on all patients (n=66) were analysed with FISH and, if metaphases could be obtained, with cytogenetic analysis. Fresh tumour material was obtained within 1 hour after enucleation and processed for FISH and/or cytogenetic analysis as (Naus et al., 2001). Conventional histopathological examination was performed on all tumours and confirmed the origin of the tumour. Cytogenetical studies were also carried out on

stimulated peripheral blood samples of each patient to exclude the presence of congenital chromosome abnormalities. Follow-up data from the time of diagnosis to the end of the study in September 2000 were obtained by reviewing each patient's charts and/or contacting their general practitioner. One patient was lost to follow-up. The remaining 80 patients consisted of 43 men and 37 women. The age at time of diagnosis ranged from 21 to 87 years (mean 60). The mean period from time of diagnosis to the presence of metastases or end of the study, was 27 months (range 1-87 months). The mean tumour diameter and thickness were 12,0 mm (range 1.5-22) and 7,0 mm (range 4.5-10), respectively. Thirteen tumours showed involvement of the ciliary body and 67 were located in the choroid.

Cytogenetic and FISH analysis

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

Dual colour FISH on uncultured tumour material using centromeric and locus specific cosmid, P1 or YAC probes for chromosome 1, 3, 6 and 8 was performed as described previously (Hagemeijer et al., 1998). The concentration for centromeric probes was 5 ng per slide; for cosmids, P1 and YAC probes 50 to 75 ng per slide were used. Eight probes were used: p1-79 (mapped to chromosome band 1p36), Pα 3.5 (centromere 3), YAC 827D3 (3q24), cos85 (6p21) and cos52 (6q23) (Prof. Y Nakamura, Tokyo, Japan), cos105H8 (8p11) and D8Z2 (centromere 8) and ETO (8q22). After hybridisation and washing, slides were counterstained with 4,6-diamidino-2-phenylindole and mounted in anti-fade solution (Dabco-Vectashield 1:1). Signals were counted in 300 interphase nuclei according to the criteria of Hopman et al. (Hopman et al., 1988). Cut-off limits for deletion (15% of the nuclei with one signal) or amplification (>10% of the nuclei with 3 or more signals) were adapted from the available literature (van Dekken et al., 1990).

Data classification

We subdivided the variation in chromosomes 1p, 3, 6p, 6q and 8q using cytogenetic and FISH analysis into 5 categories: loss of one copy, normal copy numbers (two copies), gain of one copy, gain of two copies, and gain of more than two copies. Using FISH, monosomy 3 was defined when there was only one signal seen for the centromere 3 and 3q24 probes. Gain of chromosome 8, when all three probes for chromosome 8 (i.e. 8p11, centromere 8 and 8q22) showed more than 2 signals; gains of 6p and 8q were scored when more than 2 signals were found for each of the 6p21 and 8q22 probes, and loss of 1p and 6q when the probes for 1p36 and 6q23 showed only one signal.

When different subclones were identified, only the FISH findings of the largest clone were classified. Cytogenetic results were classified for the regions studied with FISH analysis. All major chromosomal changes detected by cytogenetic analysis could also be detected by FISH analysis. Therefore, we classified only the uncultured direct FISH data if both FISH and cytogenetic analyses were available.

Statistical analysis

Disease free survival was the time from enucleation to the development of metastatic disease. The influence of single prognostic factors on disease free survival was assessed using Kaplan-Meijer analysis and the log rank test (for categorical variables) or Cox proportional hazard analysis (for continuous variables) (Table 2.1). Comparisons of the distributions of clinical and chromosomal variables were performed with Fisher's exact test (for categorical variables) and the Mann-Whitney test (for continuous variables) (Table 2.2). The independent value of the prognostic factors on disease-free survival was assessed by multivariate Cox proportional hazard analysis and the likelihood ratio test. Included in the model were age at time of diagnosis, cell type (spindle cell vs. mixed/epithelioid cell), largest tumour diameter, mutual loss of chromosome 1p and 3, gain of 6p and gain of 8q. All tests were two-sided. An effect was considered significant when the p-value was 0.05 or less. The statistical analyses were performed with SPSS-9 software.

RESULTS

A total of 80 uveal melanoma cases was analysed for chromosomal changes using cytogenetic and/or FISH analyses. Thirteen patients had died from metastatic disease and four were suffering from metastases at time of evaluation. Cytogenetic analysis was successful in 49 out of 80 tumours. For 37 tumours there were cytogenetic and fluorescent *in situ* hybridisation (FISH) data available, and in 31 tumours only FISH was performed. Not all probes could be tested on all patients because of lack of material. The mean number of probes successfully used for FISH was 6. Combining both cytogenetic and FISH data, chromosomal abnormalities were found in 88 percent of all tumours.

Univariate analysis of the single prognostic factors showed significantly lower disease free survival for patients with loss of chromosome 3 or gain of 8q in the tumour and for patients with a mixed or epithelioid tumour cell type (compared to patients without these chromosomal changes or with a spindle cell type) (Table 1). Other potential prognostic factors, such as gender, age at time of diagnosis, tumour diameter, thickness, and tumour location (i.e. involvement of ciliary body) did not reach significance. Chromosomal changes such as loss of chromosome band 1p, gain of chromosome 6p or 8 and loss of chromosome 6q were not significantly associated with disease free survival. To examine the possibility that other chromosomal variations may affect the prognosis of the monosomy 3 patients, we constructed Kaplan Meijer curves of chromosome 3 changes stratified for the other chromosomal changes and performed log rank tests to test for statistical significance. We found that the effect of monosomy 3 on disease free survival was largely modified by changes in number of copies of chromosome 1p. In tumours with normal copy numbers of chromosome 1p, no significant difference in disease free survival was observed between those patients with and without loss of chromosome 3 (p=0.9841) (Fig.2.1a), whereas this difference was significant in patients with tumours with loss of chromosome 1p (p=0.0095) (Fig. 2.1b).

TABLE 2.1 RESULTS OF THE UNIVARIATE ANALYSIS OF PROGNOSTIC MARKERS AND DISEASE FREE SURVIVAL IN 80 EYE MELANOMA

VARIABLE	No. of patients (%)/Mean	p-value
Male gender	43 (54)	0.754*
Age at time of diagnosis (yrs)	60	0.502#
Mixed/epithelioid cell type	57 (71)	0.015*
Tumour thickness (mm)	7	0.363#
Largest tumour diameter (mm)	12	0.330#
Involvement of ciliary body	13 (16)	0.414*
Genetic changes (%):		
loss of 1p	26/77 (34)	0.178*
loss of chromosome 3	32/71 (45)	0.004*
gain of chromosome 6p	19/63 (30)	0.696*
loss of chromosome 6q	21/65 (32)	0.896*
gain of chromosome 8	14/44 (32)	0.090*
gain of chromosome 8q	39/70 (56)	0.050*

* Log-rank test; # Cox-regression analysis

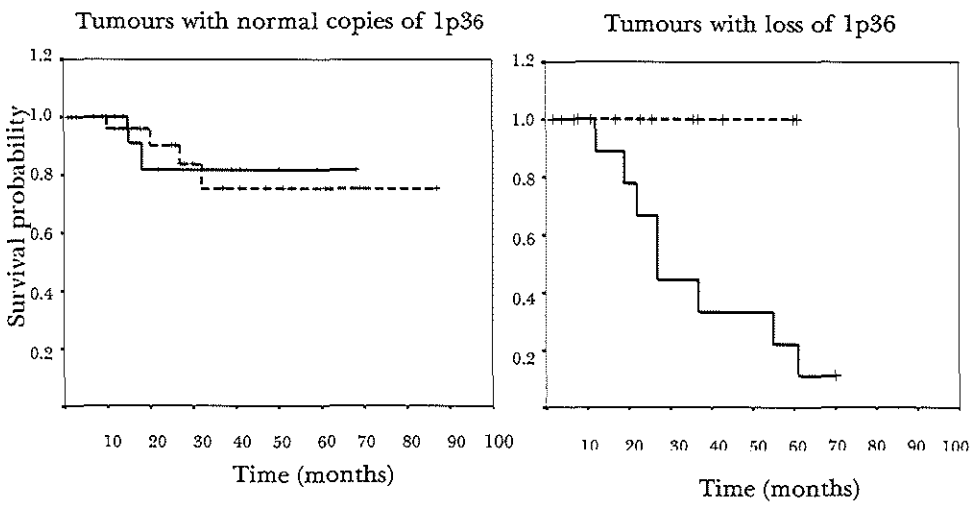


Fig. 2.1 Kaplan Meijer survival curves of loss of chromosome 3 stratified for chromosome 1p. Left: Survival curves of tumours with normal copies of chromosome 1p36, with and without chromosome 3 loss. Right: Survival curves of tumours with loss of chromosome 1p36 with and without chromosome 3 loss.

TABLE 2.2 CORRELATION BETWEEN CHROMOSOMAL ABNORMALITIES AND CLINICAL DATA

CLINICAL DATA		CHROMOSOME 1P LOSS [1]			CHROMOSOME 3 LOSS			CHROMOSOME 6P GAIN			CHROMOSOME 8q GAIN		
		-	+	P	-	+	P	-	+	P	-	+	P
Gender[2]	male	28	12	0.330	24	16	0.348	26	9	0.410	18	20	0.634
	female	19	14		15	16		17	10		13	19	
Mean age (yrs)[3]		62	60	0.557	56	68	0.001	61	55	0.079	58	61	0.467
Cell type[2]	spindle	14	7	1.000	17	4	0.005	7	11	0.002	13	6	0.013
	mixed/epithelioid	33	19		22	28		36	8		18	33	
Mean tumour thickness (mm)[3]		7.0	7.4	0.373	7.1	6.7	0.614	7.0	7.8	0.109	6.8	7.6	0.228
Mean tumour diameter (mm)[3]		11.9	12.7	0.286	11.9	12.2	0.647	11.8	13.9	0.031	10.9	13.8	0.001
Involvement of ciliary body[2]	no	40	20	0.524	34	27	0.746	34	16	0.740	27	31	0.529
	yes	7	6		5	5		9	3		4	8	

[1] Chromosome locus at which the abnormality is absent (-) or present (+).

[2] The p value is for the comparison among different subgroups within a chromosome aberration group and was calculated by Fisher's exact test.

[3] The p value is for the comparison of means among different subgroups within a chromosome aberration group and was calculated by the Mann-Whitney test.

In addition, the interaction term between loss of chromosome 1p and chromosome 3 was found to be significant (Cox proportional hazard analysis P=0.04). No interactions between chromosome 3 and the other chromosomes (6 and 8) were found. Moreover, we compared the disease free survival of patients with a concurrent loss of chromosome 1p and chromosome 3 with that of the other subgroups of patients (i.e. patients with tumours with normal copies of chromosome 1p and 3 or with either 1p or 3 loss). This difference in survival was found to be highly significant (p=0.0003).
Considering the strong interaction between chromosome 1p and 3 loss, we validated whether this concurrent loss is an independent parameter for disease free survival in a multivariate model. Confounding variables were considered age at time of diagnosis, cell type, tumour diameter and chromosome 6p and 8q gains. After correcting for these variables, we found that patients with tumours having concurrent loss of chromosomes 1p and 3 had an almost 18 times higher chance of developing metastases compared to those without these losses or with either 1p or 3 loss (P=0.0065) (Table 2.3).

TABLE 2.3 MULTIVARIATE ANALYSIS OF PROGNOSTIC MARKERS AND DISEASE FREE SURVIVAL IN 80 EYE MELANOMA (FINAL MODEL)*

VARIABLE	HAZARD RATIO	P-VALUE#
Loss of chromosome 1p with loss of 3	17.82	0.007
Mixed/epithelioid cell type	16.53	0.098
Largest tumour diameter	1.12	0.237
Age at time of diagnosis	0.98	0.465
Gain of chromosome 6p	0.51	0.531
Gain of chromosome 8q	0.27	0.274

* Cox proportional hazard analysis

Likelihood ratio test

DISCUSSION

In uveal melanoma it is known that loss of chromosome 3 is a prognostic marker for decreased survival of the patient (Prescher et al., 1996; Sisley et al., 1997; White et al., 1998). Using *univariate* analysis, we indeed found that loss of chromosome 3 was a good prognostic factor predicting the outcome of the patients. The present study is the largest series described up until now, and allows more extensive multivariate statistical analysis. We demonstrated that only those patients with a concurrent loss of chromosomes 3 and 1p are at high risk for developing metastatic disease.
The molecular genetic changes that underlie these chromosomal changes still have not been determined. However, the fact that both chromosomes 1p and 3 must be lost to affect survival could suggest that an interaction between genes on these two chromosomes is necessary for the development of metastatic disease. Chromosome 1p36 is frequently deleted in many solid tumours like skin melanoma and neuroblastoma. In the latter tumour type, loss of chromosome 1p is known to be a predictor of an

unfavourable outcome of the patient (Caron et al., 1996). A putative tumour suppressor gene, *p73,* is located in this region (Levrero et al., 2000). This gene is a homologue of *p53*, which is altered in approximately 50% of all human cancers. Another homologue of *p*53, *p*63, is located on chromosome 3q27, the other chromosome of interest (Yang et al., 1998). Very few or no mutations in human malignancies have been described in *p*73 or *p*63, other molecular mechanisms, such as a dominant negative effect of isoforms of *p73* and *p63* on itself and on *p53*, might be responsible for the inactivation of these genes. Both genes are known to interact with each other and with *p53* (Yang et al., 1999) pointing to a possible role for these genes in eye melanoma tumorigenesis.
Gain of chromosome 8q, which has been suggested to correlate with survival (Sisley et al., 1997), was only significant in our univariate analysis. In our multivariate analysis it was not found to be an independent prognostic marker and we demonstrated a strong correlation between the largest tumour diameter and the presence of chromosome 8q abnormalities. Also the abnormalities of chromosome 6 were not independently associated with survival, in contrast to what has been previously claimed (White et al., 1998). We found a strong correlation between the gain of chromosome 6p and a spindle cell type. As far as we know, the chromosomal changes (i.e. loss of chromosome 3, gain of chromosome 8q and abnormalities of chromosome 6) found by Sisley and White and co-workers and which they associated with prognosis, were not corrected for tumour diameter or cell type as in the present study. This could have influenced their findings. Another known prognostic marker for a poor outcome of uveal melanoma patients is the presence of epithelioid cells. Although in our series this cell type was significantly associated with decreased disease free survival in the univariate analysis, it was not in the multivariate analysis. Moreover, we found a strong correlation between chromosomal aberrations (chromosomes 3, 6 and 8) and cell type (Table 2.2).
This present study on chromosomal abnormalities in eye melanoma is, to our knowledge, the largest reported in the literature. However, it is still a relatively small series with a short follow-up time. Furthermore, our study may be biased since we examined only tumours from patients treated by enucleation, as no tumour material is available from patients treated with radiotherapy protocols. These findings need to be assessed in a large unselected prospective trial, which can be carried out since we have already shown that chromosome analysis can be performed on fine needle aspiration biopsies of eye melanoma using simple FISH techniques (Naus et al., submitted). However, our results do demonstrate a prognostic role of concurrent loss of chromosomes 1p and 3, which may aid clinicians in identifying those patients at a high risk of metastatic disease, and could lead to the justification of the application of adjuvant therapies to these patients.

CHAPTER 2

CHARACTERIZATION OF COMPLEX CHROMOSOMAL ABNORMALITIES IN UVEAL MELANOMA BY FLUORESCENT IN SITU HYBRIDIZATION, SPECTRAL KARYOTYPING AND COMPARATIVE GENOMIC HYBRIDIZATION

Nicole C. Naus[1,2], Ellen van Drunen[2], Annelies de Klein[1,2], Gregorius PM Luyten[1], A Dion A. Paridaens[5], Janneke C Alers[3], Bruce R Ksander[6], H Berna Beverloo[2], Rosalyn M Slater[2,4]

From the Departments of [1]Ophthalmology, [2] Cell Biology & Genetics, [3]Pathology, [4]Clinical Genetics, Erasmus University Rotterdam, Rotterdam, The Netherlands, [5]Rotterdam Eye Hospital, Rotterdam, The Netherlands, [6]Schepens Eye Research Institute, Boston, USA

Genes, Chromosomes and Cancer (2001) 30(3): 267-273

ABSTRACT

Several non-random recurrent chromosomal changes are observed in uveal melanoma. Some of these abnormalities, e.g. loss of chromosome 3, gain of the q arm of chromosome 8 and chromosome 6 abnormalities, are of prognostic value. Cytogenetic analysis and/or fluorescent *in situ* hybridization (FISH) are used to detect these changes. In some cases, however, detailed cytogenetic analysis is not possible due to the presence of complex abnormalities. To more accurately define these cytogenetic changes, we have applied comparative genomic hybridization (CGH) and/or spectral karyotyping (SKY) to two uveal melanoma cell lines and five primary uveal melanomas, with partially defined and/or complex abnormalities. SKY provided additional information on 34/39 partially defined aberrant chromosomes and revealed a new abnormality, a der(17)t(7;17)(?;q?), that had not been recognized by conventional cytogenetics. Additionally, using SKY, abnormalities involving chromosome 6 or 8 were found to be twice as common as observed with cytogenetic analysis. CGH was especially useful in assigning the abnormalities identified by SKY to specific chromosomal regions, and in addition, resulted in the detection of a small deletion of chromosome region 3q13-3q21. We conclude that SKY and CGH, as complementary methods to cytogenetic and FISH analysis, provide more complete information on the chromosomal abnormalities occurring in uveal melanoma.

INTRODUCTION

In human cancers non-random chromosomal abnormalities reflect tumor-specific events at the molecular level. These recurrent chromosomal abnormalities can provide starting points for positional cloning strategies. Furthermore, an accurate identification of the specific cytogenetic abnormalities can be of diagnostic and prognostic value.
In uveal melanoma several recurrent chromosomal abnormalities have been described. Loss of chromosome band 1p36, monosomy 3, abnormalities of chromosome 6 and/or gain of the q arm of chromosome 8 is observed in most tumors (Horsman and White, 1993; Prescher et al., 1995; Sisley et al., 2000). Loss of chromosome 3 and gain of the q arm of chromosome 8 associate strongly with a decreased survival of the patient (Prescher et al., 1996; Sisley et al., 1997). In contrast, chromosome 6 abnormalities are associated with a better prognosis (White et al., 1998). Since most of these reported abnormalities involve the loss or gain of whole chromosomes or chromosome arms, it is difficult to determine which specific regions carry genes of particular importance to tumorigenesis. Karyotyping and/or FISH analyses are used to detect chromosomal changes in uveal melanoma. However, in some cases, detailed cytogenetic analysis can be complicated by the presence of complex abnormalities.
We have applied comparative genomic hybridization (CGH) and/or spectral karyotyping (SKY) to two uveal melanoma cell lines and five primary uveal melanomas, alongside conventional karyotyping and FISH, in order to more accurately define the regions involved in the development of uveal melanoma. SKY is especially useful in recognizing the origin of structural abnormalities (Schrock et al., 1996), whereas CGH provides information on net losses and gains of chromosomal regions (Kallioniemi et al., 1992).

MATERIALS AND METHODS

Patients and cell lines

Cytogenetic and FISH studies are routinely carried out in our laboratory on fresh uveal melanoma and uveal melanoma cell lines. Five fresh tumors (EOM 159, 165, 178, 182 and 191) from eyes enucleated at the Rotterdam Eye Hospital and two cell lines (Mel202 and Mel270) were selected for the present study due to the presence of partially defined and/or complex cytogenetic abnormalities. Sterile tumor specimens were collected after enucleation at the department of pathology and processed as described previously (Luyten et al., 1996). In only three instances (Mel202, Mel270 and EOM191) was there sufficient material to carry out CGH analysis.

TABLE 3.1 CLINICAL, CYTOGENETIC AND SKY DATA OF TWO UVEAL MELANOMA CELL LINES AND 5 PRIMARY UVEAL MELANOMAS

CASE #	SEX[A] /AGE[B]	CELL TYPE	TUMOR LOCATION	KARYOTYPE [ISCN,1995]	UNDEFINED CHROMOSOME [C]	SKY
Mel202 (cell line)	F/ nr	mixed	choroid	50-53,XX[3],-X[13],add(X)(q?),+dic(1;9)(p11;p11),+5[3], add(6)(q?15),+add(6)(q?15),+7,+8,+8,del(9)(q2?1)[2],del(9)(q3?2)[2],i(9)(p10)[3],add(11)(q22),add(16)(q11),add(18)(q21),der(20)t(8;20)(q12;q13),add(22)(p11)[cp16]	add(X)(q?) add(6)(q?15)x2 del(9)(q2?1) add(11)(q22) add(16)(q11) add(18)(q21)	der(X)t(X;19)(q?;?) der(6)t(6;17)(q?15;?)x2 der(9)t(9;17)(q2?1;?) der(11)t(11;12)(q22;?) der(16)t(2;16)(?;q11) der(18)dup(18)
Mel270 (cell line)	M/ nr	mixed	ciliary body	43-48,XY,add(2)(p2?4)[4],?add(3)(q2)[5],der(6)t(6;17)(p2?5;q25)del(6)(q?),psu i dic(6)(q11),der(7)del(7)(q22q22)add(7)(q32)[3],+der(7)del(7)(q22q22)add(7)(q32)[3],?der(8)(p)[3],-9[5],?add(9)(p)[4],add(9)(p)[2],+der(9)add(9)(p)del(9)(q)[2],-10[3],add(10)(q2?)[3],add(12)(p11)[3],-13[6],add(13)(p11),+16[3],add(16)(q11),-19,add(21)(p1)[2],+mar[2][cp7]/81-87,XXYY,der(6)t(6;17)(p2?5;q25)del(6)(q?),psu i dic(6)(q11)x2,der(7)del(7)(q22q22)add(7)(q32)x2,add(10)(q2?),+marx2[cp2]	add(2)(p2?4) ?add(3)(q2) psu i dic(6)(q11) der(7)del(7)(q22q22) add(7)(q32) ?der(8)(p) ?add(9)(p) add(10)(q2?) add(12)(p11) add(13)(p11) add(16)(q11) 17 [d] add(21)(p1)	der(2)dup(2) der(3)t(3;8)(q2;?) der(6)i(6) der(7)del(7)(q22q22) t(7;17)(q32;?) der(8)t(8;21)(p21;?) der(9)dup(9) der(10)t(10;13)(q2?;?) der(12)dup(12) der(13)t(10;13)(?;p11) der(16)t(16;19)(q11;?) der(17)t(7;17)(?;?) der(21)t(19;21)(?;p1)
EOM159	F/38	spindle	choroid	40-46,XX,add(2)(q3?4)[6],add(5)(q34)[9],del(6)(q?)[3],der(7)t(7;8)(p21;q?),add(10)(p1?4)[2],add(11)(q1?4)[9],der(16)t(8;16)(q?;q24)[7],add(18)(q23)[1][cp19]/46,XX [1]	add(2)(q3?4) add(5)(q34) der(7)t(7;8)(p21;q?) add(10)(p1?4) add(11)(q1?4)	der(2)t(2;6)(q3?4;?) der(5)t(5;6)(q34;?) der(7)t(7;11)(p21;?) t(8;11)(q?;?) der(10)t(8:10)(?;p1?4) der(11)t(8:11)(?;q1?4)
EOM165	M/43	spindle	choroid	40-46,XY,add(6)(q21),der(7)t(1;7)(q12;q36),+8[cp20]	add(6)(q21)	der(6)t(6;6)(q16;?)
EOM178	F/73	mixed	choroid	45-47,XX,del(1)(p21),add(5)(p)[4],add(7)(q36),+8[2],-14[5],-15,add(17)(p12),+ring[3][cp10]	del(1)(p21) add(5)(p) add(7)(q36) add(17)(p12)	der(1)t(1;15)(p11;?) der(5)t(5;14)(p?;?) der(7)t(6;7)(?;q36) der(17)t(7;17)(?;p12) t(6;7)(?;?)
EOM182	F/52	spindle	choroid	46,XX,der(6)t(6;6)(q16;p12),add(22)(p11),add(22)(q13)[16]	add(22)(p11) add(22)(q13)	der(22)t(8:22)(?;p11) der(22)t(6:22)(?;q13)
EOM191	F/46	spindle	choroid	46,XX,add(1)(q42),add(4)(q3?2),del(6)(q1?4q2?5),der(8)t(6;8)(p12;q24),-16,add(16)(q?2),add(17)(p13),add(21)(p12), +mar1,+mar2[19]	add(1)(q42) add(4)(q3?2) add(16)(q?2) add(17)(p13) add(21)(p12) mar1	der(1)t(1;6)(q42;?) der(4)t(4;6)(q3?2;?) der(16)t(8;16)(?;q?2) der(17)t(10;17)(?;p13) der(21)t(21;21)(p12;q22) der(21)t(8:21)(?;p12) t(6;21)(?;q?)

[A]Abbreviations: F, female; M, male; nr, not reported; [B]Age at time of diagnosis (in years); [C] identified by conventional cytogenetic analysis; [D] cryptic aberration

Karyotyping and fluorescence *in situ* hybridization

Chromosome analyses were carried out after short term tissue culture and the findings were described in accordance with the ISCN (1995). Results of the cytogenetic analysis are shown in Table 3.1. For direct FISH analysis, uncultured cells were fixed with methanol/acetic acid (3:1) and interphase FISH with locus/band specific probes was performed with a range of probes using standard procedures (Arnoldus et al., 1990). The probes used were: pl.79 (band 1p36), Pα3.5 (centromere 3), YAC 827D3 (band 3q24) (Caltech Genome Research Laboratories, USA), cos52 (band 6q23) and cos85 (band 6p21) (Prof. Y Nakamura, Tokyo, Japan), D8Z2 (centromere 8), ETO (band 8q22), 105H8 (band 8p11). After hybridization and immunostaining, slides were counterstained with DAPI and mounted in anti-fade solution (Dabco- Vectashield 1:1). In each case, 300 nuclei per probe were scored according to the criteria of Hopman et al. (Hopman et al., 1988). The cut-off values used, were those described by van Dekken (van Dekken et al., 1990). Polysomy was defined as more than 2 hybridization signals in more than 10% of nuclei, and monosomy as a single signal in more than 15% of the nuclei. The FISH results are shown in Table 3.2. In addition, the chromosomal abnormalities identified by SKY and CGH were confirmed with additional FISH analyses on metaphases using subtelomeric probes (Collaboration, 1996), locus specific probes and whole chromosome paints.

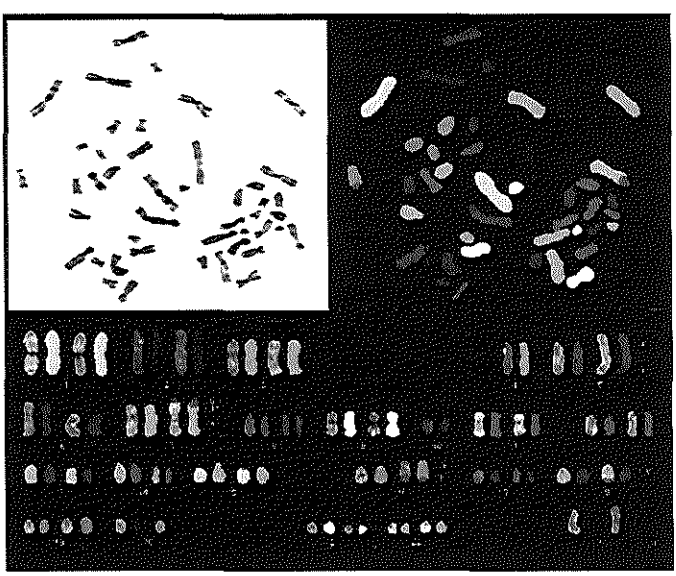


Fig 3.1 Example of SKY analysis of EOM159.

Metaphase with DAPI staining (upper left panel), metaphase showing classification colors (upper right panel) and Karyotype showing display and classification colors (lower panel). The der(2)t(2;6)(q3?4;?) and der(10)t(8;10)(?;p1?4) were not present this metaphase. Besides the described abnormalities, this metaphase showed also loss of chromosome 4 and 10.

Spectral karyotyping

Cytogenetic preparations were pre-treated with RNAse for 60 minutes at 37°C and digested with pepsin for 10 minutes. After washing, cells were fixed in 1% formaldehyde/50 mM $MgCl_2$ in PBS for 10 minutes, washed and dehydrated. Hybridization for SKY analysis was performed according to the manufacturer's protocol with minor adjustments (Applied Spectral Imaging, Migdal Ha'Emek, Israel). In short, after two days of hybridization, slides were washed with 55% formamide/2x SSC (pH7.0) followed by 1xSSC at 39°C and 4xSSC/0.05% Tween20 at RT. Cells were counterstained with DAPI and mounted in anti-fade solution (Dabco-Vectashield 1:1). Using the Spectra Cube 200 system and the Skyview analysis software (ASI), 6 to 11 metaphases from each tumor were examined. Sky results are summarized in Table 3.1 and an example of SKY analysis in EOM 159 is shown in Figure 3.1.

Comparative Genomic Hybridization

DNA from formalin-fixed paraffin-embedded tumor material (EOM191) was isolated from 30 5µm sections. The pigmented tumor was scrapedçoff from the glass slides using a fine scalpel. Excised material was deparaffinized in xylene and ethanol and air-dried. Isolation of the DNA was performed using the Puregene DNA isolation kit (Gentra systems, Minneapolis, MN). DNA from cell lines (Mel202 and 270) was isolated using standard techniques. Concentration was determined using a fluorometer (DyNA Quant 200™, Hoefer Biotech Inc., San Francisco, CA), whereas molecular weight of the DNA was estimated on ethidium bromide-stained agarose gels.

Labeling of the DNA and hybridization were essentially performed as described (Alers et al., 1999). Tumor DNA from EOM191 (<1kb) was labeled using the ULS biotin labeling kit (Kreatech Diagnostics, Amsterdam, The Netherlands). DNA from cell lines Mel202 and Mel270 (>1 kb) and male reference DNA (Promega) were nick translated (Nick Translation System, Gibco BRL, Gaithersburg, MD) with biotin or digoxigenin respectively (Boehringer Mannheim, Indianapolis, IN). The probe mixture of tumor and reference DNA was denatured and hybridized to normal male metaphase chromosomes (Vysis Inc. Downers Grove, IL) for three days at 37°C. After washing, detection of the biotin- and digoxigenin-labeled DNA probes was accomplished with avidin-fluorescein isothiocyanate (green) and anti-digoxigenin rhodamine (red), respectively. Samples were counterstained with DAPI in anti-fade solution.

Images were acquired with an epifluorescent microscope (Leica DM, Rijswijk, The Netherlands) equipped with a cooled CCD camera (Photometrics Inc., Tucson, AZ), three single excitation filters, a multi-band pass dichroic mirror and emission filters. For CGH analysis the Quips XL software from Vysis (version 3.1.1 Vysis Inc., Downers Grove, IL) was used. For each case 9 metaphases were analyzed. Loss of DNA sequences was defined as chromosomal regions where the mean green:red ratio was below 0.85, while gain was defined as chromosomal regions where the ratio was above 1.15. These threshold values were based on series of (paraffin embedded vs. fresh) normal controls. The CGH results are dipicted in Figure 3.2.

RESULTS

Cytogenetic analysis of these 2 uveal melanoma cell lines and 5 primary uveal melanoma revealed a total of 39 partially defined chromosome aberrations which could be refined by SKY analysis. Of particular note is the high incidence of involvement of chromosomes 6 and 8. In 34/39 aberrations SKY identified the translocation partner. In the cell lines Mel202 no other chromosome material was shown to be involved in the add(18) and add(22) and, likewise, in the add(2), ?add(9) and add(12) of Mel270, suggesting duplication of regions on these chromosomes. However, only for the add(2), did CGH analysis confirm this duplication. It is possible that the regions duplicated on the other chromosomes consisted of amplicons too small to be detected by CGH or, in case of Mel202, that the abnormalities were not shown by CGH since they were not present in every metaphase analyzed. Furthermore, our studies revealed two cytogenetic changes not identified by conventional chromosome analysis. In Mel270 SKY showed a cryptic aberration where an apparently normal chromosome 17 was found to consist of a translocation der(17)t(7;17)(?;q?) (Fig. 3c). In addition, CGH unexpectedly revealed a deletion of chromosome region 3q13~21 in this cell line (Fig. 3.2). Examples of the results obtained from combining cytogenetic, SKY and CGH analysis are shown in Figure 3.3.

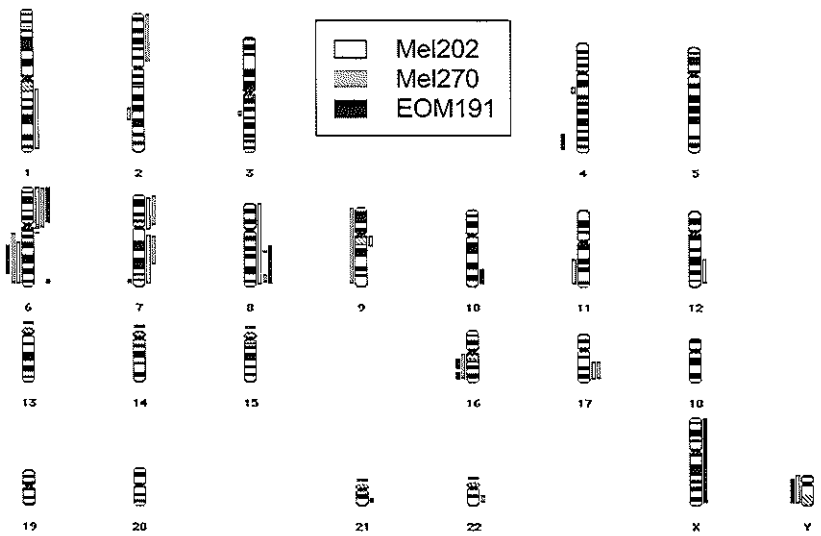


Fig. 3.2 Results from CGH analysis on two uveal melanoma cell lines and one primary tumor.
Lines on the right side of the ideogram reflect copy number increases; lines on the left represent copy number decreases.

Variation of chromosomes 6 and 8

In these seven uveal melanomas we observed frequent aberrations involving chromosome 6. Combining the SKY results with interphase FISH, most of these abnormalities could be further refined. In EOM65, EOM178 and EOM182, the extra copies of chromosome band 6p21 found by FISH, suggested the involvement of this region in the translocations of chromosome 6. However, in EOM159, a der(2)t(2;6)(q3?4;?) and der(5)t(5;6)(q34;?) were found by conventional cytogenetics and SKY (Fig. 3.2) but FISH analysis revealed gain of only one copy of 6p21. Additional FISH analysis on metaphases revealed involvement of band 6p21 only in the der(2). Consequently, other chromosome 6 regions have to be involved in the der(5). In EOM191, chromosome 6 material was translocated to marker 1 and chromosomes 1 and 4. FISH analysis refined marker 1 as a der(21)t(8;21) (?;p12)t(6;21)(p?;q?) and the der(1) and der(4) as der(1)t(1;6)(q42;q?) and der(4)t(4;6)(q3?2;q?), respectively.

TABLE 3.2 RESULTS OF ROUTINE FISH ANALYSES ON INTERPHASE CELLS[a]

CELL LINE /TUMOR	FISH ON *INTERPHASE* CELLS[b]															
	1p36		cen3		3q24		6p21		6q23		8p11		cen8		8q22	
	O[c]	E[d]	O	E	O	E	O	E	O	E	O	E	O	E	O	E
Mel202	2	2	2	2	2	2	2,3,4	3	1,2	1	4,5	5	4,5	5	5	6
Mel270	2	*2,4*	2	*2,4*	2	*1,2,4*	3,4	*4,8*	1,2	1,2	2,3	*2,4*	2,3	*2,4*	2,3	*2,4*
EOM159	2	2	2	2	2	2	3	2	1,2	1,2	2	2	2	2	2	2,4
EOM165	2	2	2	2	ND	2	2,3	2	1	1	3	3	3	3	3	3
EOM178	1	1	2	2	ND	2	4	2	2	2	2	2,3	2,3	2	2,3	2,3
EOM182	2	2	2	2	2	2	3,4	3	1,2	1	2	2	2	2	2	2
EOM191	2	2	2	2	2	2	4	3	1,2,3	1	2	2	2	2	3	2

[a] abbreviations: ND, not done; [b] Only significant cell populations (loss in >15% or gain in >10% of the cells) are indicated; [c] Observed copy number by FISH analysis; [d] Expected copy number based on cytogenetic data; [e] Tetraploid subclone is indicated in italics

Chromosome 8 abnormalities were also found to be more common than expected from cytogenetic data. In Mel270, EOM159, EOM182 and EOM191 translocations involving chromosome 8 were detected with SKY. The FISH results for chromosome region 8q22 showed, however, no unexpected copy numbers except for EOM191 (Table 2), In the other cases, FISH with the 8q22 probe was negative, indicating that other regions of chromosome 8 are the target of amplification. In one instance (EOM182) this was shown to involve the q arm of chromosome 8 since a subtelomeric probe for chromosome 8q hybridized to the der(22)t(8;22)(?;p11).

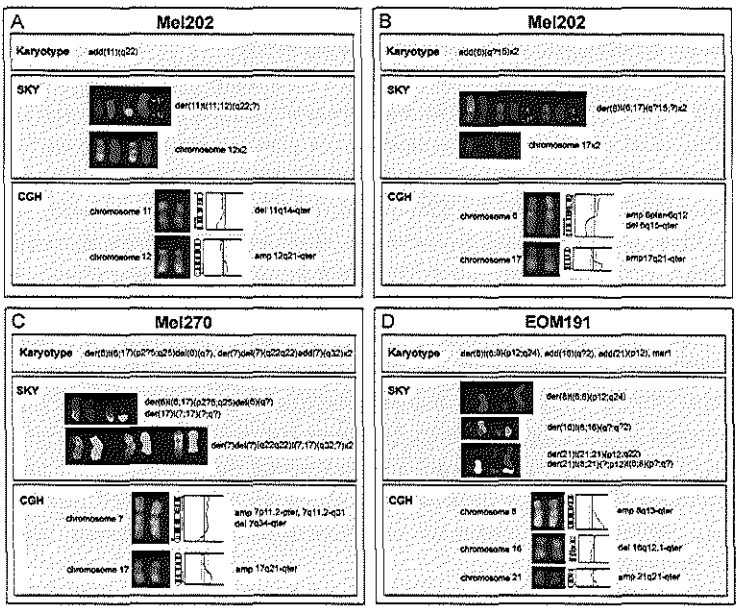


Fig. 3.3 Examples of cytogenetic, SKY and CGH results in two uveal melanoma cell lines (A-C) and one primary uveal melanoma (D). In each panel the partial karyotype describes specific chromosomal abnormalities as found by conventional cytogenetics. In the SKY and CGH boxes the chromosomes found to be participating in the abnormalities are shown.

DISCUSSION

Our findings indicate that abnormalities involving subregions of chromosomes 6 and 8 are far more common in uveal melanoma than previously assumed. Additional material from chromosome 6 was revealed by SKY to be more than twice as common as identified by cytogenetic analysis. Strikingly, in 11 out of 14 translocations involving chromosome 6, FISH analysis showed involvement of at least band 6p21. CGH results on two cell lines and one tumor showed gain of a whole short arm of chromosome 6, with loss of chromosome region 6q16~24. Structural variation involving chromosome 8 was identified by SKY in four tumors. This involved a total of seven different derivative chromosomes. In only one instance (EOM191) was this variation detected by the 8q22 FISH probe. These findings indicate that band 8q22 is probably not the target region in uveal melanoma. The small CGH study would suggest that the chromosome regions 8q21.1~21.2 and 8q23~24 may be involved, since these regions were lost in all samples analyzed. Overrepresentation of chromosome region 8q23-24~qter has also been found to be the smallest overlapping region in other studies using CGH or karyotyping

(Speicher et al., 1994; Prescher et al., 1995). All these results have narrowed down the regions of interest of chromosome 6 and 8 but the predictive value of these loci in prognostic terms needs to be evaluated in a larger study.

In contrast to what is known from the literature (Horsman and White, 1993; Prescher et al., 1995; Sisley et al., 2000), we did not observe monosomy 3 in any of the cases. However, using CGH we did detect a deletion of chromosome region 3q13~q21 in one cell line. Other studies have not reported finding this deletion (Speicher et al., 1994; Ghazvini et al., 1996; Becher et al., 1997) and it may be significant with respect to the demarcation of a region on chromosome 3 harboring a potential uveal melanoma tumor suppressor gene. As of yet, no known tumor suppressor genes have been mapped to this region and more studies are required to determine the significance of these data. The lack of monosomy 3 cases could be the result of non-random sampling, since the tumors and cell lines were selected on the basis of partially defined and/or complex karyotypes, and on the availability of metaphases necessary for the SKY analyses. The data in the present study support the earlier observations of (Parrella et al., 1999), of a mutual exclusivity of monosomy 3 and aberrations of the short arm of chromosome 6 in uveal melanoma. However, in non-selected series, we (unpublished results) and others (White et al., 1998), have observed combinations of concurrent aberrations of chromosomes 3, 6 and 8.

We have successfully applied spectral karyotyping and comparative genomic hybridization alongside conventional cytogenetics and FISH to unravel the genetic changes in seven cases of complex uveal melanoma. We have shown that the great advantage of combining cytogenetics with FISH, SKY and CGH is that it provides complementary data that increase the level of resolution of the genetic studies.

CHAPTER

4

DETECTION OFGENETIC PROGNOSTIC MARKERS IN UVEAL MELANOMA BIOPSIES USING FLUORESCENT IN SITU HYBRIDIZATION

Nicole C Naus[1,2], Ellen van Drunen[2], Rosalyn Slater[2,3], Anette CA Verhoeven[2], Cornelia M Mooy[4], A Dion A Paridaens[5], Gregorius PM Luyten[1], Annelies de Klein[1,2]

From the Departments of [1]Ophthalmology, [2]Cell Biology & Genetics, [3]Clinical Genetics and [4]Ophthalmopathology, Erasmus University Rotterdam, The Netherlands and [5]The Rotterdam Eye Hospital, The Netherlands

Submitted for publication

ABSTRACT

In uveal melanoma specific chromosomal abnormalities are known to correlate with the risk of metastases. Usually, karyotyping and fluorescence *in situ* hybridization (FISH) analysis are used to detect these abnormalities and recent publications indicated that loss of chromosome 3 and gain of chromosome 8q correlate strongly with a decreased survival of the patient, whereas chromosome 6 abnormalities are associated with a better prognosis. However, the evaluation of these chromosomal changes can be compromised in patients treated with eye-retaining treatment protocols due to the lack of tumor material.

In order to validate the use of fine needle aspiration biopsies (FNABs) and FISH for the analysis of genetic prognostic markers, we analyzed 40 of uveal melanoma biopsies and the corresponding main tumor with FISH. When possible cytogenetic analysis of the tumor was also performed.

All biopsies were found to contain tumor cells and FISH analyses of the samples were successful in all cases. Statistical analysis showed a very good agreement between the FISH results from the biopsies and those from the main tumor. In only 2 out of 249 hybridizations (0.8%) did we find a small variation that could have led to a misclassification. In one case, cytogenetic analysis pointed to relative losses of chromosome region 1p36 and chromosome 3 in a triploid tumor.

Our results indicate that the application of FISH to fine needle aspiration biopsies is a reliable method for assaying genetic prognostic parameters such as chromosome 3 loss and/or chromosome 8q gain. Implementation of this method in a diagnostic setting means that we are able to identify patients at risk of developing metastatic disease not only in enucleated patients but also in cases treated with conservative treatment modalities such as radiotherapy.

INTRODUCTION

Uveal melanoma is the most common primary intraocular tumor with an annual incidence of 0.7/100,000 in the Western population (Egan et al., 1988). Although less than 2% of the patients have clinically detectable metastases at presentation, approximately 50% of all patients ultimately die of metastatic disease. The median survival after the diagnosis of metastasis is extremely poor. No effective treatment exists for metastatic disease, but new approaches for the management of metastases, involving interferon, interleukin and a combined chemo- and immuno-therapy are under study (Albert et al., 1992; Nathan et al., 1997).

The predictive value of classic histological prognostic parameters such as tumor size, vascular patterns and histologic cell type has been analyzed in several retrospective studies. Additional clinical parameters associated with a poorer prognosis are extrascleral growth, tumor location (ciliary body), older age and male gender (Mooy and De Jong, 1996). Prescher et al. (Prescher et al., 1996) showed that loss of chromosome 3, in comparison to tumor location or tumor diameter, is a better prognostic parameter of relapse-free and overall survival. Sisley et al. (Sisley et al., 1997) confirmed the prognostic value of monosomy 3 and, in addition, demonstrated a strong inverse correlation between the presence of additional copies of 8q and survival. In contrast, patients with tumors having chromosome 6 abnormalities appear to have a better prognosis (White et al., 1998).

Although enucleation is still indicated in large uveal melanoma, radiotherapy (such as proton beam irradiation or plaque therapy, with or without transpupillary thermotherapy) has become the first choice of treatment for patients with smaller melanomas (Freire et al., 1997). A clear advantage for the patient is saving the eye and vision, but the lack of histological specimens compromises the evaluation of the prognostic markers. Since fine needle aspiration biopsies (FNABs) have been used successfully for several years for intraocular tumor diagnosis (Char et al., 1991; Shields et al., 1993), the analysis of FNABs offers an attractive and safe alternative in these cases.

In the present study we have investigated the possibility of applying fluorescence *in situ* hybridization (FISH) analysis to FNABs. FNABs were obtained *ex vivo*, i.e. after enucleation of the tumor-containing eye, and directly used for FISH analyses. To investigate whether these FNAB samples are a good representation of the main tumor, larger samples of the main tumor were processed for direct FISH analysis and conventional cytogenetics. The results of the FNAB were compared with those of the main tumor.

MATERIAL AND METHODS

Patients and tumor samples

From January 1997 to December 1999 we have collected FNABs and matched tumor material from forty uveal melanoma patients. These patients were referred for enucleation of the affected eye to the Department of Ophthalmology, University Hospital Rotterdam and the Rotterdam Eye Hospital. Informed consent was given prior to enucleation and the study was performed according to the tenets of the Declaration of Helsinki. The mean age was 60.5 years (range 34-85); 19 patients were male and 21 female. The mean tumor diameter was 12.9 mm. Five tumors were derived from the ciliary body and 35 from the choroid. Immediately after enucleation transvitreal FNABs were taken and both the FNABs and fresh tumor material were processed for FISH and/or cytogenetic analysis according to standard procedures. Histopathological diagnosis and treatment of the patient were not compromised by biopsy collection. Cytogenetic studies were carried out on peripheral blood samples of each of the patients to exclude the presence of congenital balanced chromosome abnormalities.

Fine needle aspirations biopsies

After enucleation the bulbus was first transilluminated to define the location of the tumor. A 25-gauge needle attached to a 10 ml syringe was inserted through the sclera into the tumor and suction was applied to the syringe. After sampling, the pressure was equalized before removal of the needle in order to avoid seeding of tumor cells. Cells were collected in culture medium, fixed in methanol/acetic acid and FISH preparations were made as described (Hagemeijer et al., 1998).

Fresh tumor material

Tumor specimens, collected after *ex vivo* FNABs, were processed as described (Luyten et al., 1996). For direct FISH, one ml of the cell suspension was fixed and the remaining cell suspension of the tumor was cultured. For cytogenetic analysis, cells were incubated with colcemid (0.15 μg/ml) for 6 hours at 37 °C and fixed. The chromosome preparations were stained with acridine orange or atebrine to obtain R or Q banding. Cytogenetic abnormalities were described in accordance with the ISCN (Basel, 1995). Remaining cultures were stored in N_2 and in one instance (EOM-121) used for additional FISH studies. FISH analysis on metaphases using locus specific probes and whole chromosome paints was carried out to further characterize the abnormalities in cases where cytogenetic analyses revealed the presence of partially defined chromosomes.

Fluorescence *in situ* hybridization

Dual color FISH was performed using centromeric and locus specific cosmid, P1 or YAC probes for chromosome 3 and 8. Probes for chromosome 1 and 6 were used if sufficient material was available. FISH was performed using standard procedures (Hagemeijer et

al., 1998). The concentration for centromeric probes was 5 ng per slide; for cosmids, P1 and YAC probes 50 to 75 ng per slide were used. The probes used were: p1-79 (1p36), Pα 3.5 (centromere 3), YAC 827D3 (3q24), cos85 (6p21) and cos52 (6q23) (Prof. Y Nakamura, Tokyo, Japan), Cos105H8 (8p11) and D8Z2 (centromere 8) and ETO (8q22). After hybridization and washing, slides were counterstained with 4',6-diamidino-2-phenylindole and mounted in anti-fade solution (Dabco-Vectashield 1:1). Signals were counted in 300 interphase nuclei according to the criteria of Hopman et al (Hopman et al., 1988). Cut-off limits for deletion (15% of the nuclei with one signal) or amplification (>10% of the nuclei with 3 or more signals) were adapted from the available literature (van Dekken et al., 1990). Variation found by FISH (in FNAB and the main tumor) were subdivided into 5 categories (Fig. 4.1): loss of one copy, normal copy numbers (two copies), gain of one copy, gain of two copies, and gain of more than two copies. When different subclones were identified, only the FISH findings of the largest clone were used to classify the material.

Statistical analysis

To evaluate the agreement between the FISH results obtained from the FNAB and those obtained from the direct preparations of the tumor, we calculated the overall kappa (κ), as well as the kappa of all probes separately. Kappa below 0.20 was considered as a poor strength of agreement, 0.21-0.40 as fair, 0.41-0.60 as moderate, 0.61-0.80 as good and 0.81-1.00 as very good. The Wilcoxon's matched pairs signed rank sum test was used to compare the number of FISH probes successfully used on the FNAB samples with the number of probes used on the direct preparation.

RESULTS

Figure 4.1 shows the results of the FISH analyses performed on forty FNAB specimens taken from enucleated eyes of uveal melanoma patients and the corresponding primary tumors. The findings of the conventional karyotype studies on the primary tumors are given in Table 4.1. Examples of karyotype analysis and FISH of patient EOM-121 are shown in Figure 4.2. In this case no direct preparations could be made because of shortage of material and a short-term culture (passage 1) was used for FISH and karyotype analysis of the main tumor.

TABLE 4.1 RESULTS OF THE CYTOGENETIC ANALYSIS OF THE MAIN TUMOR.

TUMOR	CYTOGENETIC ANALYSIS OF MAIN TUMOR (ISCN, 1995)
EOM 121	46-47,XY,del(1)(p31p36),-3,der(4)t(1;4)(q12;q21),+8,+21[cp3]/45,X,-Y[3]/46,XY[8]
EOM 123	46,XY[22]
EOM 125	72-76,XXX,dic(1;7)(p10;p14),+dic(1;7)(p10;p14),-3,+4,+6,-7,+i(8)(q10),+9,-11,-15,+16,+18,+20, +?21,+22[cp12]/49-54,idem [cp2]
EOM 130	41-48,XX,der(1)t(1;6)(p11;p?2),add(4)(q1?2),-5,-6,+7,+8,+8,-9,add(11)(q13~14),add(11)(q13~14),-13, -16,+22, +mar,ish der(6)(wcp6+),+mar,ish der(16)t(6;16)(wcp6+,wcp16+) [cp6]
EOM 131	46,XX[11]
EOM136	41-44,XX,der(1;8)(q10;q10),ish;der(1;8)(wcp8+,2053b3+,p1.164+,D8Z2+,puc1.77+,wcp1+),-3, +der(8),ish;der(8)ins (p?21q?23q?24.1)del(8)(q22q22)(wcp8+,114C11+,105H8+,p1.164+,2053b3+),-15,del(16)(q11q1?3),ish;del(16) (wcp16+,pHUR195-)[cp15]
EOM141	46,XY,+2,dic(6;13)(q12;p10),dic(6;14)(q12;p10)[16]
EOM147	44~47,XY,del(1)(p2?)[5],add(7)(p?2)[4],+9[2],-15[4],add(19)(q1?3)[3],+mar[2][cp6]/46,XY[3]
EOM148	47,XY,+?der(2)[2]/46,XY,add(8)(q10),der(15)t(1;15)(q11;p11)[1]/45,X,-Y[3]/ 46,XY[10]
EOM150	47,XY,+9,der(10)t(6;10)(p12;q26)[4]/47,XY,+9[3]/46,XY[4]
EOM151	46,XX,der(20)t(6;20)(p12;p12)[5]/47,idem,+8[4]/47,idem,+8,psudic(17;15)(p13;p11)[3]/46,XX[3]
EOM152	45~48,XX,-3,i(8)(q10),+i(8)(q10),+i(8)(q10)[cp5]/47~49,XX,+3[3],+6[2],+6[2][cp4]/46,XX[4]
EOM157	47,XX,+8[7]/46,XX[10]
EOM158	45,X,-Y[11]/46,XY[4]
EOM 159[a]	40~46,XX,der(2)t(2;6)(q3?4;?),der(5)t(5;6)(q34;?),del(6)(q?)[3], der(7)t(7;11)(p21;?)t(8;11)(q?;?), der(10)t(8;10) (?;p1?4),der(11)t(8;11)(?;q1?4),der(16)t(8;16)(q?;q24)[7], der(18)t(17;18)(?;q23)[cp19]/46,XX[1]
EOM 160	40~42,XX,del(1)(p21),-3,-6,i(8)(q10)[1],-12,-18[cp5]/47,XX,del(1)(q?),der(1)t(1;8)(p?;q?),+7,-8,-9,del(11p)[1]/46,XX[10]
EOM165[a]	40~46,XY,der(6)t(6;6)(q16;?),der(7)t(1;7)(q12;q36),+8[cp20]
EOM166	47,X,-X,-3,+7,i(8)(q10)[1]/idem tetraploid[1]/46,XX[4]
EOM174	75,XXY,add(1)(p),-3,+i(6)(p),i(8)(q),add(9)(p)[1]
EOM177	45~47,XY,-5[2],+8[2],add(8)(p22),+add(8)(p22)[4],-9[2],del(13)(q?14q?21),der(17)ins(17;13) (q12;?q14q21)del(17)(q22q23),-19[2],-22,+mar[cp7]
EOM178[a]	45~47,XX, der(1)t(1;15)(p11;?),der(5)t(5;14)(p?;?), der(7)t(6;7) (?;q36),+8[2],-14[5],-15,der(17)t(7;17)(?;p12)t(6;7)(?;?),+ r(5) [cp10]
EOM179	46,XY[15]
EOM180	43~45,X,-X,-3,i(8)(q10),+i(8)(q10),+i(8)(q10)[2],der(16;21)(q10;q10),der(22)[1][cp5]/tetraploid,idem, inc[2]
EOM182[a]	46,XX,der(6)t(6;6)(q16;p12), der(22)t(8;22)(?;p11), der(22)t(6;22)(?;q13)[16]
EOM187	45,X,-Y,-3,-4,i(8)(q10),+i(8)(q10),+mar[1]
EOM189	44,XY,-1,-2,-3,dic(1;6)(q10;q10),+ring[5]/45,idem,+8,[12]/90,XXYY,idem, +8,+8[1]

[a]In these cases conventional cytogenetics were supplemented with spectral karyotyping (Naus et al., 2001)

FISH results on the FNABs versus direct preparations of the main tumor.

All FNABs yielded sufficient tumor cells to allow FISH analysis. The number of probes used in the FISH analysis on the FNABs was not significantly different from the number of probes used on the direct tumor preparations (Wilcoxon's matched pairs signed rank sum test p=0.197). In 8 instances all 8 probes could be tested on the FNAB and the main tumor. In 39 out of 40 FNABs, the FISH of both chromosomes 3 and 8 could be analyzed. In one case (EOM138) the FNAB was of low quality and as a result only chromosome regions 1p36 and 3q24 could be analyzed.

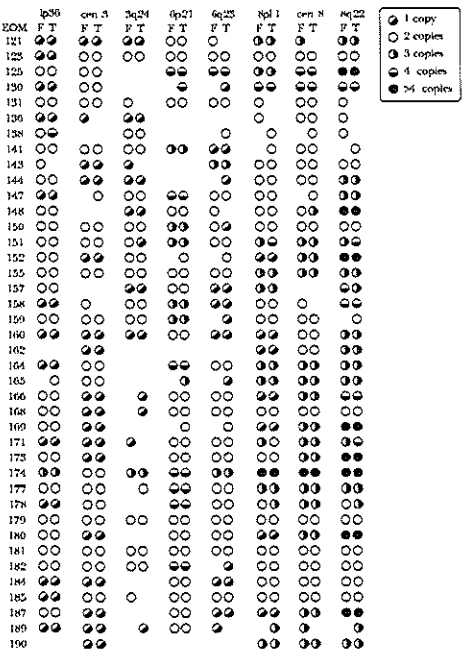


Fig. 4.1 Results of the FISH studies carried out on 40 fine needle aspiration biopsies of uveal melanoma and the corresponding tumor.

In 11/249 hybridizations discrepancies between the FISH results of the FNAB and those of the corresponding tumor were detected (summarized in Table 4.2). In three instances (regions 8p11/8q22 (EOM151), 8q22 (EOM157) this variation was due to the classification (three versus four copies of chromosome 8) and did not represent real differences. In one tumor (EOM171), FISH results of the FNAB suggested an additional chromosome 8 (gain of all chromosome 8 probes), whereas the tumor suggested the formation of an isochromosome 8q (normal chromosome 8p11, gain of centromere 8 and gain of two copies of chromosome 8q22). Thus, in both cases, gain of chromosome 8q was observed. In 6/249 hybridizations FISH showed normal copy numbers in the FNAB but abnormal numbers in the tumor samples. In 5 of these 6 hybridizations

(EOM148 (centromere 8), 150 (6q23), 151 (3q24) and 178 (8p11/8q22)) this concerned small abnormal subclones (12-17% of nuclei) that were detected in the main tumor, whereas they remained undetected in the FNAB. In EOM138 a subclone of 32% of nuclei showing gain of chromosome region 1p36 in the main tumor was not found when analyzing the FNAB. To investigate the agreement between the FISH results of the FNAB and those of the direct tumor preparation, we calculated the weighted kappa of all probes separately and an overall weighted kappa. The overall weighted kappa was 0.95 (range for the probes separately 0.90-1.00), indicating a very good agreement between the FISH results obtained from FNABs and those obtained from the main tumor.

TABEL 4.2 FISH RESULTS OF CASES WITH DISCREPANCIES BETWEEN FNAB AND TUMOR[A]

TUMOR	LOCUS	MATERIAL[B]	COPY NUMBER DETECTED BY FISH (% IN FNAB/ TUMOR)[C]				
			1	2	3	4	>4
EOM138	1p36	F/T	2 / 0	88 / 61	1 / 6	8 / 32	1 / 1
EOM148	cen 8	F/T	0 / 2	90 / 83	2 / 12	8 / 3	0 / 0
EOM150	6q23	F/T	4 / 17	91 / 77	3 / 6	2 / 0	0 / 0
EOM151	3q24	F/T	5 / 16	79 / 83	10 / 1	6 / 0	0 / 0
	8p11	F/T	0 / 0	22 / 8	41 / 15	36 / 77	1 / 0
	8q22	F/T	0 / 0	23 / 6	35 / 10	29 / 65	13 / 19
EOM157	8q22	F/T	0 / 0	59 / 60	15 / 25	26 / 15	0 / 0
EOM171	8p11	F/T	0 / 0	8 / 96	91 / 3	1 / 1	0 / 0
	8q22	F/T	0 / 0	7 / 2	89 / 13	4 / 84	0 / 1
EOM178	8p11	F/T	2 / 0	98 / 87	0 / 12	0 / 1	0 / 0
	8q22	F/T	0 / 0	96 / 83	4 / 15	0 / 2	0 / 0

[a] only cases in which differences in chromosome copy numbers were observed between FNAB and corresponding tumor are indicated in this table.

[b] F, FNAB; T, tumor

[c] percentage of cells in FNAB / tumor showing 1, 2, 3, 4 or >4 FISH signals, respectively, for the investigated chromosome locus

FISH results versus cytogenetic analysis of main tumor.

Cytogenetic analysis of the melanomas could be performed in 26/40 cases. We found variation between the FISH findings of the FNABs and main tumors and those expected from the cytogenetic analysis. In most instances (EOM 130, 147, 159, 165, 177, 178 and 182) the variation was due to the presence of partially defined or complex cytogenetic abnormalities and could be resolved by applying metaphase FISH using whole chromosome paints, or spectral karyotyping (Naus et al., 2001). In other cases (EOM123, EOM157 and EOM158) normal karyotypes were found after culturing, whereas FISH revealed abnormalities. In two cases (EOM174 and EOM187) only one metaphase could be analyzed due to poor tumor growth in vitro, and in 5 other cases (EOM148, EOM150, EOM151, EOM152 and EOM160) minor variations were found between the cytogenetic and FISH results. In EOM125 the FISH analyses revealed 2 copies for the chromosome 1p36 and centromere 3 probes. However, cytogenetic studies showed a triploid karyotype with only two copies of chromosome region 1p36 and

disomy for chromosome 3. This indicates a relative loss of these chromosome regions in this tumor, although the FISH had shown no abnormalities.

DISCUSSION

In this study we describe the application of fluorescence *in situ* hybridization for determining of the presence of genetic abnormalities in fine needle aspiration biopsies of uveal melanoma. The results of our study indicate that the tumor cells obtained by the FNAB are representative of the main tumor and that the chromosomal aberrations detected by FISH in these FNAB specimens are concordant with the major clonal genetic changes observed by conventional cytogenetics or FISH analysis of the main tumor.

In 249 out of 293 hybridizations we investigated both FNAB and the matched tumor and statistically we found a very good agreement between the FISH results of the FNAB and those of the main tumor. Variation between these results was observed for individual probes in 11 hybridizations, a total of seven cases. However, because more than one probe had been used for the identification of each chromosome, in six of these tumors (EOM148, 150, 151 (chromosome 8), 157 ,171 and 178) the presence of prognostically significant genetic variation could still be established. The results of only in 2 out of 249 hybridizations (0.8%) would have led to the misclassification of the tumor. In EOM138 we only observed a gain of chromosome region 1p36 in 32% of the main tumor cells and not in the FNAB. Gain of chromosome 1 is a very rare finding in uveal melanoma and the prognostic relevance of this abnormality is unknown. In EOM151 only the main tumor showed a subclone with loss of chromosome region 3q24, whilst the centromeric probe for chromosome 3 showed normal copy numbers in both tumor and FNAB samples. In one case, EOM125, the relative loss of chromosome 1p36 and chromosome 3 could not be demonstrated by FISH alone (both in tumor and FNAB) and cytogenetic analysis was necessary to reveal the hypertriploid karyotype. The identification of such cases could be improved by the use of a reference probe for DNA ploidy, for example centromeric probes for chromosomes 2, 12, or 13, chromosomes, which are rarely involved in numerical abnormalities in uveal melanoma.

The differences between FISH and cytogenetic analysis can be explained by culture artifacts since the overgrowth of normal cells is not rare when culturing tumor cells. Additionally, FISH has an increased level of resolution for the detection of genetic abnormalities that may not be visible by conventional chromosome studies.

Sisley recently demonstrated, using cytogenetic techniques, that all major clonal alterations were detectable in both FNAB and the main tumor (Sisley et al., 1998). They showed that with short time cultures of FNABs, conventional cytogenetic analysis was possible in 60% of the cases. The advantage of FISH for identifying genetic variation, as we have demonstrated in the present study, is that it is easier to perform and the risk of selecting particular cell populations during culture is avoided.

Folberg et al. (Folberg et al., 1985) found only a modest correlation (r=0.57) between the standard deviation of the nucleolar area measured in FNABs and that measured in the matched enucleation sample and concluded that the FNABs were unsuitable for determining prognosis using these parameters. However, the differences in sample size between tumor (200 cells per sample) and FNAB (50 cells per sample) in their study could explain their results.

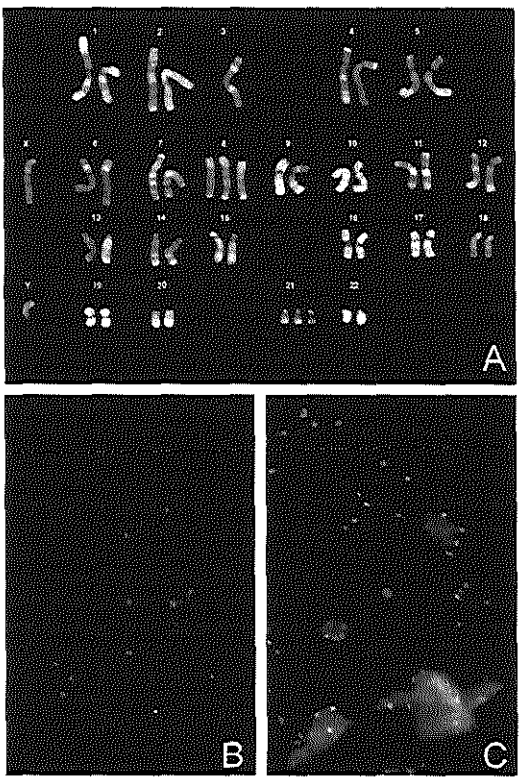


Fig. 4.2 Examples of cytogenetic and FISH analysis in uveal melanoma and FNAB. A) Representative karyotype of patient EOM121: 47 XY,del(1)(p31p36),-3,der(4)t(1;4) (q12;q21), +8,+21. B) Nuclei isolated from short-term culture of the tumor specimen hybridized with chromosome 3q24 (green) and 1p36 (red) probes. C) Nuclei isolated from FNAB hybridized with probes for centromere 3 (green) and centromere 8 (red).

One must keep in mind that these FNAB were performed *ex vivo* under ideal circumstances and we were able to use up to 8 probes to compare the FISH results of the FNAB with those of the matched tumor. When these biopsies are taken under less favorable i.e. *in vivo* conditions, FISH analysis using probes for chromosome 3 and 8 will be sufficient to predict the outcome of the patient since it is generally accepted that variation of these chromosomes are of high prognostic significance.

Up until now, no specific regions or genes on chromosome 3 and 8 involved in the tumorigenesis of uveal melanoma have been identified. However, from our comparative genomic hybridization and spectral karyotyping study, we do have indications that region 8q21.1~21.2/8q23~24 and 3q13~q21 may be involved (Naus et al., 2001). Should these regions prove to be the target regions, more specific FISH probes can be designed in order to improve the predictive outcome of this technique.
Our results clearly demonstrate that FISH analysis of FNAB specimens can be used to examine for the presence of specific chromosomal abnormalities in the tumor cells of uveal melanoma patients. This method is particularly suitable in cases where no tumor tissue has been resected and/or when patients are treated with eye-retaining treatment protocols such as irradiation or radioactive plaque therapy. Detection of these abnormalities provides important additional information for predicting the outcome of these patients and will help to recognize those individuals particular at risk of developing metastatic disease. The earlier detection of metastases and, if available, the introduction of appropriate adjuvant therapies during primary treatment could contribute to a better survival rate of uveal melanoma patients.

ACKNOWLEDGEMENTS

The authors thank Dr Carolien Klaver for her help with the statistical analyses.

CHAPTER

5

FISH ANALYSIS ON FINE NEEDLE ASPIRATION BIOPSIES IN 3 CASES OF SUSPECT UVEAL MELANOMA

Gregorius P.M. Luyten[1] Nicole C. Naus[1,2], Cornelia M. Mooy[1,3], Ellen van Drunen[2], Rosalyn Slater[2,4], Annelies de Klein[1,4]

From the Departments of Ophthalmology[1], Cell Biology & Genetics[2], Pathology[3] and Clinical Genetics[4], University Hospital Rotterdam and the Erasmus University Rotterdam, The Netherlands.

Submitted for publication

ABSTRACT

Objective To investigate whether chromosomal abnormalities can be detected by fluorescent *in situ* hybridization (FISH) on fine needle aspirate biopsies (FNAB) of suspect small pigmented uveal lesions in order to differentiate between choroidal nevus and melanoma.

Methods Transvitreal FNAB of three suspect small melanocytic uveal lesions with presence of clinical high-risk factors and no documented growth, were analyzed by fluorescent *in situ* hybridization (FISH). In addition, diagnostic cytology was performed.

Results FISH revealed extra copies of chromosome 8 in the biopsy sample of one patient who was subsequently treated by enucleation. Histopathology confirmed the diagnosis of malignant melanoma in a pre-existing nevus. Cytology could not differentiate between nevus and melanoma in all cases. In two other biopsies no chromosomal abnormalities were detected by FISH. These patients were followed for two years without any sign of tumor growth.

Conclusion FISH analysis of FNAB should be considered in cases of small melanocytic choroidal lesions to differentiate between nevus and melanoma and to guide the ophthalmologist in the management of small melanocytic uveal lesions.

INTRODUCTION

Management of small melanocytic choroidal lesions has been a matter of controversy for a long time. In the recent past, observation of small melanocytic lesions was advised until tumor growth was documented. At present, early treatment is advised (Shields and Shields, 1993). Shields found in a large series of small melanocytic choroidal lesions that tumor thickness greater than 2 mm, posterior margin touching the disc, visual symptoms, orange pigment and subretinal fluid, predict an increased risk for tumor growth up to 56% if all risk factors are found (Shields et al., 1995; Shields et al., 2000). Risk factors for distant metastases are tumor thickness, documented growth, distance to the optic disc, and ocular symptoms (Shields et al., 1995). These observations can guide the ophthalmologist in deciding whether to treat the patient or not (Shields et al., 1995; Shields et al., 2000). The overall risk of developing metastatic disease from a small melanocytic choroidal lesion is 3% within 5 years. However, patients with a tumor carrying all high-risk factors do have 19% risk of developing distant metastases (Shields et al., 1995). In a meta-analysis of small melanomas a mortality rate of 16% was found after five years (Diener-West et al., 1992). Despite the definition of these risk factors, the distinction between a benign nevus and a malignant melanoma remains difficult.

Cytogenetic studies on enucleated eyes of patients with a uveal melanoma have revealed that specific chromosomal abnormalities, i.e. loss of chromosome 3 and gain of chromosome 8q, are associated with poor prognosis (Prescher et al., 1996; Sisley et al., 1997; White et al., 1998). In most of the studies, classic cytogenetic analysis has been used to investigate the chromosomal abnormalities which is informative in only 60 to 70% of the cases. Fluorescent *in situ* hybridization (FISH) can be used to study the presence of chromosomal variation in interphase nuclei. We recently demonstrated that FISH is suitable for the detection of chromosomal abnormalities in paraffin-embedded tissue and fine needle aspiration biopsies of uveal melanoma. FISH is informative in 100% and chromosomal abnormalities are detected in 81% of the cases (Naus, submitted).

In the present study, three patients with a small melanocytic choroidal lesion are described, who presented with clinical risk factors for tumor growth and metastasis but without documented tumor growth over a prolonged period of time. Because of these discrepancies, we performed transvitreal fine needle aspiration biopsies (FNAB) to investigate the nature of these lesions by cytology. Since only a small sample of material is needed for FISH, FISH analysis was performed on the FNAB before therapeutic interventions were considered.

PATIENTS AND METHODS

In three patients, referred to our clinic between 1992 and 1994, a suspected melanocytic lesion was found and at that time, observation was advised. After re-evaluation of these patients between October 1997 and March 1998, we decided to perform transvitreal FNAB in order to differentiate between a benign choroidal nevus or a small uveal melanoma and to assess chromosomal abnormalities in order to predict the risk of metastases. Prior to FNAB an oncologic examination was performed in all patients including indirect ophthalmoscopy, fluorescent angiography (FAG) and an A- and B-scan ultrasonography (USG). To exclude distant metastases or other primary tumors, the patients were examined by the oncologist, including X-thorax, liver ultrasonography and blood tests. All patients gave informed consent.

Transvitreal FNAB procedure

A pars plana vitrectomy was performed under general anesthesia with the use of a three port vitrectomy system. All vitreous in front of the tumor was removed. For the biopsy, a special 25 gauge biopsy needle connected to a 20-ml syringe via a silicon tubing was used (Medical Workshop, Groningen, The Netherlands) (Figure 5.1). After perforating the retina the needle was inserted into the tumor and careful suction was applied. Under high magnification of the operating microscope, the needle was axially rotated with great care to release tumor cells from the tumor, suction was then released and the needle was withdrawn from the eye. The FNAB material was collected in culture medium (RPMI 1640/HEPES supplemented with 10% fetal calf serum and 1% penicillin/streptomycin) to prevent lysis of the cells, and processed for cytology and FISH analysis.

Cytology and FISH analysis

A single cells suspension was prepared from the FNAB material. For the cytology study of the specimens, drops of the single cells suspension were placed on a slide, air dried, fixed in methanol) and stained with Giemsa. The rest of the single cells suspension was treated with a hypotonic solution (0.075M KCl), fixed three times with methanol/acetic acid (3:1), and dropped onto clean glass slides which were air-dried overnight. Dual color FISH was performed using specific DNA centromeric and/or cosmide probes for chromosomes 3 and 8 and, when sufficient material was available, also for chromosomes 1 and 6, using standard procedures (Hagemeijer et al., 1998). The centromeric probes Pα3.5 (Waye and Willard, 1989) for chromosome 3 and D8Z2 (Donlon et al., 1987) for chromosome 8 were used at concentration of 5 ng per slide. The cosmid probes P1.164 (8q22) (Sacchi et al., 1995), cos52 (6q23) (Prof. Y. Nakamura, Tokyo, Japan) and p1-79 (1p36) (Buroker et al., 1987) were used at higher concentrations of 50 to 75 ng per slide. After hybridization and washing, the slides were counterstained with DAPI and mounted in anti-fade (Dabco-Vectashield 1:1).

Monosomy was defined when >15% of the cells showed loss of one signal; polysomy when >10% of the cells showed gain of a copy.

REPORT OF CASES

Case 1

A 47-year-old woman complained of an irritating right eye at consultation in October 1994. At that time the visual acuity was finger counting in the right eye and 20/20 in the left eye. A melanocytic lesion with a tumor diameter of 7.5 mm was found in the macula region, 1.5 mm from the optic disc. The melanocytic lesion showed drusen and subretinal fluid, but no orange pigment (Figure 5.2a). The USG showed a tumor thickness of 3.8 mm with a low internal reflectivity and a choroidal excavation. The tumor was clinically diagnosed as a lesion suspect for a small choroidal melanoma, but no treatment was applied. The subretinal fluid was treated by low dose radiotherapy (15 Gy in five fractions). During 3 years of follow-up, progression of the lesion could not demonstrate by serial FAG and USG. In October 1997 the patient was re-evaluated and it was decided to perform a transvitreal biopsy because of high risk of metastatic disease (tumor thickness > 3 mm, ocular symptoms and distance to the optic disc < 3 mm). Furthermore, local subretinal fluid did not respond to low dose radiotherapy.

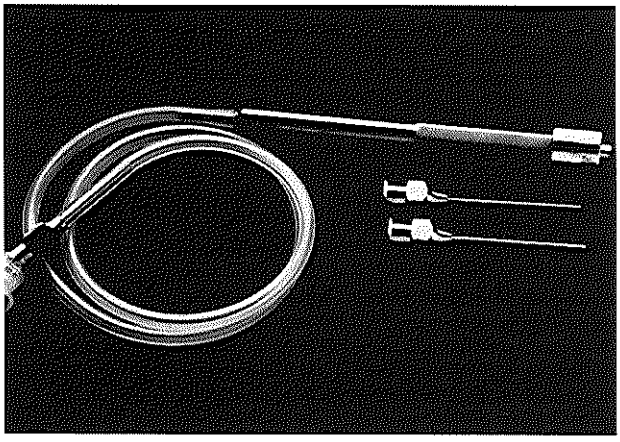


Fig 5.1 A 25 gauge biopsy needle connected to a 20-ml syringe via a silicon tubing

Case 2

A 66-year-old woman presented in 1992 with a 9 mm melanocytic lesion in the inferior-temporal quadrant, 1.5 mm from the fovea of her left eye. The tumor showed some drusen and subretinal fluid but no orange pigment (Figure 5.2b). Her visual acuity was 20/20 in both eyes. The tumor showed a tumor thickness of 3.8 mm with low-reflectivity

and choroidal excavation as measured by USG. Although treatment was considered, the tumor showed no progression on examination by serial FAG and USG; therefore observation was continued. In March 1998 the patient was re-evaluated and was considered as having increased risk of growth (based on tumor thickness and distance to the fovea). However, tumor growth could not be demonstrated and the patient had no visual complaints. At that time it was decided to perform a FNAB to verify the malignant nature of this tumor.

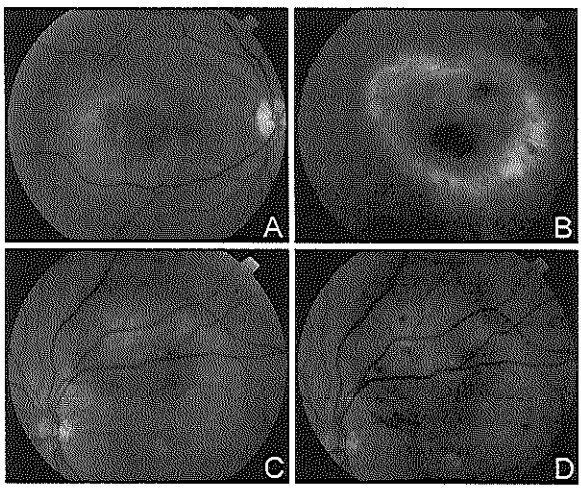


Fig 5.2 Fundus photographs of case 1, 2 and 3. A) Case 1. Right fundus October 1994; B) Case 2. Fundus photograph of the left eye C) Case 3. Fundus photograph of the left eye; D) Case 3. Choroidal nevus 1 day after FNAB; notice limited pre-retinal blood

Case 3

A 48-year-old woman was referred to our department in 1992 with a melanocytic lesion in the posterior pole of the left eye. The tumor had tumor diameter of 7.5 mm, showed subretinal fluid and was located 2 mm from the optic disc (Figure 5.2c). The tumor thickness was 2.8 mm as measured by USG. Her visual acuity was 20/20 in the right eye and 20/60 in the left eye. Tumor growth could not be demonstrated and observation was advised. At the time of re-evaluation in January 1998, a transvitreal biopsy was advised because of the tumor size, the subretinal fluid, visual symptoms, and distance to the optic disc and fovea. One-day postoperatively, some pre-retinal blood was found, but this subsided after 1 week (Figure 5.2d).

RESULTS

Case 1

The cytology specimen showed partly cohesive cells and single cells. All cells showed a polygonal appearance with round to oval nuclei and abundant cytoplasm. Melanocytic granules were scattered within the cells. Prominent nucleoli could not be demonstrated (Figure 3a). Three spots of the centromeric region of chromosome 8 were found in 26 of 100 nuclei (Figure 3b), with no loss of chromosome 3 (Table 5.1). Gain of chromosome 8 is suspect for malignant transformation and therefore prompt treatment was advised. The tumor-containing eye was enucleated and histopathologic examination confirmed the diagnosis choroidal melanoma. The tumor contained spindle cells as well as epithelioid cells in a pre-existing nevus (Figure 3c). FISH analysis on the tumor specimen confirmed the FISH findings of the FNAB. After 36 months of follow-up the patient showed no local recurrences or distant metastases.

TABLE 5.1 COPY NUMBERS CHROMOSOMAL REGIONS DETECTED BY FISH

CASE 1							CASE 2				CASE 3			
FNAB				TUMOR			FNAB				FNAB			
PROBE	NO. CELLS	COPY NUMBER	%	NO. CELLS	COPY NUMBER	%	PROBE	NO. CELLS	COPY NUMBER	%	PROBE	NO. CELLS	COPY NUMBER	%
		1	1		1	6			1	4			1	8
cen 3	100	2	87	50	2	92	1p36	142	2	96	1p36	38	2	89
		3	2		3	0			3	0			3	0
		4	10		4	2			4	0			4	3
		1	1		1	0			1	3			1	3
		2	62		2	64			2	95			2	94
cen 8	100	3	26	50	3	34	cen 3	121	3	1	cen 3	37	3	0
		4	2		4				4	1			4	3
		>4	9		>4									
					1	1			1	4			1	5
6q23				100	2	92	cen 8	121	2	94	cen 8	37	2	92
					3	6			3	0			3	0
					4	0			4	2			4	3
									1	0			1	3
									2	10			2	94
							8q22	142		0	6q23	38		
									3	0			3	0
									4	0			4	3

Case 2

On cytological examination, the major part of the cells was disrupted and therefore cell shape could not be determined. The background showed melanocytic granules. The nuclei were round to oval without prominent nucleoli. Malignant transformation of this tumor could not be demonstrated by cytology. FISH analysis (Table 1) showed no chromosomal abnormalities for chromosome 1, 3, and 8. Thus no chromosomal abnormalities indicating a high risk for metastases were detected. After 32 months of observation there are no signs of tumor growth or distant metastases.

Case 3

Cytology showed abundant melanocytic granules with normal appearing nuclei. FISH analysis on only 37-38 nuclei revealed no chromosomal abnormalities for chromosome 1, 3, 6, and 8 (Table 5.1). Treatment was not applied. Thirty-five months after the procedure tumor growth or distant metastases could not be demonstrated.

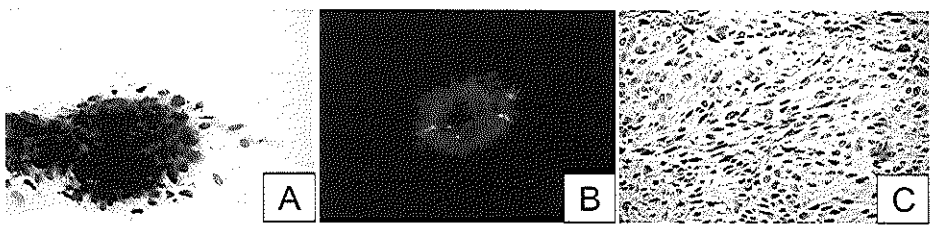


Fig 5.3 Case 1 A) Cytology of FNAB specimen; B) FISH analysis: 3 green spots for chromosome 8 and 2 red spots for chromosome 3; C) Enucleation specimen; epithelioid melanoma cells in a pre-existing nevus.

COMMENT

The distinction between choroidal melanoma and choroidal nevus can be very difficult based on the clinical parameters alone. Clinical risk factors predicting tumor growth and metastasis in small uveal melanoma, can help the clinician in the management of these lesions (Shields et al., 1995; Shields et al., 2000). Due to improvement in the knowledge about defining patients at risk and the increased experience in specialized oncology units, the rate of misdiagnoses has been decreased to less than 0.5%. In our patients clinical differentiation between choroidal nevus and small melanoma could not be made. At the time of presentation, observation was advised unless documented growth could be demonstrated. After three, five and six years, respectively of follow-up tumor growth could not be demonstrated. The present clinical practice, however, advocates treatment of small melanocytic tumors based on the ocular symptoms, tumor thickness, subretinal fluid, orange pigment, and distance to the optic disc and fovea.

The discrepancy between the presence of clinical risk factors for growth and metastases on one hand and the absence of growth over a three to six year period on the other hand, caused a therapeutic dilemma in our patients. Therefore, we decided to perform a transvitreal fine needle aspiration biopsy (FNAB) in these patients for cytological examination and to investigate the presence of chromosomal abnormalities by FISH analysis.

Cytological examination was not conclusive in all three patients which is in accordance with the relative contraindication for FNAB of a suspected intraocular tumor to differentiate between a large nevus and a small melanoma (Shields and Shields, 1993; Char and Miller, 1995; Char et al., 1996). However, FISH analysis was helpful in all cases: In first patient FISH analysis showed extra copies of chromosome 8q in the FNAB. Since in 81% of uveal melanomas chromosomal abnormalities can be found and extra copies of chromosome 8q are associated with high risk for metastatic death in uveal melanoma, prompt treatment was applied. Although radiotherapy was advised the patient preferred to have an enucleation. The FISH analysis of the paraffin-embedded tumor confirmed the findings of the FISH on the FNAB. In patient 2 and 3 the FISH analysis did not reveal chromosomal abnormalities. This does not imply that these lesions are benign tumors, since in some cases of large uveal melanoma no chromosomal abnormalities have been detected. We therefore cannot exclude a malignant melanoma based on our FISH findings. However, since no chromosomal aberrations were found, we classified these patients as low-risk for metastatic disease and further observation was advised until tumor growth is documented. Although the follow-up time after the procedure is relatively short (32 and 35 months respectively) no tumor growth or distant metastases have been found in these patients.

Fine needle aspiration biopsy (FNAB) is a safe method of obtaining tumor material from patients with a suspect melanocytic choroidal lesion (Char et al., 1989; O'Hara et al., 1993). Performing FNABs on malignant tumors always raises the question whether there is an increased risk of spreading melanoma cells in the vitreous and at the vitrectomy ports. From previous reports we know that the relative risk for vitreous seeding and recurrences is very rare (Shields et al., 1993; Char and Miller, 1995). In one study, however, the authors did find tumor cells in the fine needle tract in 8 out 15 biopsies (Glasgow et al., 1988). This study was performed on enucleated eyes, without proper visual control under the operating microscope and no vitrectomy was performed prior to biopsy. The risk for serious complications is relatively low(Shields et al., 1993; Char and Miller, 1995). In a larger series no retinal detachments were documented. Subretinal and vitreous hemorrhages are described, but are of no significance after one week postoperatively. Only in case 3 we did experience some pre-retinal blood, which subsided after one week.

FISH analysis is a reliable method for detecting the abnormalities in chromosome 3 and 8 in FNABs. At our institute, *ex vivo* FNABs (after enucleation or local resection) in a series of 40 patients were informative in 100% of the cases and the FISH analysis of the FNAB were concordant with the FISH analysis in the whole tumor (Naus, submitted). In a recent study, Sisley and coworkers, found also chromosomal abnormalities in FNAB with a cytogenetic analysis with short time culture (Sisley et al., 1998). FISH has been used in one other study of McNamara for the detection of loss of chromosome 3 in fresh uveal melanoma specimens (McNamara et al., 1997).

These three cases illustrate that FISH analysis on FNAB can be helpful in the management of patients with a small melanocytic choroidal lesion resulting in reduced morbidity. Furthermore, FISH analysis on FNAB samples from classified uveal melanoma could be used to improve the prognostic accuracy prior to treatment. Char et al, in a study of cytopathologic analysis of FNABs before radiotherapy, found a correlation between the detection of epithelioid cells in the FNAB and survival of the patients (Char et al., 1996). By defining the risk of metastases in FNABs, treatment of uveal melanoma might be optimized and adjusted for an individual patient. Whether the detection of these chromosomal abnormalities will eventually lead to a change in the management of uveal melanoma, requires future investigations.

PART 2

MOLECULAR GENETIC ABNORMALITIES IN UVEAL MELANOMA

CHAPTER

6

ESTABLISHMENT AND CHARACTERIZATION OF PRIMARY AND METASTATIC UVEAL MELANOMA CELL LINES

Gregorius P.M. Luyten[1], Nicole C. Naus[1], Cornelia M. Mooy[2], Anne Hagemeijer[3], June Kan-Mitchell[4], Ellen van Drunen[3], Vojislav Vuzevski[2], Paulus TVM de Jong[5] and Theo M Luider[1]

[1]Institute of Ophthalmology and [2]Department of Pathology and [3]Cell Biology and Genetics, Erasmus University Rotterdam, The Netherlands, [4]Department of Pathology, University of Southern California at San Diego, La Jolla, California, USA and [5]The Netherlands Ophthalmic Research Institute, Amsterdam, The Netherlands

International Journal Cancer, 1996;66:380-387

ABSTRACT

We report on the establishment and characterization of two primary (EOM-3, EOM-29) and three metastatic uveal melanoma cell lines (OMM-1, OMM-2, OMM-3), and further cytogenetic characterization of a previously described primary uveal melanoma cell line (OCM-1). Only a few long-term growing primary uveal melanoma cell lines have as yet been established, while of metastatic uveal melanoma cell lines we have found no descriptions. The morphology of the in vitro cultured cells varied from spindle to epithelioid. The cell lines were characterized by immunocytochemistry, electron microscopy and cytogenetical analysis. The relative growth rate was determined by bromodeoxyuridine (BrdU) incorporation. The melanocytic origin of the cell lines was determined by the positive staining with antibodies identifying melanoma associated antigens. Melanosomes and pre-melanosomes were indeed observed by electron microscopy in all cell lines. The stem cell karyotype was found to be normal in three cell lines (EOM-29, OMM-2, OMM-3) and abnormal in three others (EOM-3, OCM-1, OMM-1) showing a net loss of chromosome 6. The OCM-1 and the OMM-1 cell lines even demonstrated a large amount of structural chromosomal aberrations, the former being near tetraploid and the latter triploid. The EOM-29 cell line, cultured from an ciliary body melanoma, did not show the previously described chromosome 3 and 8 abnormalities.

INTRODUCTION

Uveal melanoma is the most common primary intraocular tumour in adults (Egan et al., 1988). These tumours spread haematogeneously and preferentially to the liver. A 50 % overall incidence of metastasis occurs within 15 years after initial treatment by enucleation or radiotherapy of the tumour-containing eye (Kath et al., 1993). After clinical diagnosis of hepatic metastases, median survival is extremely poor: between two and seven months (Kath et al., 1993). The biological and molecular properties of the successive steps involved in uveal melanoma progression and metastasis could be studied in detail once established uveal melanoma cell lines were available. A limited number of cell lines obtained from primary uveal melanoma have indeed been described (Kan-Mitchell et al., 1989; Aubert et al., 1993; Massarelli et al., 1994; De Waard-Siebinga et al., 1995), but not from metastatic uveal melanoma.
In this study, we report on the successful establishment of two primary (EOM-3, EOM-29) and three metastatic uveal melanoma cell lines (OMM-1, OMM-2, OMM-3) in our institute. These cell lines were characterized by immunocytochemistry, light and electron microscopy, and cytogenetic analyses. The relative growth rate was determined by bromodeoxyuridin (BrdU) incorporation. In addition, a previously described primary uveal melanoma cell line, OCM-1 (Kan-Mitchell et al., 1989), was further characterized by extended karyotypic analysis.

MATERIAL AND METHODS

Tumour material

After enucleation of the uveal melanoma containing eye, part of the ocular tumour was taken for cell culture. The eye was further histologically examined to confirm the diagnosis. When, during follow-up of the patient, metastatic disease was suspected, a biopsy was taken for diagnosis and for cell culture. All patients had given full informed consent. Between January 1992 and May 1993, cells of the primary tumours of 16 patients and of the metastatic tumours of four patients were processed for culture. The patients with primary uveal melanoma ranged in age from 22 to 87 (mean: 56.8); nine were female and seven were male; eight patients had a choroidal and eight patients had a ciliary body melanoma; three patients had a spindle-cell tumour, nine had a mixed-cell tumour and four had an epithelioid-cell tumour; tumour diameter ranged from 3 to 22 mm (mean: 11.6 mm). The patients were followed until May 1995; two patients died of metastatic disease and one is alive, 6 months after detection of a metastatic lesion in the subcutis. From all four patients with metastatic disease tumour material

was obtained from sub-cutaneous lesions. From one patient the primary as well as the metastatic tumour were available.

Culturing methods

The tumour material was processed and cultured within one to three hours after dissection as described by Luyten *et al* (Luyten et al., 1993). In brief, the tumour-containing eye or the metastatic lesion was dissected under sterile conditions. A full thickness biopsy was taken and transported in Dulbecco's-modified-Eagle's medium supplemented with 10% fetal calf serum, and 1% penicillin/streptomycin (full DMEM). The tumour material was suspended with a small pair of scissors and strained through a linen cloth by continuous irrigation with full DMEM. The resulting suspension of single cells and to a lesser extent of small clumps of tumour cells, was then washed two or three times with full DMEM. This washing was repeated depending on the amount of pigment. The cells were seeded in a culture flask (Falcon plastic T-30) with 5 ml full DMEM at 5% CO_2 (37°C). After one week the medium was renewed while the non-attached cells were discarded. Subsequently, the culture medium was changed twice a week. At reaching confluence, the cells were detached with 0.05% trypsin and 0.5 mM Na_2.EDTA and subsequently subcultured. Depending on the growth rate the cultures were passaged at 1:2 to 1:10 dilutions.

BrdU incorporation

To measure the logarithmic growth rate of the cells in culture, the cells were incubated in culture flasks for six hours with 1 mM BrdU (Boehringer Mannheim, Almere, The Netherlands). After being washed and detached, the cells were centrifuged onto object glasses, fixed in 2 M HCL at 37°C for 30 minutes and then incubated in 0.1 M borate buffer pH 8.5 at room temperature for five minutes. The cells were subsequently washed twice with PBS and incubated for 30 minutes with a peroxidase-conjugated immunoglobulin of a mouse monoclonal antibody specific for BrdU (Boehringer Mannheim; dilution 1:33) in 1% BSA/PBS. After washing with PBS again, the incorporated BrdU was visualized with 3'3 diaminobenzidine and ureum hydroxide (Sigma, St. Louis, MO). The percentage of BrdU incorporated cells could then be counted in one low power field (10X) using an eye piece grid.

Immunocytochemistry

The cells were cultured on a coverslip, washed twice with PBS at 37°C and subsequently fixed in cold acetone at -20°C for 10 minutes. Endogenous peroxidase was inhibited by incubation in 0.3% hydrogen peroxide/methanol for 15 minutes. Subsequently the coverslip was incubated in 2% fetal calf serum/PBS for five minutes. The primary antibodies, S100 (Dako, Glostrup, Denmark), NKI-C3 (Dako), HMB-45 (Biogenex, San Ramou, CA) and HNK-1 (Becton Dickinson, San Jose, CA) were incubated at dilutions of 1:600, 1:20, 1:50, 1:10, respectively for 1 hour. For S100, swine anti-rabbit peroxidase-conjugated immunoglobulin was used as a second step antibody (Dako; dilution 1:100). For the other three antibodies rabbit anti-mouse peroxidase-conjugated immunoglobulin (Dako; dilution of 1:100) was used. Both secondary antibodies were incubated for one hour. The activity of the peroxidase was then visualized using tablets of 3,3' diaminobenzidine HCL and hydrogen ureaperoxide (Sigma). Finally, the cells

were counterstained with haematoxylin for 10 seconds. Between each of the incubation steps the coverslip was rinsed with 0.1% Tween-20/PBS.

Antibodies

The S100 antibody recognizes an acidic intracellular Ca^{++} binding protein and is a sensitive but non-specific marker for melanoma cells (Moore, 1965). Monoclonal antibody NKI-C3 and HMB-45 binds both a cytoplasmic antigen produced by fetal melanocytes and melanoma cells of adults (Vennegoor et al., 1985; Gown et al., 1986). The monoclonal antibody HNK-1 recognizes a family of cell adhesion molecules (CD 57), migrating neural crest cells and a series of neural crest derivatives including tumours of neural crest origin (Abo and Balch, 1981).

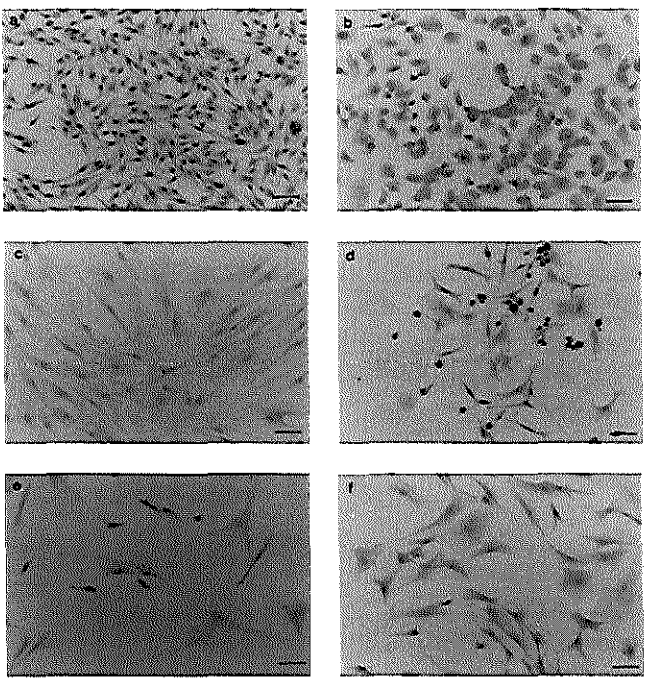


Fig 6.1 Light microscopy (scale bar 50 µm). A) The OCM-1 cell line has small and large rounded cells, with one or more nuclei, growing upon a stellate and elongated cell layer with dendritic extensions; B) The EOM-3 cell line shows a monolayer of plumb polyhedral anaplastic, epithelioid cells with abundant cytoplasm, large nuclei and prominent nucleoli; C) The EOM-29 cell line forms spindle-like patterns with large round nuclei; D) The OMM-1 cell line: cluster of large round cells (mono- and multinucleated), irregularly shaped stellate and elongated cells, very large cells with abundant cytoplasm and cytoplasmic inclusions were observed; E) The OMM-2 cell line showed a monolayer characterised by predominantly elongated cells with large nuclei, abundant cytoplasm and long dendritic extensions, forming a bundle-like, spindle-cell pattern; F) The OMM-3 cell line has spindle-cell

appearance with dendritic extensions; the nuclei are large with abundant cytoplasn and prominent nucleoli.

MHC class I expression

Sub-confluent cell cultures were detached with trypsin/EDTA, after which cytocentrifuge slides could be prepared. The major histocompatibility complex (MHC) class I expression was determined using the standard NIH micro-cytocytoxity test with locally obtained reagents.

Electron microscopy

After detachment by use of trypsin/EDTA, the cultured cells were centrifuged and fixed in 3% glutaraldehyde at room temperature for 30 minutes. Then they were post-osmificated for 30 minutes in 1% OsO4, and subsequently embedded in Epon. Ultrathin sections were prepared and counterstained with uranyl acetate and lead citrate.

Cytogenetic analysis

In order to obtain metaphases, cultured cells in the logarithmic phase of proliferation were treated with colcemid for one to three hours. After trypsinization, the cells were subjected to a hypotonic (0.075 M KCL) solution. Finally the cells were gradually fixed with cold methanol/acetic acid (3:1 v/v). Air-dried slides were banded by the reverse method.

To identify the marker chromosomes, fluorescence *in situ* hybridization (FISH) was used. Slides were then hybridized with chromosome-specific libraries (Pinkel et al., 1988), kindly provided by Dr. J. Gray. The results presented conform to the recommendations of the International System for Human Cytogenetic Nomenclature (ISCN) (Mittelman, 1985).

RESULTS

Primary and metastatic uveal melanoma cell lines

Out of the 16 cultures of the primary uveal melanomas, two cell lines could be established successfully. The remaining cell cultures stopped growing after being passaged two to five times. Of the cultures of the metastatic uveal melanomas, three out of four resulted in a stable growing cell line. The cell cultures from one patient, from whom both the primary and corresponding metastatic tumour were available, were not successful. The patient data and tumour data from the established cell lines are summarized in Table 6.1.

The primary uveal melanoma cell line OCM-1, described by Kan-Mitchell and associates (Kan-Mitchell et al., 1989), has been growing for more than seven years without morphological changes (Figure 6.1a). The EOM-3 cell line was established from a mixed cell type primary uveal melanoma. During the first six passages, the cultured cells had a

spindle cell type appearance. However, a small clone of epithelioid cells started to grow and subsequently overgrew the rest of the tumour cells. The epithelioid-cell culture underwent no further morphological changes and has been growing now for two years (Figure 6.1b). EOM-29 was established from a large extra-ocular extension of a ciliary body melanoma (epithelioid cell type) and could be passaged once a week. Gradually the passage time increased with a decreasing proliferation rate. After passage ten, the cells had a spindle like appearance in culture (Figure 6.1c) and continued to grow without demonstrating changes in their morphology.

TABLE 6.1 PATIENT AND TUMOUR DATA OF SUCCESSFUL ESTABLISHED UVEAL MELANOMA CELL LINES

CELL LINE	PATIENT GENDER/ AGE	SURVIVAL	TUMOUR LOCATION	HISTOLOGY	CELL LINE MORPHOLOGY (LM)	PASSAGE
OCM1	F/nr	nr	Posterior	Spindle B	Mixed	>50
EOM3	M/62	29	Posterior	Mixed	Epithelioid	20
EOM29	M/87	14	Ciliary body	Epithelioid	Spindle	15
OMM1	M/74	343[1]	Subcutis metastasis	Mixed	Mixed	42
OMM2	M/72	40[1]	Subcutis metastasis	Epithelioid	Spindle	23
OMM3	M/72	41[1]	Subcutis metastases	Mixed	Spindle	19

[1] Tumour related death

nr, not reported

A subcutaneous metastatic lesion (mixed cell type) was excised from a uveal melanoma patient (OMM-1), who had undergone an enucleation of his primary tumour 29 years earlier. The primary culture of this metastatic lesion and the first ten passages consisted of adherent and non-adherent cells. Gradually more cells became adherent, showing a heterogeneous morphology of spindle and epithelioid cells. This morphological heterogeneous spectrum remained the same over 2.5 years (passage 42) (Figure 6.1d).
The primary culture (OMM-2) of a sub-cutaneous metastatic lesion (epithelioid cell type) had a spindle cell pattern in cell culture, and did not show morphological changes for 2 years. The cells have been growing slowly but continuously, with a passage time of one month (Figure 6.1e). The most recently established metastatic cell line (OMM-3) was cultured from a large, partly amelanotic sub-cutaneous metastatic lesion (mixed cell type) with a spindle cell appearance in cell culture. This cell line has now been growing for more than 1 year and no morphological changes have been observed (passage 19) (Figure 6.1f)

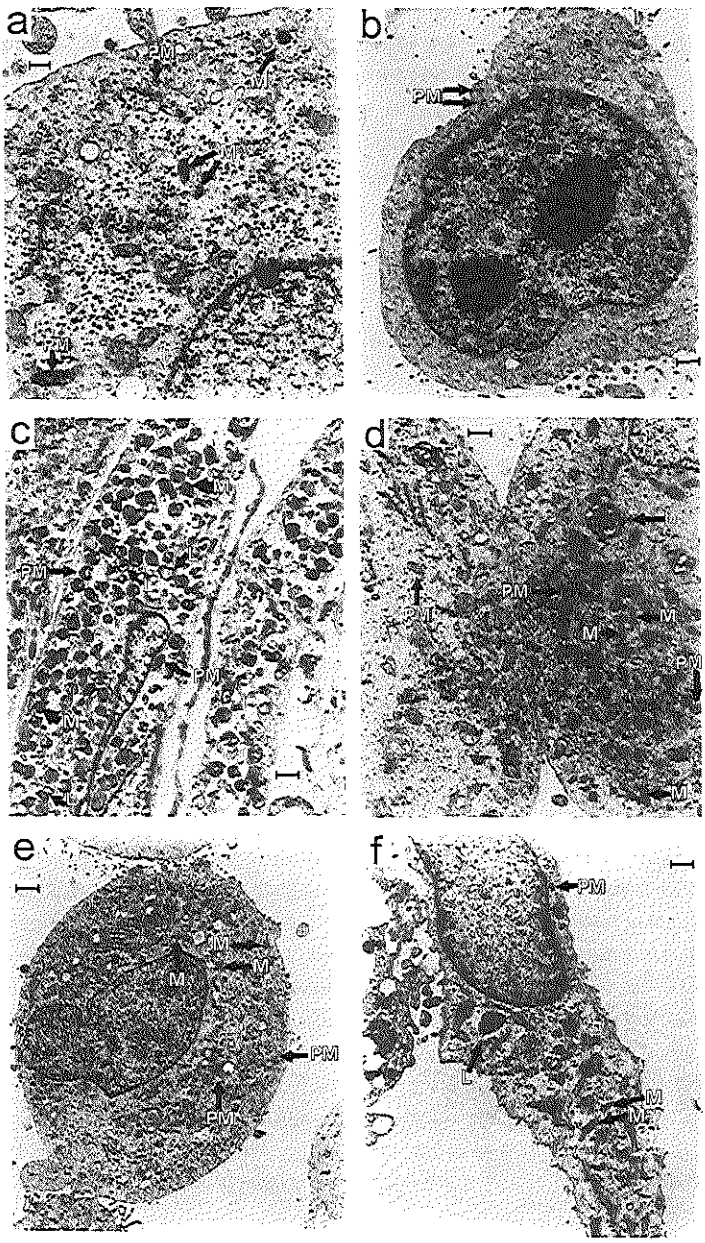


Fig 6.2 Electron microscopy. A) OCM-1: part of an uveal melanoma cell. The cytoplasm contains a few melanosomes (M) and pre-melanosomes type I (PM) and sporadic type II (scale bar, 0.350 μm); B) EOM-3: very primitive, immature cell with a large nucleus and 2 prominent nucleoli. Only a few pre-melanosomes type I (PM) are present (scale bar, 1 μm); C) EOM-29: heavily pigmented cells with abundant cytoplasm in which there are many melanosomes (M) and pre-melanosomes type III (PM) and lysosomal structures (L) (scale bar, 1 μm); D) OMM-1: 2 melanoma cells contain in the cytoplasm a large amount of osmiophylic (dense) melanosomes (M) and pre-melanosomes type III and IV, and only a few

type II (PM). Note the presence of lysosomes (L) (scale bar, 0.583 μm); E) OMM-2: melanoma cell with an aberrant cytoplasm in which dense fusiform melanosomes (M) and pre-melanosomes (type II, III and IV) (PM) are observed (scale bar, 1 μm); F) OMM-3: spindle melanocytic cells. The cytoplasm contains large amounts of rough endoplasmatic reticulum and pre-melanosomes types II and III (PM); a few lysosomes (L) are also present (scale bar, 1 μm).

Electron microscopy, immunocytochemistry

Electron microscopical morphology of the six cell lines is shown in Figure 6.2a-f. All figures are representative for the cultured cells examined and contained melanosomes and pre-melanosomes (Table 6.2). The immunocytochemical staining of the five cell lines with NKI-C3, HMB-45, S100, HNK-1 and BrdU incorporation is summarized in Table 6.2. The NKI-C3 stained all five cell lines; expression of the other three markers appeared to be facultative. The cell lines EOM-3 and OMM-1 showed a relatively high BrdU incorporation, thus showing a high frequency of cell division. The more slowly growing cell lines EOM-29, OMM-2 and OMM-3 had indeed a lower BrdU incorporation. The primary cell lines OCM-1 and EOM-29 were found to express MHC-class-I molecules, whereas in the EOM-3 cell line, no detectable MHC-class-I expression was observed. All three metastatic cell lines were found to express MHC-class-I antigens.

TABLE 6.3 CYTOGENETIC DATA OF UVEAL MELANOMA CELL LINES

CELL LINE	PASSAGE	NO OF METAPHASES ANALYSED	MODAL CHROMOSOMES	KARYOTYPE
OCM-1	>50	14	90-97	94,<4>,XX,-X,-X,del(1)(p21)[40%],der(2)t(1;2)(p31;q37), add(3)(q12), +del(3)(q13q23),del(4) (q12)[60%],-4[40%], add(5)(p11) [40%],add(5)(q13),-6[60%],add(7)(q35), del(7)(q33),-7,add(8)(q24)x2,-9,-11,inv(11)(p15q24),del(12) (p12)x3,-12,add(13)(q31),-13 or t(13q;14q)[60%],-15, add(16)(p13),-16[40%],-18[60%],-19[60%],add(20)(q13)x2 or x3,-20,add(21)(q21),-21,-21,del(22)(q11)x2,iso(22q)x2,+6-12 markers
EOM-3	10	22	46	46,X,-Y,+5,-6,+18
EOM-29	5	10	46	46,XY
OMM-1	8-10	22	60-68	64,<3>,X,-X,-Y,der(1)t(1;3)(p31;p13),der(2)t(2;5) (q32;q14?)x2,t(2;10)(q32;q25),+3[50%],-4,del(5)(q13q24), +7,add(8)(p11),-9,-11,+12,inv(13)(q12q34),-15,add(16)(p12), -17,del(19)(p12),+20,add(21)(p13),-21,+1-3 markers
OMM-2	11-14	19	46	46,XY[11]/44-45, with random loss of 1 or 2 chromosomes, [8 of which 2x -6]
OMM-3	1-4	21	46	46,XY

Cytogenetic analysis

The karyotypes of the six cell lines are described in Table 6.3. The karyotypes were obtained from 10 to 25 metaphases from each cell line at different passages. Cells from cell line EOM-29 and OMM-3 displayed a normal karyotype. The stemline karyotype of

OCM-1 was found to be tetraploid and to have many structural chromosomal aberrations (Figure 6.3a). These, among other changes, have resulted in a net loss of 3q and of the distal part of chromosome 8 (8q24-qter); monosomy 6 was observed in 60% of the metaphases of OCM-1. The stemline karyotype of EOM-3 was found to be pseudodiploid with numerical changes: a loss of Y, monosomy 6, trisomy 5 and 18 (Figure 6.3b). The stemline karyotype of cell line OMM-1 was near triploid with many structural and numerical changes, a net gain of chromosome 3, 7, 12 and 20, and loss of 4, 8p, 9, 11, 15, 17, 21, among others (Figure 6.3c). Cell line OMM-2 showed no clonal aberrations, but some non-clonal rearrangement, such as a monosomy 6 in 2 cells and an unbalanced translocation (14;22) in one cell.

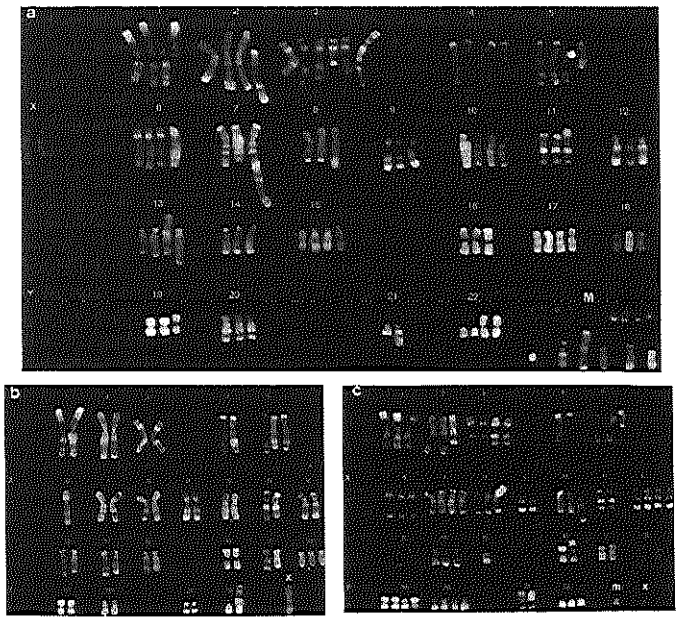


Fig 6.3 Karytypes of the cell lines. A) OCM-1; B) EOM-3 and C) OMM-1

DISCUSSION

Culturing of human uveal melanoma cells was first attempted in 1929 by Kirby (Kirby, 1929), but until the present date only a few cell lines from primary melanomas (Albert et al., 1984; Kan-Mitchell et al., 1989; Aubert et al., 1993; Massarelli et al., 1994; De Waard-Siebinga et al., 1995), and no cell lines from metastatic uveal melanomas have been described. The relative paucity of established uveal melanoma cell lines can be explained partly by the low incidence (1:100.000) of uveal melanoma and by difficulties

in the culturing of primary uveal melanoma tissue (Egan et al., 1988). Lack of therapeutic modalities for patients with hepatic metastases has led to a high mortality among them within a very short survival time, and hence provides a problem with respect to the availability of fresh metastatic tissue as well. In this study we were able to establish two primary uveal melanoma cell lines and three metastatic uveal melanoma cell lines. Although a limited overall number of tumours could be cultured at our institute, the success rate in the establishment of long-term growing cell lines was relatively high for metastatic uveal melanomas (3/4) as compared with primary uveal melanoma (2/16). Metastatic lesions originate from selected cells that have proved to be capable of growing in secondary organs. This could be the reason for the high success rate in culturing metastatic cell lines. The present availability of primary and metastatic uveal melanoma cell lines make it possible to study the differences between primary and metastatic uveal melanoma *in vitro*.

To ascertain the melanocytic origin of the cultured cells, the cells were immunocytochemically studied by the use of melanoma-associated antibodies. S100, NKI/C3 and HMB-45 are known to be powerful markers for differentiating tumours of melanocytic lineage from other anaplastic tumours. In addition, electron-microscope studies revealed melanosomes and pre-melanosomes in all the cell lines described. The cell lines showed a considerable variation in passage time as measured by BrdU incorporation. The cell lines EOM-3 and OMM-1 had relatively high BrdU incorporation and were shown to have high growth rates with short passage times in culture. The cell lines EOM-29, OMM-2 and OMM-3 had low levels of BrdU incorporation and were also more difficult to maintain in culture. However, the cell lines OMM-2 and OMM-3 were stable over a long period of culture without showing any morphological changes. The cell line EOM-29 has been in culture for a relatively short period, showing no morphological changes, but immortality is not yet established. No difference in growth rate between primary and metastatic cell lines could be demonstrated by the BrdU incorporation.

Although epithelioid cells have often been regarded as the most malignant cell type, three out of six cell lines in our study, were of the spindle cell type (EOM-29, OMM-2, OMM-3). This in contrast to the histological cell type of the original tumour of the patient. This can probably be explained by the selective outgrowth of certain tumour cells in a favourable culture medium such that used by us. In case of the cell line EOM-3, the spindle cells of the fresh tumour culture were gradually overgrown by undifferentiated, anaplastic cells at passage five. After that time the uniform morphological cell type of EOM-3 remained unaltered. Whether the phenotypical change of the cell line EOM-3 is accompanied by genetical instabilities, reflecting one or more steps in tumour progression, or by simple selection is not known. Both cell lines OCM-1 and OMM-1 consist of a heterogeneous population of cells with a high growth rate, showing signs of selection of one of the morphological cell types present in the original tumour.

The normal expression of MHC class I molecules in five of the six cell lines indicates a possibility of a MHC class I restricted T-cell response in metastatic uveal melanoma patients. In the EOM-3 cell line, MHC-class-I expression is down-regulated and, as a result of this, increased susceptibility to natural-killer-cell activity has been demonstrated (Ma et al., 1995). These data indicate that enhancement of the T-cell

response by the use of allogenic irradiated cell lines might be one of the most promising ways to treat patients with metastatic disease.
In cytogenetical analyses of primary uveal melanoma, the most frequently described chromosomal abnormalities found are: monosomy of chromosome 3, net loss of genetic material of chromosome 6q and 8p, and gain of 6p and 8q (Horsman and White, 1993; Singh et al., 1994). It has been suggested that monosomy 3 and the isochromosome 8q are associated with ciliary-body melanoma and poor prognosis (Sisley et al., 1990). Cytogenetic analysis of our cell lines, showed a normal karyotype in three cell lines and karyotypic abnormalities in the other three, with partial loss of chromosome 6 among multiple other aberrations. OCM-1 and OMM-1 were near-tetraploid and triploid, respectively. In these cell lines, partial loss of 3q and 8p were seen, as a consequence of structural changes. In contrast to other results on cytogenetical abnormalities in ciliary-body melanoma, our cell line EOM-29, which was derived from a massive extrascleral growth of a ciliary-body melanoma, did not demonstrate the chromosome-3 and -8 abnormalities. We have found however no systematic differences in the karyotypes of the cultured cells of primary versus metastatic tumours. The karyotyping was assessed after establishment of the cell lines and thus, some chromosomal abnormalities could be the consequence of *in vitro* clonal selection and progression, instead of a representation of the situation of the tumour *in vivo*. In future studies, cytogenetic analysis of primary uveal melanoma cell cultures will hopefully provide more information on the karyotype of the tumour cells. Furthermore, complementary studies using molecular cytogenetic approaches and comparative genomic hybridization could give more detailed information on chromosomal abnormalities in fresh tumour and cell lines in culture (Speicher et al., 1994).

CHAPTER

METHYLATION IS THE MAJOR CAUSE OF *P16*^{INK4A} INACTIVATION IN UVEAL MELANOMA CELL LINES

Nicole C. Naus,[1,2] Anette C.A. Verhoeven,[1,2] Manja J.M. Muijtjens,[2] Riena Aliredjo,[2] Gregorius P. M. Luyten,[1] Annelies de Klein,[3]

From the Departments of [1]Ophthalmology, [2]Cell Biology & Genetics, [3] Clinical Genetics Erasmus University Rotterdam, The Netherlands.

Submitted for publication

ABSTRACT

Purpose Uveal melanoma is the most common primary malignant intraocular tumor in the Western world. In cutaneous melanoma deletions or mutation of the $p16^{INK4a}/p14^{ARF}$ gene are frequently found. In our effort to unravel the mechanisms of uveal melanoma tumorigenesis, we determined the possible involvement of these genes in uveal melanoma cell lines.

Methods Nine uveal melanoma cell lines were investigated for deletions, mutations, methylation and expression of the $p16^{INK4a}$ gene. In addition, the expression of its alternatively spliced gene product, $p14^{ARF}$, was determined.

Results In contrast to what is known from cutaneous melanoma, no homozygous deletions were detected in these nine cell lines. LOH was observed in 3 out of nine cell lines. Two of these cell lines also had a mutation the $p16^{INK4a}$ gene. Furthermore, four out of nine uveal melanoma cell lines showed methylation of the promotor region resulting in the absence of $p16^{INK4a}$ mRNA.

Conclusions In total, six out of nine cell lines showed aberrant $p16^{INK4a}$ mRNA or absence of expression. Our finding suggest that methylation, instead of homozygous deletion is the major cause of $p16^{INK4a}$ inactivation in uveal melanoma cell lines.

INTRODUCTION

Uveal melanoma is the most common primary malignant intraocular tumor in adults in the western world and research has been concentrating on the identification of (cyto)genetic changes in order to unravel the molecular pathogenesis. Cytogenetic and fluorescent *in situ* hybridization (FISH) analyses have shown that non-random abnormalities (e.g. monosomy 3, gain of chromosome 8q and abnormalities of chromosome 6) do occur in uveal melanoma (Prescher et al., 1996; Sisley et al., 1997; White et al., 1998), but genes involved in the tumorigenesis are yet to be identified. In cutaneous melanoma, the $p16^{INK4a}$ gene (also known as *CDKN2A*, *p16* or *MTS1*), located on chromosome 9p21, plays an important role in tumorigenesis and is involved in both familial and sporadic tumors (Piepkorn, 2000). $P16^{INK4a}$ evokes a G1 cell cycle arrest by inhibiting the cyclin-dependent kinases 4 and 6 (cdk4/cdk6) and can be inactivated by mutations, homozygous deletions or by methylation of the promotor region (reviewed by Piepkorn et al. (Piepkorn, 2000)). In cutaneous melanoma cell lines homozygous deletions or intragenic mutations of the $p16^{INK4a}$ gene are found in about 50% and 20% of the cell lines, respectively (Walker et al., 1998). Furthermore germline mutations are found in approximately 20% of the familial cutaneous melanoma (Greene, 1999). The $p16^{INK4a}$ locus harbors also a related gene, $p14^{ARF}$ which uses an alternative exon 1 (1β) and a different reading frame of exon 2 and 3 of $p16^{INK4a}$, generating a structurally different protein. $P14^{ARF}$ binds and inhibits MDM2, thereby preventing p53 degradation. In contrast to $p16^{INK4a}$, to our knowledge, no $p14^{ARF}$ mutations have been detected in familial or sporadic cutaneous melanoma. Since both cutaneous and uveal melanomas originate from neural crest derived melanocytes this prompted us to investigate the role of the $p16^{INK4a}$ gene and the expression of $p14^{ARF}$ in uveal melanoma cell lines. In the present study we searched for deletions, mutations and methylation of the $p16^{INK4a}$ gene and determined the expression of both genes in 9 uveal melanoma cell lines.

METHODS

Cell lines

Nine uveal melanoma cell lines were used in the present study. EOM3, EOM29, OCM1, Mel202, 92.1 and Mel270 are cell lines established from primary uveal melanomas (De Waard-Siebinga et al., 1995; Luyten et al., 1996; Verbik et al., 1997). OMM1, OMM2 and OMM3 are metastatic uveal melanoma cell lines derived from metastases of 3 different uveal melanoma patients (Luyten et al., 1996). OMM2.2, OMM2.3 and OMM2.6 are metastatic cell lines derived from separate tumor nodules in the liver of the same patient as Mel270 (Verbik et al., 1997). All cell lines were grown in RPMI1640 (with

HEPES and glutamate) supplemented with 10% fetal calf serum, 1% penicillin and 1% streptomycin and passaged depending on growth.

Fluorescence *in situ* hybridization

We examined the copy number of the $p16^{INK4a}$ /$p14^{ARF}$ locus on interphase nuclei using the biotin-labeled P1 probes 1062 and 1063 and cosmid c5 (Kamb et al., 1994) as described before (Hagemeijer et al., 1998). Per slide 75 ng probe was used. After hybridization and immunodetection, slides were counterstained with DAPI and mounted in anti-fade (Dabco- Vectashield 1:1) and 300 nuclei per probe were scored according to the criteria of Hopman et al (Hopman et al., 1988). The cut-off values used, were those described by van Dekken (van Dekken et al., 1990). Polysomy was defined as more than 2 hybridization signals in more than 10% of nuclei, and monosomy as a single signal in more than 15% of the nuclei.

Chromosome 9 marker analysis

Eight microsatellite repeats, selected from The Genome Database and depicted in Figure 7.1 were synthesized by Isogen Bioscience (Maarsen, The Netherlands) and used as described previously (van der Riet et al., 1994). Markers D9S171, D9S156 flank the $p16^{INK4a}$ /$p14^{ARF}$ locus and marker CT29 is located within this locus.

$p16^{INK4a}$ mutation analysis

Genomic DNA of the 9 cell lines was used for the mutation analyses. Analysis of exon 2 was performed by Single Strand Conformation Polymorphism (SSCP) analysis as described previously (Gruis et al., 1995). Analyses of exon 1 and 3 of the $p16^{INK4a}$ gene was performed using Restriction Endonuclease Fingerprinting (REF) (Liu and Sommer, 1995). Exon 1 was amplified using primers E1F: AGGTATTAGCTTAGGATGTGTGGC, and E1R: CTACCTGATTCCAATTCCCCTG, resulting in a fragment of 1431 bp; exon 3 was amplified with primers E3F: TCGCGCTTTCTCTGCCCTCC, and E3R: GAAAACTACGAAAGCGGGGTGGG, resulting in a fragment of 392 bp. Polymerase chain reaction (PCR) products were purified using the Qiagen PCR purification system (Qiagen Inc, Santa Clarita, USA) and digested with *Xba*I, *Dde*I/*Xma*I, and *Mse*I/*Msc*I (exon 1) or *Rsa*I/*Pst*I, *Rsa*I/*Sau*3A, *Xcm*I/*Pst*I (exon 3). After digestion and heat-inactivation and fragments were end-labeled with 6 µCi [γ-^{32}P] dATP. Products were loaded on a non-denaturing MDE gel (J.T. Baker Inc, Philipsburg, USA) containing 0% or 5% glycerol (exons 1 and 3) or 10% glycerol (exon 2). The gels were run at 6W for 14 hours at RT or 4°C. After electrophoresis, the gels were transferred to blotting paper, dried and exposed to a Kodak X-Omat AR film for 24 hours. All aberrant bands were sequenced (Base Clear, Leiden, The Netherlands).

Methylation specific PCR

Bisulfite modification and methylation specific PCR (MSP) was carried out on all cell lines as described previously (Herman et al., 1996). Cell line T47D (methylated) and Hela (unmethylated), were used as positive and negative control respectively. Furthermore, the methylation status was investigated in 5 primary retinal pigment epithelium (RPE) cultures, in 3 primary strains derived from uveal melanocytes and in lymphocytes of 5

uveal melanoma patients. PCR products were loaded onto 2.5% agarose gels, stained with ethidium bromide, and directly visualized using UV illumination.

Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR) of *p16^{INK4a}* and *p14^{ARF}*

RNA was isolated using GTC-lysates. RNA extraction was performed using the RNeasy kit (Qiagen Inc, Santa Clarita, USA). cDNA was synthesized from 1-3 µg of total RNA using 2 µl of a random primer (0.5 µg/ µl) and 10 U super RT (HT Biotechnology LTD, Cambridge, England). For *p16^{INK4a}*, primers 57F GGAGCAGCATGGAGCCTT and E3R GAAAACTACGAAAGCGGGGTGGG generating a product of 566 bp, were used. For p14^{ARF} primers (OL27 and OL28, generating a product of 291 bp) and running conditions were those described by Gardie et al. (Gardie et al., 1998). Amplification products were separated on a 1% agarose gel.

RESULTS

Cytogenetic and FISH analyses

Cytogenetic and FISH analysis, using locus specific probes was performed to detect structural and/or numerical chromosomal abnormalities, which could disrupt chromosome 9p21. All cell lines, have been maintained in vitro for many years and are characterized by cytogenetically heterogeneous clonal cell populations (De Waard-Siebinga et al., 1995; Luyten et al., 1996; Naus et al., 2001). Chromosome 9 abnormalities were found in three cell lines (Table 7.1). In OCM1 and OMM1 a relative loss of chromosome 9 was found. In more than 70% of the metaphases of Mel270 this loss was also observed. In other metaphases of this cell line derivatives of chromosome 9p were found. Loss of 9p21 was also observed in the three metastatic cell lines of Mel270 (OMM2.2, 2.3 and 2.6) using FISH analysis. Nevertheless, we searched for deletions in 26 non-cultured sporadic uveal melanoma and detected loss of one copy of chromosome 9p21 in only one case: a uveal melanoma patient with a nevus of Ota (Naus et al., unpublished results).

Neither in the cell lines nor in the primary uveal melanoma homozygous deletions were observed.

Chromosome 9 marker

To investigate the possibility of smaller deletions or the presence of isodisomy of chromosome 9, we performed microsatellite analysis with markers distributed on chromosome 9 and around the *p16^{INK4a}* locus in particular (Figure 7.1). Homozygosity was found in Mel270 and its metastases, in OMM1 and Mel202. The observed LOH in these latter two indicated the formation of an isodisomy of chromosome 9(p) (Table 7.1). In these three cases all 9(p) markers showed evidence for one type of allele, suggesting loss of the other chromosome 9(p). In all other cases heterozygosity of marker CT29 was observed although homozygosity for some other markers did occur. Since no normal DNA is available from these cell lines, a possible deletion of these loci

can not be excluded. Again, no homozygous deletions of the *p16*^{INK4a} locus could be detected in any of the cell lines.

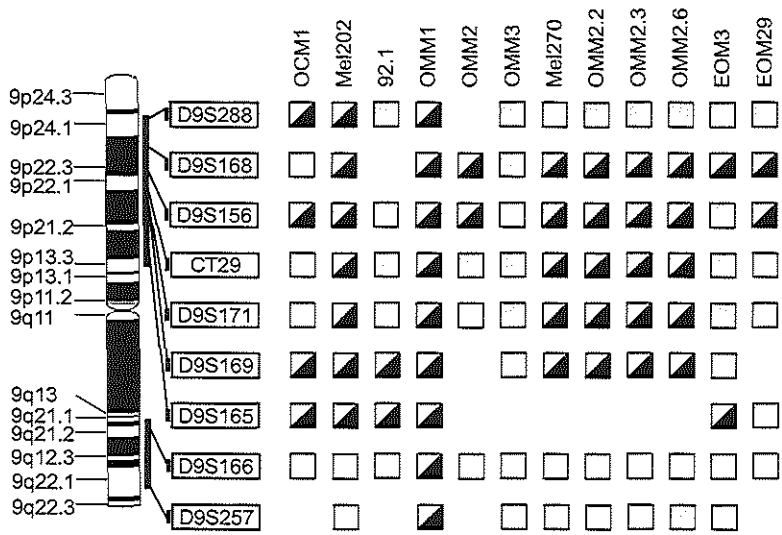


Fig 7.1 chromosome 9 marker analysis in 12 uveal melanoma cell lines. All markers are listed from telomere to centromere with the approximate map location indicated to the left. Grey boxes, the retention of heterozygosity; black-gray boxes, homozygosity of the marker.

Mutation analyses

To search for gene inactivating events other than homozygous deletions, mutation analysis of the *p16*^{INK4a} gene was performed. Results of this mutation analysis are summarized in Table 7.1. In 4 uveal melanoma cell lines (OCM1, Mel202, Mel270 and OMM3) aberrant SSCP patterns of *p16*^{INK4a} were found (Figure 7.2). In the Mel270-related metastatic cell lines (OMM2.2, OMM2.3 and OMM2.6) the same SSCP pattern was found as in the parental cell line. Sequence analysis of Mel270 and its metastatic derivatives revealed a C→G substitution in the 3' untranslated region (position 540, cDNA; GenBank Identification number 558656). Also in OMM3 a single basepair substitution (G→A at position 580, cDNA) was found in the 3' untranslated region. Both substitutions were found to be relative common polymorphisms in cutaneous melanoma and are, in these tumors, associated with a shorter progression time to metastatic (Sauroja et al., 2000). Furthermore, a 8 bp deletion in exon 2 of Mel202 (position 232-240, cDNA) and a 28 bp deletion, starting at position 496 (cDNA) extending in intron 2 thus including the intron 2 splice-donor site of OCM1 were found. These mutations were not described previously (http://www.uwcm.ac.uk/uwcm/mg/search/335362.html). To evaluate the mutation frequency in uncultured primary uveal melanoma, we analyzed 30 primary uveal melanoma with SSCP analysis for mutation in exon 2 of the *p16*^{INK4a} gene but no mutations or (homozygous) deletions were found.

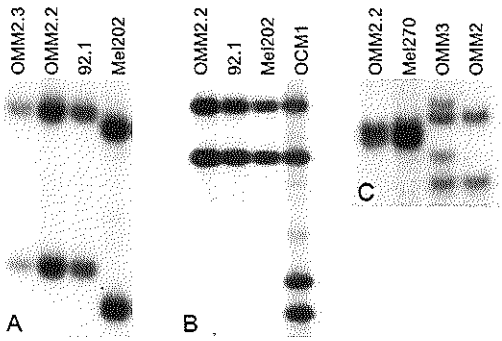


Fig 7.2 Examples of aberrant SSCP patterns of p16^{INK4a} in uveal melanoma cell lines. (A) Mutation of Mel202 and (B) of OCM1 in exon 2 of *p16^{INK4a}*. (C) Polymorphisms in OMM3, Mel270 and OMM2.2 in exon 3. Note the homozygous aberration of Mel202, Mel270 and OMM2.2. In OMM3 and OCM1 heterozygous aberrations are observed.

Methylation specific PCR

Elimination of the *p16^{INK4a}* function can also be achieved by methylation of the promotor region. To determine the methylation status of these uveal melanoma cell lines, we performed a methylation specific PCR. In four out of 8 cases a product was amplified using methylation-specific primers, whereas no product was amplified in these cases with primers specific for unmethylated DNA (Figure 7.3). This indicates the detection of methylated *p16^{INK4a}* alleles in these cell lines. Four cases were only positive with primers for unmethylated DNA. In one case (EOM29) no PCR products could be amplified with either of the primers. In non-tumor samples (RPE cell lines, melanocyte cell lines and lymphocytes of uveal melanoma patients) the methylation specific PCR indicated that the p16^{INK4a} promotor was unmethylated (data not shown).

RT-PCR

To investigate the expression of the gene in the uveal melanoma cell lines, we performed a RT-PCR using primers for *p16^{INK4a}* and *p14^{ARF}* respectively. *P16^{INK4a}* expression was found in all cell lines with non-methylated promotor regions and in EOM29. In OCM1 a 28 bp deletion including the intron 2 spice-donor site was detected and the use of a cryptic splice-donor site in exon 2 resulted in a frame-shift and a assumed protein with a substitution of the last 28 amino acids by HPRLKEPERL. As is shown in Figure 7.3, we detected both the wild type and truncated mRNA in OCM1. In Mel202 the deletion of 8 basepairs resulted in a frame-shift of the *p16^{INK4a}* gene causing in a stop codon at position 388 and an assumed protein of hybrid *p16^{INK4a}/p14^{ARF}* protein of 115 aminoacids. The deletion was to small to be visible using gelelectrophoresis. In cases where methylation was detected, no expression of the gene was found. In all cell lines normal *p14^{ARF}* expression was detected (Figure 7.3).

TABLE 7.1 SUMMARY OF THE MARKER ANALYSIS, MUTATION ANALYSES, METHYLATION SPECIFIC PCR AND RT-PCR FOR THE P16^{INK4A}

CELL LINE	COPY NUMBER OF 9P21 LOCUS[2]	PLOIDY, CHROMOSOME 9 ABNORMALITIES[3]	LOH *P16^{INK4A}* LOCUS	ABERRANT SSCP PATTERN *P16^{INK4A}*	METHYLATION	EXPRESSION *P16^{INK4A}* (RT-PCR)
EOM3	2,3	pseudodiploid	-	-	+	-
EOM29	2	pseudodiploid	-	-	nr	+
OCM1	2,3	hypertetraploid, -9	-	exon 2; 28 bp deletion	-	+
92.1	2,4	hyperdiploid/ hypertetraploid	-	-	+	-
Mel202	2,3	hyperdiploid	+	exon 2; 8 bp deletion	-	+
Mel270	1,2	hypodiploid, ç9 [5], add(9)(p) [2] / hypotetraploid [2]	+	exon 3; polymorphism C540G	+	-
OMM2.2[1]	1,2	nd	+	exon 3; polymorphism C540G	+	-
OMM2.3[1]	1,2	nd	+	exon 3; polymorphism C540G	+	-
OMM2.6[1]	1,2	nd	+	exon 3; polymorphism C540G	+	-
OMM1	2,3	hypertriploid, -9	+	-	+	-
OMM2	2,3	pseudodiploid	-	-	-	+
OMM3	2,3,4	pseudodiploid	-	exon 3; polymorphism G580A	-	+

Abbreviations: nd, not done; nr, no result. [1] Metastatic cell lines derived from Mel270. [2] Detected by FISH. Only significant cell populations (loss in >15% or gain in >10% of the cells) are indicated. The main cell population is indicated in bold. [3] Ploidy and chromosome 9 abnormalities as observed by cytogenetic analysis

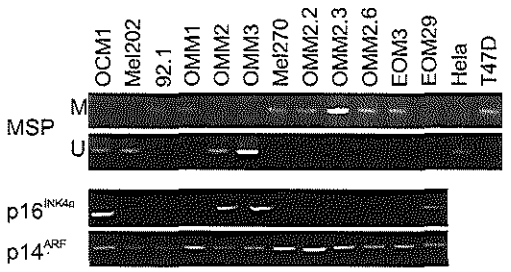


Fig 7.3 Methylation Specific PCR and RT-PCR of 12 uveal melanoma cell lines. The upper two panels represent the methylation specific PCR. M: methylated; U: unmethylated. The lower two panels show the results of the RT-PCR of $p16^{INK4a}$ and $p14^{ARF}$ in 12 uveal melanoma cell lines. In OCM1 both the wild-type (566 bp) and mutated products (492 bp) are visible. In Mel202 the deletion of 8 bp is to small to discriminate on agarose gel.

DISCUSSION

Inactivation of tumor suppressor genes, by homozygous deletions, mutations or methylation of promotor regions is a common event in human cancers. In contrast to many tumors, such as cutaneous melanoma, in which mutations in specific genes are found, the molecular pathogenesis in uveal melanoma is largely unknown. In our search for genes involved in the tumorigenesis of uveal melanoma we studied nine uveal melanoma cell lines for deletions, mutations, methylation and expression of the *$p16^{INK4a}$ gene*.

Using cytogenetic and FISH analysis no homozygous deletions were found in the uveal melanoma cell lines and primary tumors. However, in only three out of nine cell lines (EOM29, OMM2 and OMM3) we found an apparent normal expression of *$p16^{INK4a}$*. Thus in the other 6 cell lines alternative mechanisms are involved in the inactivation of this gene. Four out of nine cell lines (EOM3, 92.1, Mel270 and OMM1) showed methylation of the promotor region resulting in a total absence of the *$p16^{INK4a}$* transcript. In OMM1 and Mel270 we, additionally, observed LOH of the locus. Aberrant *$p16^{INK4a}$* expression was also found in Mel202 where LOH and a 8 bp deletion in exon 2 were detected resulting in an assumed protein of hybrid $p16^{INK4a}/p14^{ARF}$ protein of 115 aminoacids, thereby disrupting not only $p16^{INK4a}$ but also $p14^{ARF}$. Whether this affects the ability of the $p14^{ARF}$ protein to cause cell cycle arrest is unclear since others have shown that sequences encoded by exon 1β alone are sufficient for ARF function (Quelle et al., 1997). In OCM1 a 28 bp deletion including the intron 2 spice-donor site was detected and the use of a cryptic splice-donor site in exon 2 resulted in a frame-shift

and a assumed protein of 138 bp. In all cell lines normal expression of exon 1β ($p14^{ARF}$) was detected using RT-PCR.
Homozygous deletions of the *p16*^{INK4a} gene are the most common cause of gene inactivation in cutaneous melanoma cell lines (Foulkes et al., 1997) and also in primary cutaneous loss of chromosome region 9p21 is frequently observed. However, the incidence of homozygous deletions in uncultured sporadic cutaneous melanoma is lower compared to the rate in melanoma cell lines. This is also seen in other tumor types and has led to the suggestion that these abnormalities were the result of in vitro selection (Cairns et al., 1994). However, this controversy was laid to rest by the discovery of microdeletions that encompassed p16 and because of the presence of normal cells within primary tumor specimens hampering the detection of deletions (Cairns et al., 1995). In contrast to cutaneous melanoma, our results indicate that homozygous deletions are rare or absent in uveal melanoma cell lines as well as in primary uveal melanoma. Although, mutations were detected in two uveal melanoma cell lines, in 30 primary uveal melanoma we did not detect any mutations in exon 2. This low frequency of mutations in primary uveal melanoma are concordant to those published by others (Ohta et al., 1994; Singh et al., 1996a; Merbs and Sidransky, 1999). A recent publication of Merbs et al. (Merbs and Sidransky, 1999) suggested that homozygous deletions or methylation were found in sporadic uveal melanoma in 12% and 6% respectively. Our series on uveal melanoma cell lines showed that methylation was the most common mechanism for *p16*^{INK4a} inactivation i.e. four out of nine cell lines. This is also likely to occur in primary uveal melanoma since others have shown that methylation status is not influenced by cell culture (Gonzalgo et al., 1997). Furthermore, we found no methylation in cultures derived from retinal pigment epithelium and uveal melanocytes. In cutaneous melanoma, methylation of the *p16*^{INK4a} promotor is infrequent and whether uveal melanoma are more prone to methylation of *p16*^{INK4a} than to homozygous deletions, compared to cutaneous melanoma, has to evaluated in a larger study.

ACKNOWLEDGEMENTS

The authors thank Heleen van de Bosch and Yvonne Mijnsbergen for their technical assistance and Dr. N. Gruis for help with the *p16*^{INK4a} mutation analysis.

CHAPTER

8

MUTATION ANALYSIS OF THE *PTEN* GENE IN UVEAL MELANOMA CELL LINES

Nicole C. Naus[1,2], Wieke Zuidervaart[1], Nazik Rayman[2], Rosalyn Slater[2,3], Ellen van Drunen[2], Bruce Ksander[4], Gregorius P.M. Luyten[1], A. de Klein[1,2]

Department of [1]Ophthalmology, [2]Cell Biology & Genetics, [3]Clinical Genetics, Erasmus University Rotterdam, Rotterdam, The Netherlands, [4]Schepens Eye Research Institute, Boston, USA

Parts of this chapter are published in
International Journal of Cancer, 2000; 87:151-153

SUMMARY

The *PTEN* gene at chromosome region 10q23 has been shown to be mutated in cutaneous melanoma cell lines and primary tumours. However, its role in the development of uveal melanoma is not known. In this study we investigated 9 uveal melanoma cell lines for genetic changes in this gene. Using cytogenetic studies, two cell lines were found to have a translocation involving chromosome 10q. Fluorescent *in situ* hybridisation (FISH) using a PAC probe containing the entire coding sequence of the *PTEN* locus revealed no abnormalities except for a hemizygous deletion in a subclone of one cell line. In all cases normal expression was detected by reversed transcriptase-PCR (RT-PCR) and no mutations in the coding region of the gene were found using single stranded conformation polymorphism analyses (SSCP). We therefore conclude that, in contrast to cutaneous melanoma, the *PTEN* gene does not appear to play a role in the pathogenesis of uveal melanoma.

INTRODUCTION

The molecular pathogenesis of uveal melanoma is largely unknown. Cytogenetic studies have shown that recurrent abnormalities do occur, including deletions of chromosome 1p, loss of chromosome 3, gain of chromosome 8q and alterations of chromosome 6 (Prescher et al., 1996; White et al., 1996; Sisley et al., 1997). However, specific chromosomal regions or genes involved in tumorigenesis have yet to be identified.
In 40% of the *cutaneous* malignant melanoma cell lines, sporadic mutations are found in the *PTEN* gene (Guldberg et al., 1997), although reports on the incidence of sporadic mutations in primary cutaneous melanoma seem to be conflicting (Boni et al., 1998; Tsao et al., 1998). The findings on the *PTEN* gene in cutaneous melanoma cell lines prompted us to investigate the role of the *PTEN* gene in uveal melanoma cell lines since both cutaneous and uveal melanoma arise from neural crest derived melanocytes. *PTEN* is a known tumour suppressor gene and sporadic mutations of the *PTEN* gene or deletions of the *PTEN* locus at chromosome 10q23 have also been found in glioblastomas (Wang et al., 1997), prostate (Cairns et al., 1997), breast (Li et al., 1997) and endometrial carcinomas (Risinger et al., 1997). Germline mutations of *PTEN* are responsible for Cowden disease and the Bannayan-Zonana syndrome (Marsh et al., 1998). The *PTEN* protein encodes a dual-specific phosphatase and plays a major role in the inhibition of cell migration and the formation of focal adhesions (Tamura et al., 1998). In the present study, we examined 9 uveal melanoma cell lines using cytogenetic analysis supplemented with FISH to search for deletions or translocations affecting the *PTEN* gene region at 10q23. The expression of the gene was investigated by RT-PCR, and SSCP analysis of all 9 coding regions was carried out to screen for the presence of small mutations.

MATERIALS AND METHODS

Uveal melanoma cell lines

Nine uveal melanoma cell lines isolated from primary or metastatic uveal melanoma were used in the present study. EOM3 and EOM29 (Luyten et al., 1996), OCM1 (Kan-Mitchell et al., 1989), 92.1 (De Waard-Siebinga et al., 1995), Mel202 and Mel270 (Chen et al., 1997) were all established from primary tumours. OMM1, OMM2 and OMM3 (Luyten et al., 1996) were derived from metastases of 3 different uveal melanoma patients. All cell lines were cultured in RPMI1640/ HEPES supplemented with 10% fetal calf serum, 1% penicillin and 1% streptomycin and passaged depending on growth. Cytogenetic data of cell line EOM3, EOM29, OCM1, OMM1, OMM2 and OMM3 have been published previously (De Waard-Siebinga et al., 1995; Luyten et al., 1996). The karyotype of 92.1 was reevaluated. Cytogenetic studies on Mel202 and Mel270 were

carried out in our laboratory using standard cytogenetic techniques.The karyotypes of all cell lines described according to the standard nomenclature (ISCN, 1995) are given in Table 8.1.

Fluorescent *in situ* hybridisation

A PAC probe, 190D6, containing the entire coding sequence of the *PTEN* locus was isolated from a PAC library and was used for FISH analysis of the cell lines. Single colour FISH using biotin-labeled PAC190D6 was performed on chromosome preparations as described before (Hagemeijer et al., 1998). Per slide 75 ng of PAC 190D6 was used and the probe was prehybridised with COT1 DNA for 30 min. After hybridisation and immunostaining, slides were counterstained with DAPI and mounted in anti-fade (Dabco-Vectashield 1:1) and 300 interphase nuclei were scored in each case. A cut-off value for deletions was calculated from hybridisation on cultured uveal melanoma cells known to have apparently normal chromosomes 10. The cut-off value was determined at 10% (mean of aberrant signals + 3 sd).

Single-strand conformation polymorphism (SSCP) analysis

The 9 exons of the *PTEN* gene were amplified using 11 pairs of primers (Vlietstra et al., 1998). Fifty ng of genomic tumour DNA was amplified as follows: Hot start of 4' 95°C followed by 30 cycles of 1' at 95°C, 90" at 55°C (exons 1-6) or 50°C (exons 7-9), 2' at 72°C. PCR was performed using 0.1U Taq polymerase (HT Biotechnology LTD, Cambridge, England), 20 pmol of each primer, 0.05 mM dATP, 0.2 mM dGTP, dTTP, dCTP and 1μCi [α-^{32}P]dATP per reaction. PCR products were loaded on a denaturing MDE gel (J.T. Baker Inc, Philipsburg, USA). The gels were run at 6W for 15 hours. After electrophoresis, the gels were dried and exposed to a Kodak X-Omat AR film for 24 hours.

RT-PCR

RNA was isolated using GTC-lysates. RNA extraction was performed using the RNeasy kit (Qiagen Inc, Santa Clarita, USA). cDNA was synthesised from 1- 3 μg of total RNA using 2 μl of a random primer (0.5 μg/ μl) and 10 U super RT (HT Biotechnology LTD, Cambridge, England). Two partially overlapping primer pairs were used to amplify the *PTEN* cDNA. Pair 1: 977F CCACCAGCAGCTTCTGCC ATCTCT and 1736R CCAATTCAGGACCCAC ACGACGG. Pair 2: 1649F GTTCAGTGGCGGAACTTGCA ATCCTCA and 2423R CCCTATACATCCACAGGGTTTTGACACTTG. The PCRs contained 0.2 mM dNTP, 20 pmol of each primer, and 0.1U Taq polymerase (HT Biotechnology LTD, Cambridge, England). Amplification was performed as follows: 1 cycle of 4' 95°C, 30 cycles of 1' 95°C, 2' 65°C, 2' 72°C followed by a final extension of 10' 72°C. Amplification products were separated on an 1% agarose gel.

RESULTS

The cytogenetic and FISH results are summarised in Table 8.1. Only one cell line (Mel270) had underrepresentation of chromosome 10 and this was present in a subclone. Two cell lines (Mel270 and OMM1) had structural abnormalities involving the long arm of chromosome 10. FISH studies using PAC probe 190D6 for the region 10q23 showed agreement between the numbers of signals seen and the reported cytogenetic findings in 6 cell lines. In 3 cell lines (OMM1, OMM2 and Mel270) variation in the number of FISH signals was observed. In OMM1 and OMM2 a small population of interphase nuclei was unexpectedly found to have 4 signals for the PAC probe 190D6. Examination of 7 metaphases from OMM1 showed the expected three FISH signals. Interphase FISH studies on line Mel270 revealed 12% of the nuclei to have only one signal for 190D6. This is above the established cut-off level of 10%. Monosomy for chromosome 10 was found in a subclone (3/7 metaphases) of this line on cytogenetic analysis.

Mutation analysis on genomic DNA was performed using exon-specific SSCP. All 9 exons and flanking sequences were amplified using 11 primer pairs. In all cases two single stranded bands were visible. We did not detect any abnormal banding patterns indicative for a mutation in the *PTEN* gene. Examples of exon 3 and 5 amplifications are shown in figure 8.1.

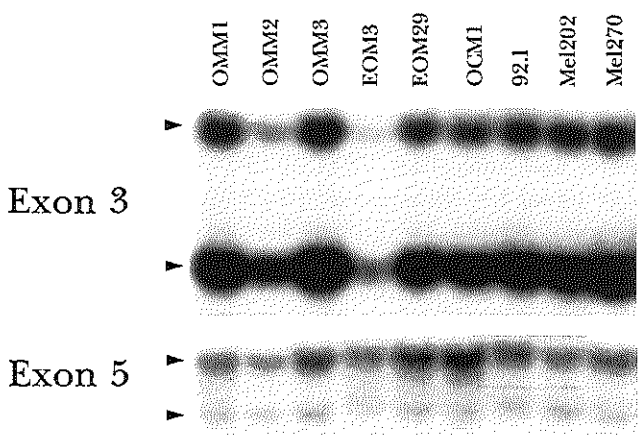


Fig 8.1 Exon-specific SSCP for exon 3 and 5 of the *PTEN* gene in uveal melanoma cell lines.
The two single stranded bands are clearly visible (arrowheads).

Although in none of the cell lines mutations could be detected in the coding region, mutations in the 5' UTR could not be excluded. Therefore we investigated the expression of the *PTEN* gene using RT-PCR. Two partially overlapping primers were used. In all cell lines both PCR products of 759 and 774 bp were amplified (figure 8.2). No abnormal sized bands were found.

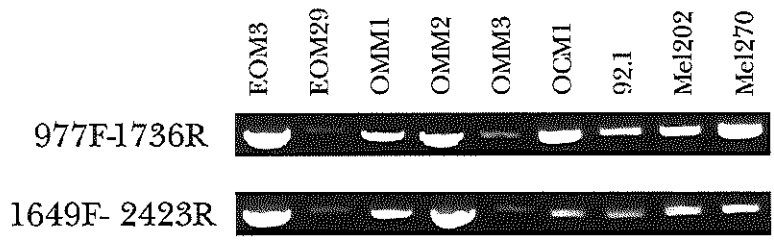


Fig 8.2 PTEN mRNA expression in uveal melanoma cell lines. Agarose gel analysis of RT-PCR amplified fragments. Two partially overlapping primers were used which amplified products of 759 (977F/1736R) and 774 bp (1649F/2423R).

DISCUSSION

Recent investigations showed that 40% of the cutaneous malignant melanoma cell lines harbour mutations of the tumour suppressor gene *PTEN* at chromosome region 10q23. This prompted us to investigate the presence of loss or mutations of the *PTEN* gene in 9 uveal melanoma cell lines using cytogenetic and molecular genetic techniques.
Cytogenetic analysis revealed 2 cell lines with structural abnormalities in the long arm of chromosome 10. In both cases FISH analysis showed that the breakpoint was not located within the *PTEN* gene. One of these cell lines had also a subclone of cells with loss of chromosome 10 and interphase FISH studies on this line revealed this subpopulation with one signal for chromosome 10q23. The discrepancy between the FISH findings of 12% monosomy for this chromosome and the cytogenetic results (43% monosomy) may be due to the fact that this particular subclone is actively proliferating. Lines OMM1 and OMM2 had small populations of interphase nuclei with 4 FISH signals for the *PTEN* gene. In both instances these subclones were not detected by chromosome analysis. This can be explained by the fact that the clones may not have been actively proliferating, or they may have later arisen in vitro since the FISH studies were carried out at a later passage than the original cytogenetic analyses.

TABLE 8.1 KARYOTYPES AND RESULTS OF THE FISH ANALYSES OF THE *PTE* GENE IN 9 UVEAL MELANOMA CELL LINES

CELL LINE	TUMOUR TYPE	KARYOTYPE (ISCN,1995)	NO. COPIES OF CHR.10[1]	NO. *PTEN* SIGNALS DETECTED WITH FISH[2]
EOM3	primary	46,X,-Y,+5,-6,+18[20][3]	2	2 (94%)
EOM29	primary	46,XY[20][3]	2	2 (87%)
OCM1	primary	94,XX,-X,-X,del(1)(p21),der(2)t(1;2)(p31;q37),add(3)(q12),+del(3)(q13q23),-4,del(4)(q12),add(5)(p11),add(5)(q13),-6,	4	4 (80%)
		-7,add(7)(q35), del(7)(q33),add(8)(q24)x2,-9,-11,inv(11)(p15q24),-12,del(12)(p12)x3,-13,add(13)(q31),-15,-		
		16,add(16)(p13),-18,-19,-20,add(20)(q13)x2, add(20)(q13),-21,-21,add(21)(q21),del(22)(q11)x2,i(22q)x2,+6-12mar[cp14][3]		
92.1	primary	47,add(X)(q2?5),+8,+8,der(11)t(1;11)(q22;q23~24),add(17)(q24~25)[4]/47,idem,add(12)(p11)[4]/94,idemx2,add(12)(p11)	2	2 (45%)
		x2[3]/97,idemx2, +7,+7,add(9)(p22)x2,+mar[cp5][4]	4	4 (43%)
MEL202	primary	50-53,XX[3],-X[13],+dic(1;9)(p11;p11),+5[3],add(6)(q?15),+add(6)(q?15),+7,+8,+8,+8,del(9)(q2?1)[2],del(9)(q3?2)[2],	2	2 (91%)
		i(9)(p10)[3], add(11)(q22),add(18)(q21),der(20)t(8;20)(q12;q13),add(22)(p11)[cp16]		
MEL270	primary	43~48,XY,add(2)(p2?4)[4],?add(3)(q2)[5],psu i dic(6)(q11),der(7)del(7)(q22q22)add(7)(q32)x2,?der(8)(p)[3],-9[5],	1	1(12%)
		?add(9)(p)[4], add(9)(p)[2],+der(9)add(9)(p)del(9)(q)[2],-10[3],add(10)(q2?)[3],add(12)(p11)[2],-13[6],add(13)(p11),	2	2 (72%)
		add(16)(q11),add(17)(p2?3)[6],-19,add(21)(p1)[2],+1~3mar[cp7]/81~87,psu i dic(6)(q11)x2,der(7)del(7)(q22q22)	4	4 (11%)
		add(7)(q32)x2,add(10)(q2?),add(17)(p2?3),+marx2[cp2]		
OMM1	metastasis	60-68,X,-X,-Y,der(1)t(1;3)(p31;p13),der(2)t(2;5)(q32;q14?)x2,t(2;10)(q32;q25),+3[3],-4,del(5)(q13q24),+7,add(8)(p11),-9,	3	3 (80%)
		-11,+12, inv(13)(q12q34),-15,add(16)(p12),-17,del(19)(p12),+20,-21,add(21)(p13),+1-3 mar[cp7][3]		4 (12%)
OMM2	metastasis	38-46,XY,-Y,-20[cp16][3]	2	2 (83%)
				4 (12%)
OMM3	metastasis	46,XY[20][3]	2	2 (93%)

[1]Expected copy number of chromosomes 10 based on cytogenetic findings

[2]Actual copy number of the *PTEN* gene detected by FISH (percentage of cells; only significant levels are given)

[3]Previously described by Luyten et al,1996

[4]Karyotype was reevaluated and described according to the ISCN1995

Except for a small proportion of cells of Mel270, no significant variation in the *PTEN* gene could be detected by FISH or cytogenetic analysis in these 9 uveal melanoma cell lines. In addition, SSCP analysis revealed no aberrant bands showing that mutations in the *PTEN* gene were not present. This is in accordance with the observations of the RT-PCR where we found expression of the *PTEN* gene in all cell lines and no abnormal sized RNA. These results are in contrast to cutaneous malignant melanoma cell lines.

The question remains, however, whether we can extrapolate our findings on uveal melanoma cell lines to fresh tumour material. The cytogenetic findings in primary uveal melanoma show, in general, a simple karyotype with only a few recurrent abnormalities as loss of chromosome 1p and chromosome 3, gain of chromosome 8q and structural abnormalities of chromosome 6 (Prescher et al., 1996; White et al., 1996; Sisley et al., 1997). Some of the uveal melanoma cell lines in our study had these genetic changes but these were accompanied by more complex chromosome variation. Whether these complex abnormalities are the result of prolonged cell culture, or due to the fact that tumours with more complex karyotype are more likely to grow in vitro is unclear. However, the fact that in our study no *PTEN* mutations were found in these uveal melanoma cell lines and that cytogenetic abnormalities involving chromosome 10q23 were not observed, and are also not common in primary uveal melanoma, suggests that *PTEN* mutations or deletions probably do not play a role in the aetiology of uveal melanoma. This is in contrast to primary cutaneous melanoma where chromosome 10q23 abnormalities have been observed, indicating the involvement of the *PTEN* gene. Other genetic differences between cutaneous and uveal melanoma have been reported. Whereas in 100% of the cutaneous melanoma involvement of the CDN2 gene or its downstream target genes has been postulated (Walker et al., 1998), CDKN2 mutations or loss is rarely observed in uveal melanoma cell lines and primary tumours (Singh et al., 1996a; Singh et al., 1996b). Also cytogenetically, there is a clear difference between uveal and cutaneous melanoma. The typical abnormalities found in uveal melanoma, such as loss of chromosome 3 or gain of 8q, are rarely observed in cutaneous melanoma. However, chromosome 1 and 6 abnormalities have also been reported in the latter.

Biological differences also exist between these two tumours. The integrin expression, which is important for the growth and metastatic capacity of melanoma cells, as well as the expression of melanoma associated antigens differ markedly between uveal and cutaneous (Mooy and De Jong, 1996; Marshall et al., 1998). All these data suggest that, despite the common embryonic origin, these tumours follow a different path towards tumorigenesis and have a different biological behaviour.

In summary, our results indicate that the *PTEN* gene is probably not involved in the pathogenesis of uveal melanoma and that the tumorigenesis of uveal melanoma involves a different set of genes to these involved in cutaneous melanoma.

CHAPTER

GENERAL CONSIDERATIONS AND FUTURE PROSPECTS

9.1 GENERAL CONSIDERATIONS

The detection of chromosomal abnormalities in solid tumours not only provides information on the outcome of cancer patients, but may also point to chromosome loci involved in tumorigenesis. As is described in chapter 1 and 2 of this thesis, predicting the survival outcome of uveal melanoma patients based on classical prognostic markers such as tumour diameter or cell type remains difficult. Cytogenetic analyses have shown that specific chromosomal changes, especially loss of chromosome 3, are strongly associated with patient survival (Prescher et al., 1996; Sisley et al., 1997; White et al., 1998). We have analysed 80 uveal melanoma for chromosomal changes in chromosomes 1, 3, 6 and 8 using both cytogenetic and FISH analyses and found that, after correcting for several other co-variables, survival was not correlated with the solitary loss of chromosome 3, but with a mutual loss of chromosome 1p36 and 3 (Chapter 2). It is most likely that the patients described in literature who succumb to metastatic disease and have tumours with loss of chromosome 3, also actually have lost the chromosome 1p36 region. In contrast to these studies, in which conventional cytogenetics and/or CGH was used, we performed FISH analysis with a probe specific for chromosome 1p36 allowing a more sensitive analysis of this chromosome band. We selected this probe based on the cytogenetic finding that loss of chromosome 1p36 was commonly found in our series of uveal melanoma. The prognostic significance of mutual loss of chromosomes 1p36 and 3, enables us to select patients with a high risk of metastatic disease more accurately and may lead to an adjustment of the treatment protocols for this specific group of patients. New treatment protocols for metastatic uveal melanoma are under investigation, but the preliminary results are, in general, disappointing. However, these trials are being applied to patients diagnosed of metastatic disease. We theorise that when these or other adjuvant therapies can be applied to high risk patients without (clinical detectable) metastases, the results may be promising.

Chromosome analysis can only be performed when tumour material is available. However, more and more patients are being treated with radiotherapy, restricting prognostic evaluation. In these cases and in patients where the diagnosis of uveal melanoma is uncertain, chromosome analysis using FISH can be applied to fine needle aspiration biopsies (chapters 4 and 5). Through our large uveal melanoma sample, we were able to show that the FISH technique was feasible to detect the genetic changes and that these biopsies were representative for the main tumour. Although these results are very promising, clinicians are stille resistant to taking a biopsy from a tumour. The potential risk of seeding tumour cells in the biopsy tract, although unlikely to cause recurrent disease, results in a wait and see attitude. Therefore, this method should also be studied on *in vivo* biopsies followed by careful patient and tumour follow-up. Only then will we be able to prove this matter beyond a doubt.

The finding that mutual loss of chromosome 1p and 3 correlates strongly with survival, not only provides information guiding ophthalmologists in the treatment of uveal melanoma patients, it also suggests that genes located on chromosome 1p and 3 interact with each other, thereby causing a more aggressive phenotype. This may help to further

delineate the chromosomal region of interest. Also, chromosome data obtained with modern cytogenetic techniques such as SKY and CGH, enable us to narrow down this region. These data point to the involvement of regions which remained undetected using conventional cytogenetics or FISH only. We found a small deletion on chromosome 3q13~21 and two regions on chromosome 8 (8q21.1~21.2 and 8q23~24). Furthermore, in these complex cases, gain of chromosome 6p and 8 occurred more frequently than can be detected by conventional analysis (chapter 3). We observed small parts of chromosome 8 being translocated to other chromosomes. Additional FISH analyses using (sub)telomeric probes should be carried out to investigate if a particular chromosome 8 region is commonly involved in these translocations or whether this is a more random phenomenon.

Monosomy 3 is suggested to be is an early event in tumorigenesis. White and coworkers found an isodisomy 3 in a non-pigmented part of a tumour wherease the pigmented part showed monosomy 3. They hypothesised that this abnormality of chromosome 3 plays a central role in the molecular pathogenesis of uveal melanoma (White et al., 1996). This hypothesis is supported by data of Presher et al, who described a uveal melanoma with two subclones, both containing monosomy 3. In one subclone there was a trisomy 8 and in the other sucblone as isochromosome 8q. The authors suggested that a stemline contained loss of chromosome 3 only and that the subclones developed independently from each other. (Prescher et al., 1994) These data furthermore suggest that gain of chromosome 8q is a subsequent event. Additionally, our own data (chapter 2) and those of Aalto et al (Aalto et al., 2001) suggest that loss of chromosome 1p plays an important role in the transition of primary uveal melanoma to metastatic disease. The role of chromosome 6 remains uncertain. Based on all these results, we can propose the following uveal melanoma progression model (Figure 9.1):

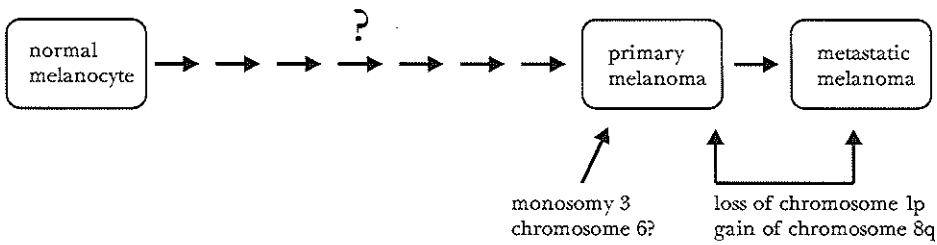


Fig 9.1 Tumour progression model for uveal melanoma melanoma.

Although chromosome 1p36 is deleted in many tumour types, as of yet, no convincing tumour suppressor gene has been identified. One of the most interesting candidate genes is *p73*, a *p53* homologue. This gene is further discussed in paragraph 10.2, Future Prospects.

Furthermore, *CMM1*, a melanoma gene, was linked to this region on chromosome band 1p36 (Bale et al., 1989). These data, could not be confirmed by other researchers, however, despite intensive efforts.

Although several tumour suppressor genes have been mapped to chromosome 3p, such as *VHL* (Von Hippel-Lindau syndrome), *TGFBR2* (transforming growth factor beta receptor 2 in colorectal cancer), *DLEC1* (deleted in lung and oesophageal cancer 1), as

of yet, no known tumour suppressor genes have been mapped to chromosome 3q13~24. Interestingly, however, this region harbours a relativaly high amount of genes involved in ocular pathology, such as *BFSP2* (beaded filament structural protein 2 involved in congenital cataract), *BPES* (blepharophimosis, epicanthus inversus and ptosis type 1), *GLC1C* (glaucoma 1C) and *RHO* (rhodopsin, involved in retinitis pigmentosa type 4) (http://nciarray.nci.nih.gov/cgi-bin/cards/). Although these syndromes give rise to clearly distinct phenotypes, it may suggest that this region may be eye specific. In order to demarcate this chromosome 3 region found by CGH, we are currently performing LOH analysis using polymorphic markers.
C-myc, located on chromosome 8q24.1, has been suggested to be a candidate oncogene because of the frequent accumulation of chromosomes 8q in uveal melanoma. Surprisingly, expression of *c-myc* was found to be correlated with an improved prognosis, in contrast to what is known from cutaneous melanoma where it was shown to be correlated with decreased survival (Chana et al., 1999). Furthermore, the *RCA1* gene (renal carcinoma, familial, associated 1), involved in renal cell carcinoma, is known to map to chromosome 8q24.1. However, our CGH results show that the critical region on chromosome 8 is probably more proximal to these genes. Unfortunately, until now, no known tumour-related genes are mapped to this regions.

In the second part of the thesis, we hypothesised that tumour suppressor genes that are known to be involved in cutaneous melanoma could also be involved in uveal melanoma tumorigenesis. We established and characterised primary and metastatic uveal melanoma cell lines to facilitate mutation analyses and expression studies of these genes (chapter 6). When using cell lines, one should remember that it is a artificial system. It is generally agreed that using cell lines has disadvantages, the main question being whether the individual cell lines are representative of the original tumour (Masters, 2000). As we have shown in chapter 6, the uveal melanoma cell lines still express the melanoma-associated antigens and (pre-)melanosomes are also present, indicating their melanocytic origin. However, this does not imply that the uveal melanoma cell lines are representative for primary uveal melanoma in general. We observed that the specific chromosomal changes found in primary uveal melanoma, such as loss of chromosome 1p36, monosomy 3 and gain of chromosome 8, were not present in our uveal melanoma cell lines. It might be that only those tumours with specific genetic aberrations are amenable for cell cultures, thereby causing selection. In addition, an accumulation of genetic abnormalities may be necessary for unlimited growth. In contrast, the main advantage and reason why cancer cell lines are so widely used is that they provide an almost unlimited supply of cells with similar genotypes and phenotypes. Moreover, cell lines can be used to compare lines derived from different tissues such as cutaneous melanoma and uveal melanoma. We used cell lines to analyse genes like $p16^{INK4a}$, $p14^{ARF}$ and *PTEN*. In contrast to cutaneous melanoma cell lines, we found no *PTEN* alterations in primary and metastatic uveal melanoma lines (chapter 8). Furthermore, no homozygous deletions of the $p16^{INK4a}$ locus were found, although this is known to be the major cause of gene inactivation in cutaneous melanoma cell lines. In 26 uncultured primary uveal melanoma, we detected a deletion of chromosome 9p21 in only one case. This patient had also a nevus of Ota, a condition which is known to associate with uveal melanoma. Uveal melanoma cell lines were

however shown to inactivate $p16^{INK4a}$ by promotor region methylation. No alterations in $p14^{ARF}$ were found (chapter 7). These and other data, such as a different chromosomal constitutions and expression of melanoma-associated antigens, suggest that cutaneous and uveal melanoma follow a different molecular path toward tumour initiation and to metastatic disease (Cree, 2000). These findings may indicate that not the origin of these melanomas, but the environment (skin versus eye), is decisive for which path a tumour cell follows to eventually result in a generalised metastatic disease.

9.2 FUTURE PROSPECTS

We have shown that FISH analysis for chromosomes 1p36 and 3 enables us to identify uveal melanoma patients with a high risk of metastatic disease. Monosomy 3 is already used by some clinicians as a marker for poor prognosis. However, we have shown that both chromosomes 1p36 and 3 should be tested for prognostic purposes.
Furthermore, this thesis provides a backbone for research into delineating the critical regions and identifying genes involved in uveal melanoma development and metastatic disease progression. We found evidence that both chromosome 1p and 3 loss contribute to a more aggressive phenotype of uveal melanoma. Furthermore, our studies point to a small deletion on chromosome 3q13-24 and two specific regions on chromosome 8q (8q21.1~21.2 and 8q23~24). To confirm these aberrations and to narrow down the regions on these chromosomes, allelic imbalance studies using polymorphic markers, have to be performed. These studies should be performed in the few cases which showed normal copies of chromosome 1p and 3 in the tumour but still developed metastatic disease. It is tempting to consider that, in these cases, homozygosity is present due isodisomy formation. Furthermore, LOH studies should be carried out in cases with partial losses of chromosomes 1p36 and 3.
Furthermore, SKY and CGH will be usefull to delineate the region of interest on chromosome 1, 3 and 8. Especially those patients whose tumours had partial losses or gains on these, should be further characterised using these techniques. CGH can even be applied in cases where no fresh tumour material is available. It would be interesting to apply CGH on paraffin embedded material from liver biopsies in cases with metastatic disease. This method can also be used to survey the numerical chromosomal changes in familial cases of uveal melanoma. Additionally, an array of BAC clones can be used for CGH analysis. This offers a better sensitivity and resolution than metaphase chromosomes, the traditional target for CGH and thereby allows a further refinement of the target regions found by conventional cytogenetics or CGH (Pinkel et al., 1998).
Other strategies to identify genes involved in uveal melanoma include the testing of putative candidate genes and the screening of micro arrays. Potential tumour suppressor genes include the p63 and p73 genes (Yang and McKeon, 2000). The involvement of p53 in uveal melanoma is uncertain but the location of the p63 and p73 on chromosome 3q27-29 and 1p36 respectively, makes them interesting candidates. Recently, it was shown that the TA-p73 and TA-p63 isotypes are capable of inducing apoptosis or, possibly, tumor suppression (Yang et al., 1998). Furthermore, it was found that primary lung cancers, squamous cell cancers and epithelial neoplastic cells predominantly express the ΔNp63 isotypes. (Nylander et al., 2000). The ΔN isoforms of

p63 are known to have dominant negative effects on both the TA-isoforms of p63 and on p53 (Yang et al., 1998). All these data suggest a complex cross-talk between the members of the p53 gene family.

Lastly, DNA chip technology can be used to analyse differences in expression patterns on large number of genes in two samples. These samples can be tumour versus normal samples or, for example, tumours with and without chromosome 1p and 3 loss. Also, genes from one particular chromosome or chromosome arm can be tested. The data obtained with this technique could provide information on tumor progression pathways, on genes involved in tumorigenesis and may allow the identification of tumour subsets with highly malignant phenotypes (Bittner et al., 2000). Although the purpose of this method seems unlimited, it will only yield useful information if the starting material is well characterised, as is the case in our samples.
In the last 6 years, we have collected well characterised tumour material and these samples provide an excellent starting point for these novel types of analyses.

REFERENCES

AALTO Y, ERIKSSON L, SEREGARD S, LARSSON O , KNUUTILA S (2001) 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 42: 313-317.

ABO T , BALCH CM (1981) A differentiation antigen of human NK and K cells identified by a monoclonal antibody (HNK-1). J Immunol 127: 1024-1029.

AKSLEN LA, MONSTAD SE, LARSEN B, STRAUME O , OGREID D (1998) Frequent mutations of the p53 gene in cutaneous melanoma of the nodular type. Int J Cancer 79: 91-95.

ALBERT DM, NIFFENEGGER AS , WILLSON JK (1992) Treatment of metastatic uveal melanoma: review and recommendations. Surv Ophthalmol 36: 429-438.

ALBERT DM, RUZZO MA, MCLAUGHLIN MA, ROBINSON NL, CRAFT JL , EPSTEIN J (1984) Establishment of cell lines of uveal melanoma. Methodology and characteristics. Invest Ophthalmol Vis Sci 25: 1284-1299.

ALERS JC, ROCHAT J, KRIJTENBURG PJ, VAN DEKKEN H, RAAP AK , ROSENBERG C (1999) Universal linkage system: an improved method for labeling archival DNA for comparative genomic hybridization. Genes Chromosomes Cancer 25: 301-305.

ALEXANDER HR, JR., BARTLETT DL , LIBUTTI SK (1998) Isolated hepatic perfusion: a potentially effective treatment for patients with metastatic or primary cancers confined to the liver. Cancer J Sci Am 4: 2-11.

ANASTASSIOU G, SCHILLING H, STANG A, DJAKOVIC S, HEILIGENHAUS A , BORNFELD N (2000) Expression of the cell adhesion molecules ICAM-1, VCAM-1 and NCAM in uveal melanoma: a clinicopathological study. Oncology 58: 83-88.

ANTLE CM, DAMJI KF, WHITE VA , ROOTMAN J (1990) Uveal malignant melanoma and optic nerve glioma in von Recklinghausen's neurofibromatosis. Br J Ophthalmol 74: 502-504.

AOYAMA T, MASTRANGELO MJ, BERD D, NATHAN FE, SHIELDS CL, SHIELDS JA, ROSATO EL, ROSATO FE , SATO T (2000) Protracted survival after resection of metastatic uveal melanoma. Cancer 89: 1561-1568.

ARNOLDUS EP, WIEGANT J, NOORDERMEER IA, WESSELS JW, BEVERSTOCK GC, GROSVELD GC, VAN DER PLOEG M , RAAP AK (1990) Detection of the Philadelphia chromosome in interphase nuclei. Cytogenet Cell Genet 54: 108-111.

AUBERT C, ROUGE F, REILLAUDOU M , METGE P (1993) Establishment and characterization of human ocular melanoma cell lines. Int J Cancer 54: 784-792.

BALE SJ, DRACOPOLI NC, TUCKER MA, CLARK WH, JR., FRASER MC, STANGER BZ, GREEN P, DONIS-KELLER H, HOUSMAN DE , GREENE MH (1989) Mapping the gene for hereditary cutaneous malignant melanoma-dysplastic nevus to chromosome 1p. N Engl J Med 320: 1367-1372.

BATAILLE V, SASIENI P, CUZICK J, HUNGERFORD JL, SWERDLOW A , BISHOP JA (1995) Risk of ocular melanoma in relation to cutaneous and iris naevi. Int J Cancer 60: 622-626.

BECHER R, GIBAS Z, KARAKOUSIS C , SANDBERG AA (1983) Nonrandom chromosome changes in malignant melanoma. Cancer Res 43: 5010-5016.

REFERENCES

BECHER R, KORN WM , PRESCHER G (1997) Use of fluorescence in situ hybridization and comparative genomic hybridization in the cytogenetic analysis of testicular germ cell tumors and uveal melanomas. Cancer Genet Cytogenet 93: 22-28.

BEDIKIAN AY, LEGHA SS, MAVLIGIT G, CARRASCO CH, KHORANA S, PLAGER C, PAPADOPOULOS N , BENJAMIN RS (1995) Treatment of uveal melanoma metastatic to the liver: a review of the M. D. Anderson Cancer Center experience and prognostic factors. Cancer 76: 1665-1670.

BIRCK A, AHRENKIEL V, ZEUTHEN J, HOU-JENSEN K , GULDBERG P (2000) Mutation and allelic loss of the PTEN/MMAC1 gene in primary and metastatic melanoma biopsies. J Invest Dermatol 114: 277-280.

BIRINDELLI S, TRAGNI G, BARTOLI C, RANZANI GN, RILKE F, PIEROTTI MA , PILOTTI S (2000) Detection of microsatellite alterations in the spectrum of melanocytic nevi in patients with or without individual or family history of melanoma. Int J Cancer 86: 255-261.

BITTNER M, MELTZER P, CHEN Y, JIANG Y, SEFTOR E, HENDRIX M, RADMACHER M, SIMON R, YAKHINI Z, BEN-DOR A, SAMPAS N, DOUGHERTY E, WANG E, MARINCOLA F, GOODEN C, LUEDERS J, GLATFELTER A, POLLOCK P, CARPTEN J, GILLANDERS E, LEJA D, DIETRICH K, BEAUDRY C, BERENS M, ALBERTS D , SONDAK V (2000) Molecular classification of cutaneous malignant melanoma by gene expression profiling. Nature 406: 536-540.

BLASI MA, ROCCELLA F, BALESTRAZZI E, DEL PORTO G, DE FELICE N, ROCCELLA M, ROTA R , GRAMMATICO P (1999) 3p13 region: a possible location of a tumor suppressor gene involved in uveal melanoma. Cancer Genet Cytogenet 108: 81-83.

BLOM DJ, LUYTEN GP, MOOY C, KERKVLIET S, ZWINDERMAN AH , JAGER MJ (1997) Human leukocyte antigen class I expression. Marker of poor prognosis in uveal melanoma. Invest Ophthalmol Vis Sci 38: 1865-1872.

BONI R, VORTMEYER AO, BURG G, HOFBAUER G , ZHUANG Z (1998) The PTEN tumour suppressor gene and malignant melanoma. Melanoma Res 8: 300-302.

BRANTLEY JR MA, JR. , HARBOUR JW (2000) Deregulation of the Rb and p53 Pathways in Uveal Melanoma. Am J Pathol 157: 1795-1801.

BUROKER N, BESTWICK R, HAIGHT G, MAGENIS RE , LITT M (1987) A hypervariable repeated sequence on human chromosome 1p36. Hum Genet 77: 175-181.

CAIRNS P, MAO L, MERLO A, LEE DJ, SCHWAB D, EBY Y, TOKINO K, VAN DER RIET P, BLAUGRUND JE , SIDRANSKY D (1994) Rates of p16 (MTS1) mutations in primary tumors with 9p loss [letter; comment]. Science 265: 415-417.

CAIRNS P, OKAMI K, HALACHMI S, HALACHMI N, ESTELLER M, HERMAN JG, JEN J, ISAACS WB, BOVA GS , SIDRANSKY D (1997) Frequent inactivation of PTEN/MMAC1 in primary prostate cancer. Cancer Res 57: 4997-5000.

CAIRNS P, POLASCIK TJ, EBY Y, TOKINO K, CALIFANO J, MERLO A, MAO L, HERATH J, JENKINS R, WESTRA W , ET AL. (1995) Frequency of homozygous deletion at p16/CDKN2 in primary human tumours. Nat Genet 11: 210-212.

CANNON-ALBRIGHT LA, GOLDGAR DE, MEYER LJ, LEWIS CM, ANDERSON DE, FOUNTAIN JW, HEGI ME, WISEMAN RW, PETTY EM, BALE AE , ET AL. (1992) Assignment of a locus for familial melanoma, MLM, to chromosome 9p13- p22. Science 258: 1148-1152.

CANTORE M, FIORENTINI G, AITINI E, DAVITTI B, CAVAZZINI G, RABBI C, LUSENTI A, BERTANI M, MORANDI C, BENEDINI V , ET AL. (1994) Intra-arterial hepatic carboplatin-based chemotherapy for ocular melanoma metastatic to the liver. Report of a phase II study. Tumori 80: 37-39.

CARON H, VAN SLUIS P, DE KRAKER J, BOKKERINK J, EGELER M, LAUREYS G, SLATER R, WESTERVELD A, VOUTE PA , VERSTEEG R (1996) Allelic loss of chromosome 1p as a predictor of unfavorable outcome in patients with neuroblastoma. N Engl J Med 334: 225-230.

CHANA JS, WILSON GD, CREE IA, ALEXANDER RA, MYATT N, NEALE M, FOSS AJ , HUNGERFORD JL (1999) c-myc, p53, and Bcl-2 expression and clinical outcome in uveal melanoma. Br J Ophthalmol 83: 110-114.

CHANG AE, KARNELL LH , MENCK HR (1998) The National Cancer Data Base report on cutaneous and noncutaneous melanoma: a summary of 84,836 cases from the past decade. The American College of Surgeons Commission on Cancer and the American Cancer Society. Cancer 83: 1664-1678.

CHAR DH, KROLL SM, MILLER T, CASTRO J , QUIVEY J (1996) Irradiated uveal melanomas: cytopathologic correlation with prognosis. Am J Ophthalmol 122: 509-513.

CHAR DH , MILLER T (1995) Accuracy of presumed uveal melanoma diagnosis before alternative therapy. Br J Ophthalmol 79: 692-696.

CHAR DH, MILLER TR , CRAWFORD JB (1991) Cytopathologic diagnosis of benign lesions simulating choroidal melanomas. Am J Ophthalmol 112: 70-75.

CHAR DH, MILLER TR, LJUNG BM, HOWES EL , STOLOFF A (1989) Fine needle aspiration biopsy in uveal melanoma. Acta Cytol 33: 599-605.

CHEN PW, MURRAY TG, SALGALLER ML , KSANDER BR (1997) Expression of MAGE genes in ocular melanoma cell lines. J Immunother 20: 265-275.

COLEMAN K, BAAK JP, VAN DIEST P, MULLANEY J, FARRELL M , FENTON M (1993) Prognostic factors following enucleation of 111 uveal melanomas. Br J Ophthalmol 77: 688-692.

COLEMAN K, BAAK JP, VAN DIEST PJ , MULLANEY J (1996) Prognostic value of morphometric features and the callender classification in uveal melanomas. Ophthalmology 103: 1634-1641.

COLLABORATION NIOHAIOMM (1996) A complete set of human telomeric probes and their clinical application. National Institutes of Health and Institute of Molecular Medicine collaboration [published erratum appears in Nat Genet 1996 Dec;14(4):487]. Nat Genet 14: 86-89.

COUPLAND SE, ANASTASSIOU G, STANG A, SCHILLING H, ANAGNOSTOPOULOS I, BORNFELD N , STEIN H (2000) The prognostic value of cyclin D1, p53, and MDM2 protein expression in uveal melanoma. J Pathol 191: 120-126.

CREE IA (2000) Cell cycle and melanoma–two different tumours from the same cell type. J Pathol 191: 112-114.

DAHLENFORS R, TORNQVIST G, WETTRELL K , MARK J (1993) Cytogenetical observations in nine ocular malignant melanomas. Anticancer Res 13: 1415-1420.

DE WAARD-SIEBINGA I, BLOM DJ, GRIFFIOEN M, SCHRIER PI, HOOGENDOORN E, BEVERSTOCK G, DANEN EH , JAGER MJ (1995) Establishment and characterization of an uveal-melanoma cell line. Int J Cancer 62: 155-161.

DI CRISTOFANO A , PANDOLFI PP (2000) The multiple roles of PTEN in tumor suppression. Cell 100: 387-390.

DIENER-WEST M, HAWKINS BS, MARKOWITZ JA , SCHACHAT AP (1992) A review of mortality from choroidal melanoma. II. A meta-analysis of 5- year mortality rates following enucleation, 1966 through 1988. Arch Ophthalmol 110: 245-250.

DOHNER H, STILGENBAUER S, BENNER A, LEUPOLT E, KROBER A, BULLINGER L, DOHNER K, BENTZ M , LICHTER P (2000) Genomic Aberrations and Survival in Chronic Lymphocytic Leukemia. N Engl J Med 343: 1910-1916.

DOLIN PJ, FOSS AJ , HUNGERFORD JL (1994) Uveal melanoma: is solar ultraviolet radiation a risk factor? Ophthalmic Epidemiol 1: 27-30.

REFERENCES

DOLIN PJ , JOHNSON GJ (1994) Solar ultraviolet radiation and ocular disease: a review of the epidemiological and experimental evidence. Ophthalmic Epidemiol 1: 155-164.

DONLON TA, BRUNS GA, LATT SA, MULHOLLAND J , WYMAN AR (1987) A chromosome 8 enriched alphoid repeat. Cytogenet Cell Genet 46: 607.

EASTON DF, STEELE L, FIELDS P, ORMISTON W, AVERILL D, DALY PA, MCMANUS R, NEUHAUSEN SL, FORD D, WOOSTER R, CANNON-ALBRIGHT LA, STRATTON MR , GOLDGAR DE (1997) Cancer risks in two large breast cancer families linked to BRCA2 on chromosome 13q12-13. Am J Hum Genet 61: 120-128.

EGAN KM, SEDDON JM, GLYNN RJ, GRAGOUDAS ES , ALBERT DM (1988) Epidemiologic aspects of uveal melanoma. Surv Ophthalmol 32: 239-251.

FARGNOLI MC, CHIMENTI S, KELLER G, SOYER HP, DAL POZZO V, HOFLER H , PERIS K (1998) CDKN2a/p16INK4a mutations and lack of p19ARF involvement in familial melanoma kindreds. J Invest Dermatol 111: 1202-1206.

FITZGERALD MG, HARKIN DP, SILVA-ARRIETA S, MACDONALD DJ, LUCCHINA LC, UNSAL H, O'NEILL E, KOH J, FINKELSTEIN DM, ISSELBACHER KJ, SOBER AJ , HABER DA (1996) Prevalence of germ-line mutations in p16, p19ARF, and CDK4 in familial melanoma: analysis of a clinic-based population. Proc Natl Acad Sci U S A 93: 8541-8545.

FOLBERG R, AUGSBURGER JJ, GAMEL JW, SHIELDS JA , LANG WR (1985) Fine-needle aspirates of uveal melanomas and prognosis. Am J Ophthalmol 100: 654-657.

FOLBERG R, RUMMELT V, PARYS-VAN GINDERDEUREN R, HWANG T, WOOLSON RF, PEER J , GRUMAN LM (1993) The prognostic value of tumor blood vessel morphology in primary uveal melanoma. Ophthalmology 100: 1389-1398.

FOULKES WD, FLANDERS TY, POLLOCK PM , HAYWARD NK (1997) The CDKN2A (p16) gene and human cancer. Mol Med 3: 5-20.

FOUNTAIN JW, BALE SJ, HOUSMAN DE , DRACOPOLI NC (1990) Genetics of melanoma. Cancer Surv 9: 645-671.

FOUNTAIN JW, KARAYIORGOU M, ERNSTOFF MS, KIRKWOOD JM, VLOCK DR, TITUS-ERNSTOFF L, BOUCHARD B, VIJAYASARADHI S, HOUGHTON AN, LAHTI J , ET AL. (1992) Homozygous deletions within human chromosome band 9p21 in melanoma. Proc Natl Acad Sci U S A 89: 10557-10561.

FREIRE JE, DE POTTER P, BRADY LW , LONGTON WA (1997) Brachytherapy in primary ocular tumors. Semin Surg Oncol 13: 167-176.

GALLAGHER RP, ELWOOD JM, ROOTMAN J, SPINELLI JJ, HILL GB, THRELFALL WJ , BIRDSELL JM (1985) Risk factors for ocular melanoma: Western Canada Melanoma Study. J Natl Cancer Inst 74: 775-778.

GAMEL JW, MCLEAN IW , MCCURDY JB (1993) Biologic distinctions between cure and time to death in 2892 patients with intraocular melanoma. Cancer 71: 2299-2305.

GARDIE B, CAYUELA JM, MARTINI S , SIGAUX F (1998) Genomic alterations of the p19ARF encoding exons in T-cell acute lymphoblastic leukemia. Blood 91: 1016-1020.

GHAZVINI S, CHAR DH, KROLL S, WALDMAN FM , PINKEL D (1996) Comparative genomic hybridization analysis of archival formalin-fixed paraffin-embedded uveal melanomas. Cancer Genet Cytogenet 90: 95-101.

GLASGOW BJ, BROWN HH, ZARGOZA AM , FOOS RY (1988) Quantitation of tumor seeding from fine needle aspiration of ocular melanomas. Am J Ophthalmol 105: 538-546.

GOLDSTEIN AM, DRACOPOLI NC, ENGELSTEIN M, FRASER MC, CLARK WH, JR. , TUCKER MA (1994) Linkage of cutaneous malignant melanoma/dysplastic nevi to chromosome 9p, and evidence for genetic heterogeneity. Am J Hum Genet 54: 489-496.

GONDER JR, SHIELDS JA, ALBERT DM, AUGSBURGER JJ , LAVIN PT (1982) Uveal malignant melanoma associated with ocular and oculodermal melanocytosis. Ophthalmology 89: 953-960.

GONZALGO ML, BENDER CM, YOU EH, GLENDENING JM, FLORES JF, WALKER GJ, HAYWARD NK, JONES PA , FOUNTAIN JW (1997) Low frequency of p16/CDKN2A methylation in sporadic melanoma: comparative approaches for methylation analysis of primary tumors. Cancer Res 57: 5336-5347.

GOWN AM, VOGEL AM, HOAK D, GOUGH F , MCNUTT MA (1986) Monoclonal antibodies specific for melanocytic tumors distinguish subpopulations of melanocytes. Am J Pathol 123: 195-203.

GREENE MH (1999) The genetics of hereditary melanoma and nevi: 1998 Update. Cancer 86: 2464-2477.

GRIFFIN CA, LONG PP , SCHACHAT AP (1988) Trisomy 6p in an ocular melanoma. Cancer Genet Cytogenet 32: 129-132.

GRUIS NA, VAN DER VELDEN PA, SANDKUIJL LA, PRINS DE, WEAVER-FELDHAUS J, KAMB A, BERGMAN W , FRANTS RR (1995) Homozygotes for CDKN2 (p16) germline mutation in Dutch familial melanoma kindreds. Nat Genet 10: 351-353.

GULDBERG P, THOR STRATEN P, BIRCK A, AHRENKIEL V, KIRKIN AF , ZEUTHEN J (1997) Disruption of the MMAC1/PTEN gene by deletion or mutation is a frequent event in malignant melanoma. Cancer Res 57: 3660-3663.

HAGEMEIJER A, DE KLEIN A, WIJSMAN J, VAN MEERTEN E, DE GREEF GE , SACCHI N (1998) Development of an interphase fluorescent in situ hybridization (FISH) test to detect t(8;21) in AML patients. Leukemia 12: 96-101.

HERMAN JG, GRAFF JR, MYOHANEN S, NELKIN BD , BAYLIN SB (1996) Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci U S A 93: 9821-9826.

HOLLY EA, ASTON DA, CHAR DH, KRISTIANSEN JJ , AHN DK (1990) Uveal melanoma in relation to ultraviolet light exposure and host factors. Cancer Res 50: 5773-5777.

HOPMAN AH, RAMAEKERS FC, RAAP AK, BECK JL, DEVILEE P, VAN DER PLOEG M , VOOIJS GP (1988) In situ hybridization as a tool to study numerical chromosome aberrations in solid bladder tumors. Histochemistry 89: 307-316.

HORSMAN DE, SROKA H, ROOTMAN J , WHITE VA (1990) Monosomy 3 and isochromosome 8q in a uveal melanoma. Cancer Genet Cytogenet 45: 249-253.

HORSMAN DE , WHITE VA (1993) Cytogenetic analysis of uveal melanoma. Consistent occurrence of monosomy 3 and trisomy 8q. Cancer 71: 811-819.

HORSTHEMKE B, PRESCHER G, BORNFELD N , BECHER R (1992) Loss of chromosome 3 alleles and multiplication of chromosome 8 alleles in uveal melanoma. Genes Chromosomes Cancer 4: 217-221.

HURKS H, METZELAAR-BLOK JA, BARTHEN ER, ZWINDERMAN AH, DE WOLFF-ROUENDAAL D, KEUNEN JE , JAGER MJ (2000) Expression of epidermal growth factor receptor: risk factor in uveal melanoma. Invest Ophthalmol Vis Sci 41: 2023-2027.

JAKOBIEC FA, COLEMAN DJ, CHATTOCK A , SMITH M (1979) Ultrasonically guided needle biopsy and cytologic diagnosis of solid intraocular tumors. Ophthalmology 86: 1662-1681.

JAY M , MCCARTNEY AC (1993) Familial malignant melanoma of the uvea and p53: a Victorian detective story. Surv Ophthalmol 37: 457-462.

JENSEN OA , ANDERSEN SR (1959) Late conplications of biopsy in intraocular tumors. Acta Ophthalmologica 37: 568-572.

KAGHAD M, BONNET H, YANG A, CREANCIER L, BISCAN JC, VALENT A, MINTY A, CHALON P, LELIAS JM, DUMONT X, FERRARA P, MCKEON F , CAPUT D (1997) Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. Cell 90: 809-819.

REFERENCES

KALLIONIEMI A, KALLIONIEMI OP, SUDAR D, RUTOVITZ D, GRAY JW, WALDMAN F , PINKEL D (1992) Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. Science 258: 818-821.

KAMB A, GRUIS NA, WEAVER-FELDHAUS J, LIU Q, HARSHMAN K, TAVTIGIAN SV, STOCKERT E, DAY RS, 3RD, JOHNSON BE , SKOLNICK MH (1994) A cell cycle regulator potentially involved in genesis of many tumor types [see comments]. Science 264: 436-440.

KAN-MITCHELL J, MITCHELL MS, RAO N , LIGGETT PE (1989) Characterization of uveal melanoma cell lines that grow as xenografts in rabbit eyes. Invest Ophthalmol Vis Sci 30: 829-834.

KATH R, HAYUNGS J, BORNFELD N, SAUERWEIN W, HOFFKEN K , SEEBER S (1993) Prognosis and treatment of disseminated uveal melanoma. Cancer 72: 2219-2223.

KAUFFMAN ML (1952) Aspiration Biopsy of Malignant Melanoma of Choroid. Arch. Ophthalmol 47: 541-542.

KIRBY DB (1929) Tissue culture in ophthalmic research. Trans Am Ophthalmol Soc 27: 334-383.

KNUDSON AG, JR. (1971) Mutation and cancer: statistical study of retinoblastoma. Proc Natl Acad Sci U S A 68: 820-823.

KNUDSON AG (1993) Antioncogenes and human cancer. Proc Natl Acad Sci U S A 90: 10914-10921.

KO LJ , PRIVES C (1996) p53: puzzle and paradigm. Genes Dev 10: 1054-1072.

KUMAR R, SAUROJA I, PUNNONEN K, JANSEN C , HEMMINKI K (1998) Selective deletion of exon 1 beta of the p19ARF gene in metastatic melanoma cell lines. Genes Chromosomes Cancer 23: 273-277.

LEVRERO M, DE LAURENZI V, COSTANZO A, GONG J, WANG JY , MELINO G (2000) The p53/p63/p73 family of transcription factors: overlapping and distinct functions. J Cell Sci 113: 1661-1670.

LEYVRAZ S, SPATARO V, BAUER J, PAMPALLONA S, SALMON R, DORVAL T, MEULI R, GILLET M, LEJEUNE F , ZOGRAFOS L (1997) Treatment of ocular melanoma metastatic to the liver by hepatic arterial chemotherapy. J Clin Oncol 15: 2589-2595.

LI J, YEN C, LIAW D, PODSYPANINA K, BOSE S, WANG SI, PUC J, MILIARESIS C, RODGERS L, MCCOMBIE R, BIGNER SH, GIOVANELLA BC, ITTMANN M, TYCKO B, HIBSHOOSH H, WIGLER MH , PARSONS R (1997) PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science 275: 1943-1947.

LI W, GRAGOUDAS ES , EGAN KM (2000) Metastatic melanoma death rates by anatomic site after proton beam irradiation for uveal melanoma. Arch Ophthalmol 118: 1066-1070.

LIU Q , SOMMER SS (1995) Restriction endonuclease fingerprinting (REF): a sensitive method for screening mutations in long, contiguous segments of DNA. Biotechniques 18: 470-477.

LOHRUM MA , VOUSDEN KH (2000) Regulation and function of the p53-related proteins: same family, different rules. Trends Cell Biol 10: 197-202.

LONG JC, BLACK WC , DANIELSON RW (1953) Aspiration biopsy in intraocular tumors. Arch Ophthalmol. 50: 303-310.

LUYTEN GP, MOOY CM, DE JONG PT, HOOGEVEEN AT , LUIDER TM (1993) A chicken embryo model to study the growth of human uveal melanoma. Biochem Biophys Res Commun 192: 22-29.

LUYTEN GP, MOOY CM, EIJKENBOOM WM, STIJNEN T, HELLEMONS LP, LUIDER TM , DE JONG PT (1995) No demonstrated effect of pre-enucleation irradiation on survival of patients with uveal melanoma. Am J Ophthalmol 119: 786-791.

LUYTEN GP, NAUS NC, MOOY CM, HAGEMEIJER A, KAN-MITCHELL J, VAN DRUNEN E, VUZEVSKI V, DE JONG PT , LUIDER TM (1996) Establishment and characterization of primary and metastatic uveal melanoma cell lines. Int J Cancer 66: 380-387.

MA D, LUYTEN GP, LUIDER TM , NIEDERKORN JY (1995) Relationship between natural killer cell susceptibility and metastasis of human uveal melanoma cells in a murine model. Invest Ophthalmol Vis Sci 36: 435-441.

MAKITIE T, SUMMANEN P, TARKKANEN A , KIVELA T (1999) Microvascular loops and networks as prognostic indicators in choroidal and ciliary body melanomas. J Natl Cancer Inst 91: 359-367.

MAKLEY JR. TA (1967) Biopsy of intraocular lesions. Am. J. Ophthalmol. 64: 591-599.

MARSH DJ, COULON V, LUNETTA KL, ROCCA-SERRA P, DAHIA PL, ZHENG Z, LIAW D, CARON S, DUBOUE B, LIN AY, RICHARDSON AL, BONNETBLANC JM, BRESSIEUX JM, CABARROT-MOREAU A, CHOMPRET A, DEMANGE L, EELES RA, YAHANDA AM, FEARON ER, FRICKER JP, GORLIN RJ, HODGSON SV, HUSON S, LACOMBE D, ENG C , ET AL. (1998) Mutation spectrum and genotype-phenotype analyses in Cowden disease and Bannayan-Zonana syndrome, two hamartoma syndromes with germline PTEN mutation. Hum Mol Genet 7: 507-515.

MARSHALL JF, RUTHERFORD DC, HAPPERFIELD L, HANBY A, MCCARTNEY AC, NEWTON-BISHOP J , HART IR (1998) Comparative analysis of integrins in vitro and in vivo in uveal and cutaneous melanomas. Br J Cancer 77: 522-529.

MASSARELLI R, BELKHOU R, DUNEL-ERB S, CHEVALIER C, ABBE JC, PIGNOL JP, MOUTAOUAKKIL M , SAHEL J (1994) Caracterisation morphologique de lignees cellulaires de melanome uveal humain. C R Acad Sci III 317: 25-33.

MASTERS JR (2000) Human cancer cell linesfact and fantasy. Nature reviews 1: 233-236.

MCKENNA RW (2000) Multifaceted approach to the diagnosis and classification of acute leukemias. Clin Chem 46: 1252-1259.

MCLEAN IW (1993) The biology of haematogenous metastasis in human uveal malignant melanoma. Virchows Arch A Pathol Anat Histopathol 422: 433-437.

MCLEAN IW , GAMEL JW (1998) Cause-specific versus all-cause survival. Ophthalmology 105: 1989-1990.

MCLEAN IW, KEEFE KS , BURNIER MN (1997) Uveal melanoma. Comparison of the prognostic value of fibrovascular loops, mean of the ten largest nucleoli, cell type, and tumor size. Ophthalmology 104: 777-780.

MCNAMARA M, FELIX C, DAVISON EV, FENTON M , KENNEDY SM (1997) Assessment of chromosome 3 copy number in ocular melanoma using fluorescence in situ hybridization. Cancer Genet Cytogenet 98: 4-8.

MERBS SL , SIDRANSKY D (1999) Analysis of p16 (CDKN2/MTS-1/INK4A) alterations in primary sporadic uveal melanoma. Invest Ophthalmol Vis Sci 40: 779-783.

MILLS AA, ZHENG B, WANG XJ, VOGEL H, ROOP DR , BRADLEY A (1999) p63 is a p53 homologue required for limb and epidermal morphogenesis. Nature 398: 708-713.

MITTELMAN F: AN INTERNATIONAL SYSTEM FOR HUMAN CYTOGENETIC NOMENCLATURE (1985) ISCN 1985. Karger, Basel, 1985.

MOORE BW (1965) A soluble protein characteristic of the nervous system. Biochem Biophys Res Commun 19: 739-744.

MOOY CM , DE JONG PT (1996) Prognostic parameters in uveal melanoma: a review. Surv Ophthalmol 41: 215-228.

MOOY CM, LUYTEN GP, DE JONG PT, JENSEN OA, LUIDER TM, VAN DER HAM F , BOSMAN FT (1995) Neural cell adhesion molecule distribution in primary and metastatic uveal melanoma. Hum Pathol 26: 1185-1190.

MOOY CM, VAN DER HELM MJ, VAN DER KWAST TH, DE JONG PT, RUITER DJ , ZWARTHOFF EC (1991) No N-ras mutations in human uveal melanoma: the role of ultraviolet light revisited. Br J Cancer 64: 411-413.

REFERENCES

MOURIAUX F, MAURAGE CA, LABALETTE P, SABLONNIERE B, MALECAZE F , DARBON JM (2000) Cyclin-dependent kinase inhibitory protein expression in human choroidal melanoma tumors. Invest Ophthalmol Vis Sci 41: 2837-2843.

MUKAI S , DRYJA TP (1986) Loss of alleles at polymorphic loci on chromosome 2 in uveal melanoma. Cancer Genet Cytogenet 22: 45-53.

NATHAN FE, BERD D, SATO T, SHIELD JA, SHIELDS CL, DE POTTER P , MASTRANGELO MJ (1997) BOLD+interferon in the treatment of metastatic uveal melanoma: first report of active systemic therapy. J Exp Clin Cancer Res 16: 201-208.

NAUS NC, VAN DRUNEN E, DE KLEIN A, LUYTEN GPM, PARIDAENS DA, ALERS JC, KSANDER BR, BEVERLOO HB , SLATER RM (2001) Characterization of complex chromosomal abnormalities in uveal melanoma by Fluorescent In Situ Hybridization, Spectral Karyotyping and Comparative Genomic Hybridization. Genes Chromosomes Cancer 30: 267-273.

NAUS NC, ZUIDERVAART W, RAYMAN N, SLATER R, VAN DRUNEN E, KSANDER B, LUYTEN GP , KLEIN A (2000) Mutation analysis of the PTEN gene in uveal melanoma cell lines. Int J Cancer 87: 151-153.

NELEN MR, VAN STAVEREN WC, PEETERS EA, HASSEL MB, GORLIN RJ, HAMM H, LINDBOE CF, FRYNS JP, SIJMONS RH, WOODS DG, MARIMAN EC, PADBERG GW , KREMER H (1997) Germline mutations in the PTEN/MMAC1 gene in patients with Cowden disease. Hum Mol Genet 6: 1383-1387.

NOMOTO S, HARUKI N, KONDO M, KONISHI H , TAKAHASHI T (1998) Search for mutations and examination of allelic expression imbalance of the p73 gene at 1p36.33 in human lung cancers. Cancer Res 58: 1380-1383.

NYLANDER K, COATES PJ , HALL PA (2000) Characterization of the expression pattern of p63 alpha and delta Np63 alpha in benign and malignant oral epithelial lesions. Int J Cancer 87: 368-372.

O'HARA BJ, EHYA H, SHIELDS JA, AUGSBURGER JJ, SHIELDS CL , EAGLE RC (1993) Fine needle aspiration biopsy in pediatric ophthalmic tumors and pseudotumors. Acta Cytol 37: 125-130.

OHTA M, NAGAI H, SHIMIZU M, RASIO D, BERD D, MASTRANGELO M, SINGH AD, SHIELDS JA, SHIELDS CL, CROCE CM , ET AL. (1994) Rarity of somatic and germline mutations of the cyclin-dependent kinase 4 inhibitor gene, CDK4I, in melanoma. Cancer Res 54: 5269-5272.

PARRELLA P, SIDRANSKY D , MERBS SL (1999) Allelotype of posterior uveal melanoma: implications for a bifurcated tumor progression pathway. Cancer Res 59: 3032-3037.

PERIS K, CHIMENTI S, FARGNOLI MC, VALERI P, KERL H , WOLF P (1999) UV fingerprint CDKN2a but no p14ARF mutations in sporadic melanomas [letter]. J Invest Dermatol 112: 825-826.

PIEPKORN M (2000) Melanoma genetics: an update with focus on the CDKN2A(p16)/ARF tumor suppressors. J Am Acad Dermatol 42: 705-722; quiz 723-706.

PINKEL D, LANDEGENT J, COLLINS C, FUSCOE J, SEGRAVES R, LUCAS J , GRAY J (1988) Fluorescence in situ hybridization with human chromosome-specific libraries: detection of trisomy 21 and translocations of chromosome 4. Proc Natl Acad Sci U S A 85: 9138-9142.

PINKEL D, SEGRAVES R, SUDAR D, CLARK S, POOLE I, KOWBEL D, COLLINS C, KUO WL, CHEN C, ZHAI Y, DAIRKEE SH, LJUNG BM, GRAY JW , ALBERTSON DG (1998) High resolution analysis of DNA copy number variation using comparative genomic hybridization to microarrays. Nat Genet 20: 207-211.

PRESCHER G, BORNFELD N , BECHER R (1990) Nonrandom chromosomal abnormalities in primary uveal melanoma. J Natl Cancer Inst 82: 1765-1769.

PRESCHER G, BORNFELD N , BECHER R (1994) Two subclones in a case of uveal melanoma. Relevance of monosomy 3 and multiplication of chromosome 8q. Cancer Genet Cytogenet 77: 144-146.

PRESCHER G, BORNFELD N, FRIEDRICHS W, SEEBER S , BECHER R (1995) Cytogenetics of twelve cases of uveal melanoma and patterns of nonrandom anomalies and isochromosome formation. Cancer Genet Cytogenet 80: 40-46.

PRESCHER G, BORNFELD N, HIRCHE H, HORSTHEMKE B, JOCKEL KH , BECHER R (1996) Prognostic implications of monosomy 3 in uveal melanoma. Lancet 347: 1222-1225.

PRESCHER G, BORNFELD N, HORSTHEMKE B , BECHER R (1992) Chromosomal aberrations defining uveal melanoma of poor prognosis. Lancet 339: 691-692.

PRONK JC, BEEMER FA, GERAEDTS JPM, LESCHOT NJ , NIERMEIJER MF: Medische Genetica, Fifth ed. Wetenschappelijke uitgeverij Bunge, Utrecht, 1994.

QUELLE DE, CHENG M, ASHMUN RA , SHERR CJ (1997) Cancer-associated mutations at the INK4a locus cancel cell cycle arrest by p16INK4a but not by the alternative reading frame protein p19ARF. Proc Natl Acad Sci U S A 94: 669-673.

RANDERSON-MOOR JA, HARLAND M, WILLIAMS S, CUTHBERT-HEAVENS D, SHERIDAN E, AVEYARD J, SIBLEY K, WHITAKER L, KNOWLES M, NEWTON BISHOP J , BISHOP DT (2001) A germline deletion of p14(ARF) but not CDKN2A in a melanoma-neural system tumour syndrome family. Hum Mol Genet 10: 55-62.

RISINGER JI, HAYES AK, BERCHUCK A , BARRETT JC (1997) PTEN/MMAC1 mutations in endometrial cancers. Cancer Res 57: 4736-4738.

RUAS M , PETERS G (1998) The p16INK4a/CDKN2A tumor suppressor and its relatives. Biochim Biophys Acta 1378: F115-177.

SACCHI N, MAGNANI I, KEARNEY L, WIJSMAN J, HAGEMEIJER A , DARFLER M (1995) Interphase cytogenetics of the t(8;21)(q22;q22) associated with acute myelogenous leukemia by two-color fluorescence in situ hybridization. Cancer Genet Cytogenet 79: 97-103.

SANDBERG AA , CHEN Z (1994) Cancer cytogenetics and molecular genetics: detection and therapeutic strategy. In Vivo 8: 807-818.

SAUROJA I, SMEDS J, VLAYKOVA T, KUMAR R, TALVE L, HAHKA-KEMPPINEN M, PUNNONEN K, JANSEN CT, HEMMINKI K , PYRHONEN S (2000) Analysis of G(1)/S checkpoint regulators in metastatic melanoma. Genes Chromosomes Cancer 28: 404-414.

SCHROCK E, DU MANOIR S, VELDMAN T, SCHOELL B, WIENBERG J, FERGUSON-SMITH MA, NING Y, LEDBETTER DH, BAR-AM I, SOENKSEN D, GARINI Y , RIED T (1996) Multicolor spectral karyotyping of human chromosomes [see comments]. Science 273: 494-497.

SCHWARTZ LH, FERRAND R, BOELLE PY, MAYLIN C, D'HERMIES F , VIRMONT J (1997) Lack of correlation between the location of choroidal melanoma and ultraviolet-radiation dose distribution. Radiat Res 147: 451-456.

SEDDON JM, ALBERT DM, LAVIN PT , ROBINSON N (1983) A prognostic factor study of disease-free interval and survival following enucleation for uveal melanoma. Arch Ophthalmol 101: 1894-1899.

SHIELDS CL, CATER J, SHIELDS JA, SINGH AD, SANTOS MC , CARVALHO C (2000) Combination of clinical factors predictive of growth of small choroidal melanocytic tumors. Arch Ophthalmol 118: 360-364.

SHIELDS CL, SHIELDS JA, KIRATLI H, DE POTTER P , CATER JR (1995) Risk factors for growth and metastasis of small choroidal melanocytic lesions. Trans Am Ophthalmol Soc 93: 259-275.

SHIELDS JA , SHIELDS CL (1993) Current management of posterior uveal melanoma. Mayo Clin Proc 68: 1196-1200.

SHIELDS JA, SHIELDS CL, EHYA H, EAGLE RC, JR. , DE POTTER P (1993) Fine-needle aspiration biopsy of suspected intraocular tumors. The 1992 Urwick Lecture. Ophthalmology 100: 1677-1684.

SINGH AD, BOGHOSIAN-SELL L, WARY KK, SHIELDS CL, DE POTTER P, DONOSO LA, SHIELDS JA, CANNIZZARO LA (1994) Cytogenetic findings in primary uveal melanoma. Cancer Genet Cytogenet 72: 109-115.

SINGH AD, CROCE CM, WARY KK, SHIELDS JA, DONOSO LA, SHIELDS CL, HUEBNER K, OHTA M (1996A) Familial uveal melanoma: absence of germline mutations involving the cyclin-dependent kinase-4 inhibitor gene (p16). Ophthalmic Genet 17: 39-40.

SINGH AD, WANG MX, DONOSO LA, SHIELDS CL, DE POTTER P, SHIELDS JA (1996B) Genetic aspects of uveal melanoma: a brief review. Semin Oncol 23: 768-772.

SINILNIKOVA OM, EGAN KM, QUINN JL, BOUTRAND L, LENOIR GM, STOPPA-LYONNET D, DESJARDINS L, LEVY C, GOLDGAR D, GRAGOUDAS ES (1999) Germline brca2 sequence variants in patients with ocular melanoma. Int J Cancer 82: 325-328.

SISLEY K, COTTAM DW, RENNIE IG, PARSONS MA, POTTER AM, POTTER CW, REES RC (1992) Non-random abnormalities of chromosomes 3, 6, and 8 associated with posterior uveal melanoma. Genes Chromosomes Cancer 5: 197-200.

SISLEY K, NICHOLS C, PARSONS MA, FARR R, REES RC, RENNIE IG (1998) Clinical applications of chromosome analysis, from fine needle aspiration biopsies, of posterior uveal melanomas. Eye 12: 203-207.

SISLEY K, PARSONS MA, GARNHAM J, POTTER AM, CURTIS D, REES RC, RENNIE IG (2000) Association of specific chromosome alterations with tumour phenotype in posterior uveal melanoma. Br J Cancer 82: 330-338.

SISLEY K, RENNIE IG, COTTAM DW, POTTER AM, POTTER CW, REES RC (1990) Cytogenetic findings in six posterior uveal melanomas: involvement of chromosomes 3, 6, and 8. Genes Chromosomes Cancer 2: 205-209.

SISLEY K, RENNIE IG, PARSONS MA, JACQUES R, HAMMOND DW, BELL SM, POTTER AM, REES RC (1997) Abnormalities of chromosomes 3 and 8 in posterior uveal melanoma correlate with prognosis. Genes Chromosomes Cancer 19: 22-28.

SPECHT CS, SMITH TW (1988) Uveal malignant melanoma and von Recklinghausen's neurofibromatosis. Cancer 62: 812-817.

SPEICHER MR, PRESCHER G, DU MANOIR S, JAUCH A, HORSTHEMKE B, BORNFELD N, BECHER R, CREMER T (1994) Chromosomal gains and losses in uveal melanomas detected by comparative genomic hybridization. Cancer Res 54: 3817-3823.

TAMURA M, GU J, MATSUMOTO K, AOTA S, PARSONS R, YAMADA KM (1998) Inhibition of cell migration, spreading, and focal adhesions by tumor suppressor PTEN. Science 280: 1614-1617.

TSAO H, ZHANG X, BENOIT E, HALUSKA FG (1998) Identification of PTEN/MMAC1 alterations in uncultured melanomas and melanoma cell lines. Oncogene 16: 3397-3402.

TUCKER MA, SHIELDS JA, HARTGE P, AUGSBURGER J, HOOVER RN, FRAUMENI JF, JR. (1985) Sunlight exposure as risk factor for intraocular malignant melanoma. N Engl J Med 313: 789-792.

VAN DEKKEN H, PIZZOLO JG, REUTER VE, MELAMED MR (1990) Cytogenetic analysis of human solid tumors by in situ hybridization with a set of 12 chromosome-specific DNA probes. Cytogenet Cell Genet 54: 103-107.

VAN DER RIET P, NAWROZ H, HRUBAN RH, CORIO R, TOKINO K, KOCH W, SIDRANSKY D (1994) Frequent loss of chromosome 9p21-22 early in head and neck cancer progression. Cancer Res 54: 1156-1158.

VAN HEES CL, JAGER MJ, BLEEKER JC, KEMME H, BERGMAN W (1998) Occurrence of cutaneous and uveal melanoma in patients with uveal melanoma and their first degree relatives. Melanoma Res 8: 175-180.

VAZQUEZ F , SELLERS WR (2000) The PTEN tumor suppressor protein: an antagonist of phosphoinositide 3- kinase signaling. Biochim Biophys Acta 1470: M21-35.

VEASEY CA (1951) Intraocular biopsy. Am. J. Ophthalmol. 34: 432-434.

VENNEGOOR C, CALAFAT J, HAGEMAN P, VAN BUITENEN F, JANSSEN H, KOLK A , RUMKE P (1985) Biochemical characterization and cellular localization of a formalin- resistant melanoma-associated antigen reacting with monoclonal antibody NKI/C-3. Int J Cancer 35: 287-295.

VERBIK DJ, MURRAY TG, TRAN JM , KSANDER BR (1997) Melanomas that develop within the eye inhibit lymphocyte proliferation. Int J Cancer 73: 470-478.

VLIETSTRA RJ, VAN ALEWIJK DC, HERMANS KG, VAN STEENBRUGGE GJ , TRAPMAN J (1998) Frequent inactivation of PTEN in prostate cancer cell lines and xenografts. Cancer Res 58: 2720-2723.

VOGELSTEIN B, LANE D , LEVINE AJ (2000) Surfing the p53 network. Nature 408: 307-310.

WALKER GJ, FLORES JF, GLENDENING JM, LIN AH, MARKL ID , FOUNTAIN JW (1998) Virtually 100% of melanoma cell lines harbor alterations at the DNA level within CDKN2A, CDKN2B, or one of their downstream targets. Genes Chromosomes Cancer 22: 157-163.

WANG SI, PUC J, LI J, BRUCE JN, CAIRNS P, SIDRANSKY D , PARSONS R (1997) Somatic mutations of PTEN in glioblastoma multiforme. Cancer Res 57: 4183-4186.

WAYE JS , WILLARD HF (1989) Chromosome specificity of satellite DNAs: short- and long-range organization of a diverged dimeric subset of human alpha satellite from chromosome 3. Chromosoma 97: 475-480.

WHITE E , PRIVES C (1999) DNA damage enables p73. Nature 399: 734-735, 737.

WHITE VA, CHAMBERS JD, COURTRIGHT PD, CHANG WY , HORSMAN DE (1998) Correlation of cytogenetic abnormalities with the outcome of patients with uveal melanoma. Cancer 83: 354-359.

WHITE VA, MCNEIL BK, THIBERVILLE L , HORSMAN DE (1996) Acquired homozygosity (isodisomy) of chromosome 3 during clonal evolution of a uveal melanoma: association with morphologic heterogeneity. Genes Chromosomes Cancer 15: 138-143.

WILTSHIRE RN, ELNER VM, DENNIS T, VINE AK , TRENT JM (1993) Cytogenetic analysis of posterior uveal melanoma. Cancer Genet Cytogenet 66: 47-53.

WOLL E, BEDIKIAN A , LEGHA SS (1999) Uveal melanoma: natural history and treatment options for metastatic disease. Melanoma Res 9: 575-581.

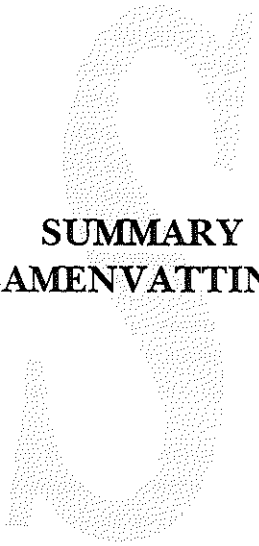
YANG A, KAGHAD M, WANG Y, GILLETT E, FLEMING MD, DOTSCH V, ANDREWS NC, CAPUT D , MCKEON F (1998) p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. Mol Cell 2: 305-316.

YANG A , MCKEON F (2000) P63 and P73: P53 mimics, menaces and more. Nature Reviews 1: 199-207.

YANG A, SCHWEITZER R, SUN D, KAGHAD M, WALKER N, BRONSON RT, TABIN C, SHARPE A, CAPUT D, CRUM C , MCKEON F (1999) p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. Nature 398: 714-718.

YANOFF M , FINE BS: Ocular pathology, a text and atlas, Third ed. J.B. Lippincott Company, Philadelphia, 1989.

ZIMMERMAN LE, MCLEAN IW , FOSTER WD (1978) Does enucleation of the eye containing a malignant melanoma prevent or accelerate the dissemination of tumour cells. Br J Ophthalmol 62: 420-425.



SUMMARY
SAMENVATTING

SUMMARY

In this thesis we describe cytogenetic and molecular genetic analyses of uveal melanoma. Uveal melanoma is the most common intraocular tumour in adults in the Western world, with an annual incidence of 7 per million people. About half of the patients die within fifteen years after time of diagnosis of metastatic disease, which is preferentially located in the liver. Several prognostic markers have been demonstrated to associate with (disease free) survival, however, the selection of patients at risk of metastatic disease remains difficult. In an attempt to more carefully predict the patients outcome, we analysed eighty uveal melanoma from patients treated with enucleation for chromosomal changes using cytogenetic and FISH techniques (**chapter 2**). We found a strong correlation between a concurrent loss of chromosomes 1p and 3 and a decreased disease free survival of the patients. Using these parameters patients at risk for metastatic disease can be selected which may justify adjuvant therapies in these cases. Furthermore, these data provide information about chromosome regions involved in uveal melanoma progression. Genes such as *p73*, mapped to chromosome 1p36, and *p63* on chromosome 3q27, will be tested for their involvement in uveal melanoma tumorigenesis.

Most uveal melanoma show simple karyotypes and cytogenetic and/or FISH analysis will be sufficient to characterise these genetic changes. However, in some cases, complex karyotypes or discrepancies between cytogenetic and FISH analyses are found. In **chapter 3**, we describe the analysis of the complex chromosomal abnormalities in five primary uveal melanoma and two uveal melanoma cell lines using conventional cytogenetic, FISH, SKY and CGH analyses. We demonstrated that, using cytogenetics or FISH only, chromosome 6 and 8 abnormalities are clearly underestimated. Furthermore we found a small deletion on chromosome 3q13-21 which may narrow down the chromosome region on chromosome 3 involved in uveal melanoma tumorigenesis.

These chromosome analyses are only applicable to patients treated with enucleation, since only then tumour material is available. However, more and more patients are treated with radiotherapy, restricting genetic prognostic evaluation. We investigated whether FISH analysis on fine needle aspiration biopsies of uveal melanoma could provide a tool for genetic studies in patients treated with eye conserving protocols (**chapter 4**). We found that FISH analysis is suitable for the detection of chromosomal changes in these *ex vivo* biopsies, and that the biopsies were representative for the original tumour. These data, indeed, suggest that this method can be used to obtain information about the chromosomal composition of these melanomas, which enables us to predict the patients outcome.

Chromosome analysis can also be applied to biopsies of lesion suspected to be uveal melanoma (**chapter 5**). In that study, we performed FISH analysis to three of such lesions and found gain of chromosome 8, in one case, suggesting malignant transformation. In the remaining cases no chromosomal changes were detected. In the first case we decided to enucleate the patient and found a small melanoma derived

from a pre-existent naevus. In the other two cases, no action was undertaken and appoximately 3 year after the procedure, no tumour progression was observed.

The second part of this thesis describes the establishment of uveal melanoma cell lines (chapter 6) and mutation analyses of potential tumour suppressor genes on these cell lines. In cutaneous melanoma cell lines, deletions or mutations in the $p16^{INK4a}$ and *PTEN* gene are frequently described. In chapter 7 we describe the mutation analysis of the $p16^{INK4a}$ and $p14^{ARF}$ gene in uveal melanoma. In contrast to what is known from cutaneous melanoma, we found that methylation is the major cause of $p16^{INK4a}$ inactivation in uveal melanoma cell lines. In the PTEN gene, no mutations were found in uveal melanoma cell lines (chapter 8). These data suggest that, despite a common origin, uveal and cutaneous melanoma follow a different route towards tumorigenesis. The general conclusions, which can be drawn from the studies described in this thesis, are discussed in chapter 9. Furthermore, in this chapter, recommendations for future research are made.

SAMENVATTING

In dit proefschrift worden de cytogenetische en moleculair genetische analyse van oogmelanomen beschreven. Het oogmelanoom is de meest voorkomende primaire maligne tumor van het oog met een jaarlijkse incidentie van 7 per miljoen mensen. De helft van deze patiënten overlijdt uiteindelijk aan de gevolgen van uitzaaiingen, waarbij vooral de lever is aangedaan. Het onderzoek naar oogmelanomen heeft zich vooral geconcentreerd op het vinden van parameters die correleren met de overleving van de patiënt. Het voorspellen van de prognose van een individuele patiënt bleef echter tot nu toe moeizaam.

In het eerste deel van dit proefschrift, wordt de nadruk gelegd op de cytogenetische afwijkingen in oogmelanomen.

In een serie van 80 tumoren hebben wij onderzocht of patiënten met een hoog risico op uitzaaiingen kunnen worden onderscheiden van patiënten met een lager risico, op basis van chromosoom afwijkingen in de tumor. Wij hebben hierbij gebruik gemaakt van cytogenetica en FISH technieken (hoofdstuk 2). Er werd een sterke correlatie gevonden tussen verlies van zowel chromosoom regio 1p36 en chromosoom 3 en de ziektevrije overleving van de patiënt. Deze prognostische marker stelt ons in staat hoog-risico patiënten te selecteren. Bij deze patiënten kunnen we overwegen aanvullende therapie te geven.

Buiten deze klinische relevantie van onze bevindingen geven deze resultaten inzicht in welke chromosoomregio's betrokken kunnen zijn bij het ontstaan of de uitbreiding van oogmelanomen. Genen zoals p73 (op chromosoom 1p36) en p63 (op chromosoom 3q27), dienen te worden onderzocht op hun betrokkenheid in oogmelanomen.

Meestal vinden we in oogmelanomen eenvoudige chromosomale afwijkingen en volstaan cytogenetica en FISH voor de analyse van deze tumoren. In sommige tumoren worden echter complexe afwijkingen gevonden. In hoofdstuk 3 beschrijven wij het gebruik van SKY en CGH als aanvullende technieken in vijf van deze gevallen en in twee oogmelanoom cellijnen. Chromosoom 6 en 8 afwijkingen werden vaker gevonden dan verwacht op basis van cytogenetica en FISH analyse. Bovendien hebben we een kleine deletie op chromosoom 3q13-24 opgespoord, hetgeen het gebied op chromosoom 3, dat mogelijk een rol speelt bij het ontstaan of de uitbreiding van oogmelanomen, verder verkleint.

De hierboven beschreven chromosoomanalyse kan alleen worden gedaan op tumormateriaal. Tegenwoordig worden veel patienten echter behandeld met bestraling van het aangedane oog waardoor er geen materiaal beschikbaar is voor analyse. Wij onderzochten of in dergelijke gevallen biopten van de melanomen genomen konden worden voor chromosoom onderzoek (hoofdstuk 4). Van veertig oogmelanomen hebben we, na enucleatie, een biopt genomen. Deze biopten hebben we, samen met een stukje van de oorspronkelijke tumor, geanalyseerd met FISH. Het bleek dat deze

biopten goed te gebruiken waren voor FISH analyse en dat ze representatief waren voor de tumor waaruit het biopt genomen was. Hoewel deze biopten genomen waren van geënucleëerde ogen, wijzen de resultaten erop dat we bij patiënten die behandeld worden met oogsparende therapieën, ook chromosoomanalyse kunnen verrichten ter evaluatie van de prognose van de betreffende patiënt.

Biopten kunnen ook genomen worden in gevallen waarbij de diagnose oogmelanoom niet geheel zeker is. Wij hebben in drie van zulke gevallen biopten genomen en chromosoomanalyse verricht met behulp van FISH analyse (hoofdstuk 5). In een van deze drie biopten vonden we een extra chromosoom 8, hetgeen suggereert dat er kwaadaardige cellen aanwezig zijn. Bij deze patiënt werd behandeling geadviseerd waarbij de patient de voorkeur gaf aan enucleatie. In de andere twee biopten werden geen afwijkingen gevonden en werden de patiënten gevolgd zonder verdere behandeling. Bij deze patienten werd in de drie jaar na het nemen van de biopten geen tumorgroei waargenomen.

Het tweede deel van dit proefschrift beschrijft de moleculair genetische analyse van oogmelanomen. In hoofdstuk 6 beschrijven wij het ontwikkelen en karakteriseren van twee primaire en drie metastatische oogmelanoom cellijnen. Deze cellijnen stellen ons in staat genen te onderzoeken die mogelijk betrokken zijn bij oogmelanomen.

Genen waarvan we weten dat zij betrokken zijn bij huidmelanomen zijn het $p16^{INK4a}/p14^{ARF}$ gen en het *PTEN* gen. In tegenstelling tot huidmelanomen, waar mutaties en verlies van het $p16^{INK4a}$ gen frequent gevonden worden, vonden wij dat in oogmelanomen dit gen meestal (in 4 van de 9 cellijnen) uitgeschakeld was door middel van methylering (hoofdstuk 7). Ook werden in deze oogmelanoom cellijnen geen mutaties in het PTEN gen gevonden (hoofdstuk 8).

De conclusies die getrokken worden uit het onderzoek beschreven in dit proefschrift worden uiteengezet in hoofdstuk 9. Bovendien bevat dit hoofdstuk aanbevelingen voor verder onderzoek.

COLOUR ILLUSTRATIONS
CHAPTERS 3, 4 & 5

CHAPTER 3

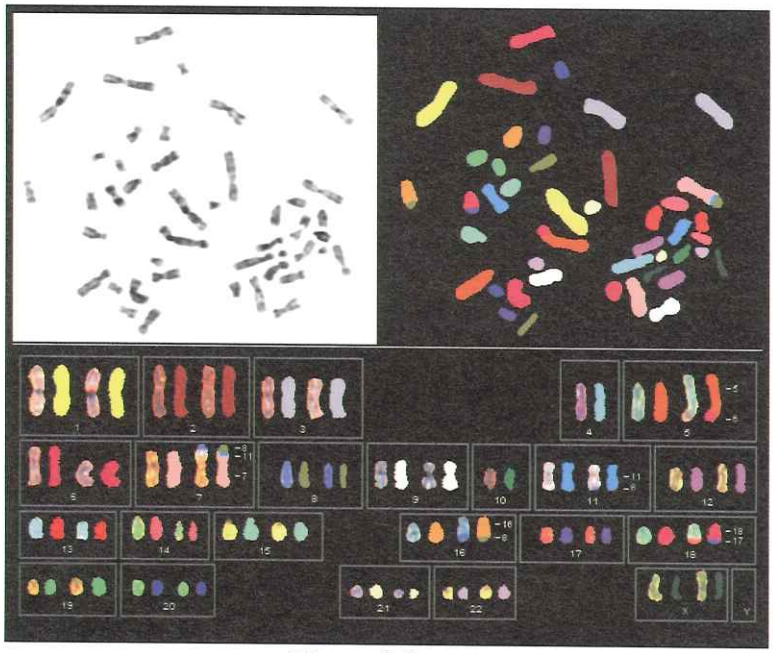


Figure 3.1

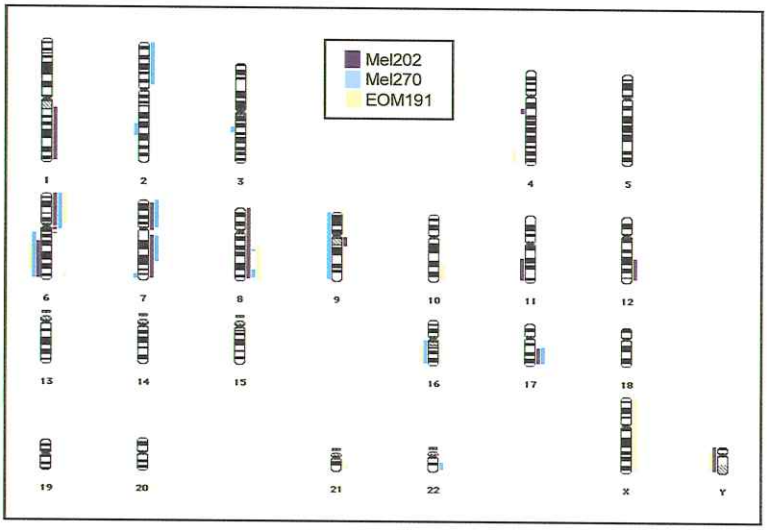


Figure 3.2

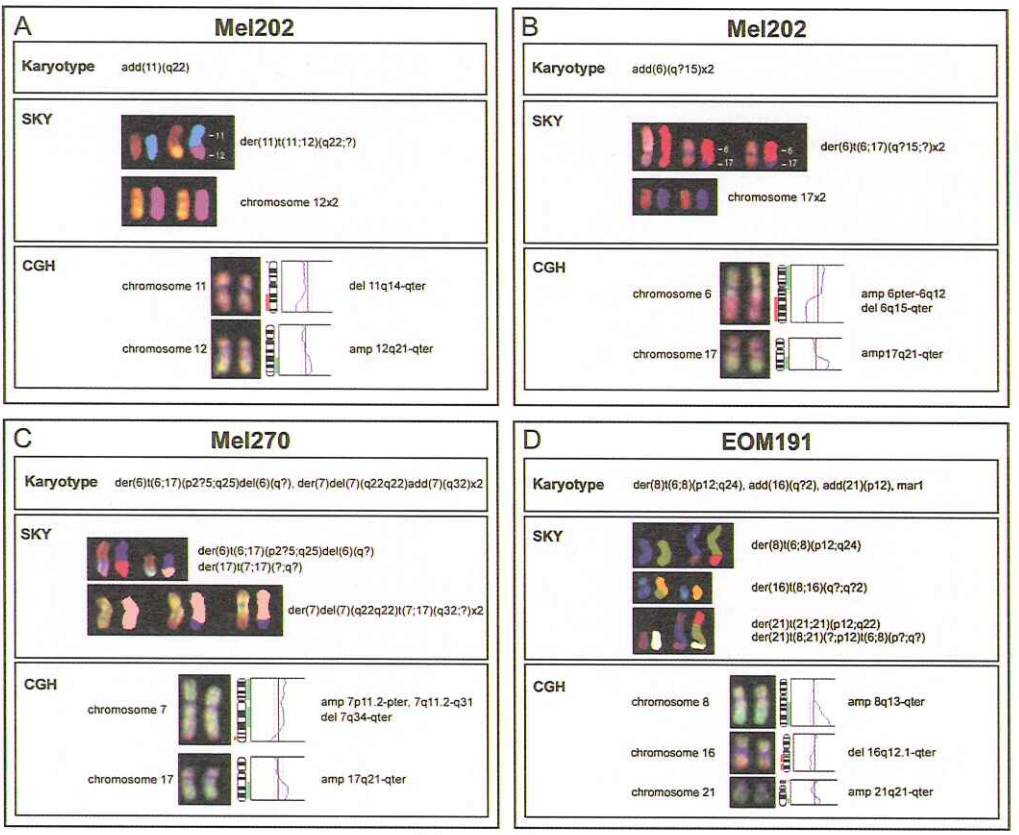

A
Mel202
Karyotype
add(11)(q22)
SKY
der(11)t(11;12)(q22;?)
chromosome 12x2
CGH
chromosome 11
del 11q14-qter
chromosome 12
amp 12q21-qter
B
Mel202
Karyotype
add(6)(q?15)x2
SKY
der(6)t(6;17)(q?15;?)x2
chromosome 17x2
CGH
chromosome 6
amp 6pter-6q12
del 6q15-qter
chromosome 17
amp17q21-qter
C
Mel270
Karyotype
der(6)t(6;17)(p2?5;q25)del(6)(q?), der(7)del(7)(q22q22)add(7)(q32)x2
SKY
der(6)t(6;17)(p2?5;q25)del(6)(q?)
der(17)t(7;17)(?;q?)
der(7)del(7)(q22q22)t(7;17)(q32;?)x2
CGH
chromosome 7
amp 7p11.2-pter, 7q11.2-q31
del 7q34-qter
chromosome 17
amp 17q21-qter
D
EOM191
Karyotype
der(8)t(6;8)(p12;q24), add(16)(q?2), add(21)(p12), mar1
SKY
der(8)t(6;8)(p12;q24)
der(16)t(8;16)(q?;q?2)
der(21)t(21;21)(p12;q22)
der(21)t(8;21)(?;p12)t(6;8)(p?;q?)
CGH
chromosome 8
amp 8q13-qter
chromosome 16
del 16q12.1-qter
chromosome 21
amp 21q21-qter

Figure 3.3

CHAPTERS 4 & 5

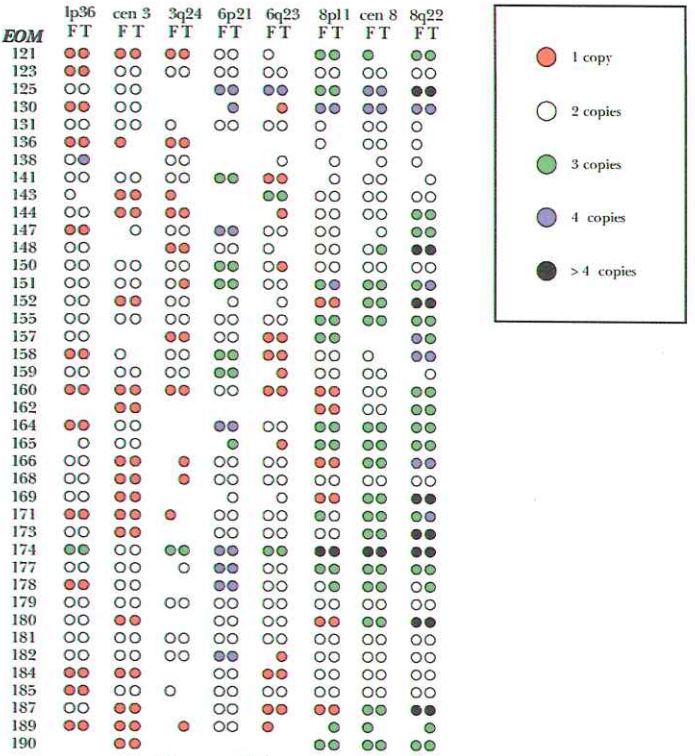


Figure 4.1

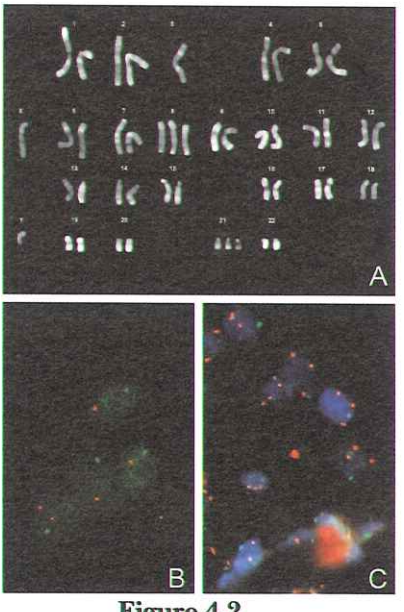


Figure 4.2

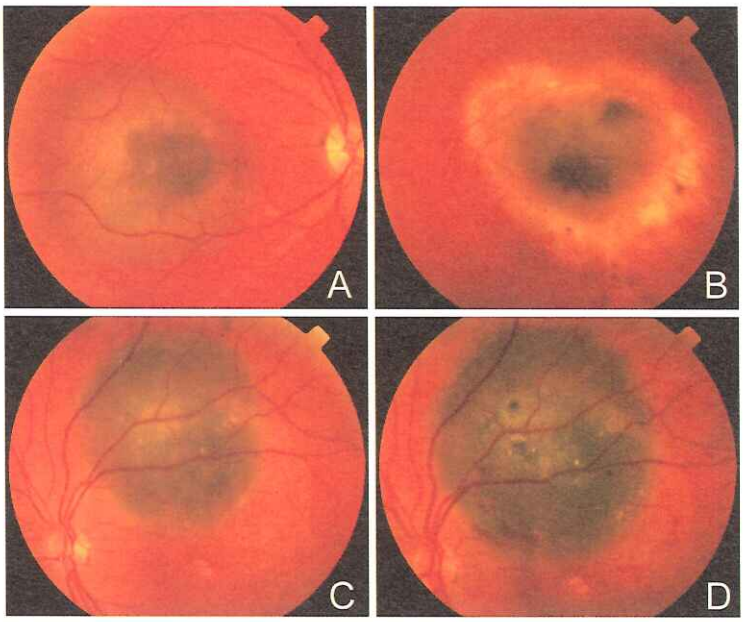


Figure 5.2

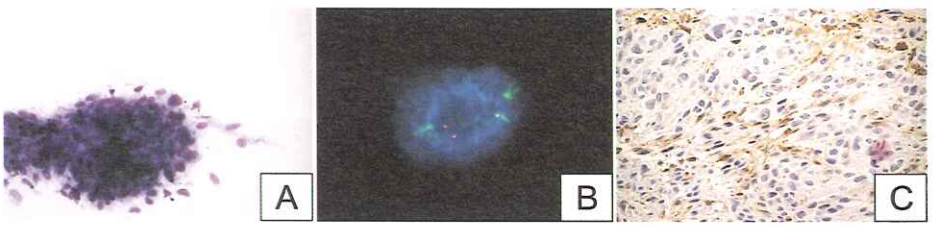
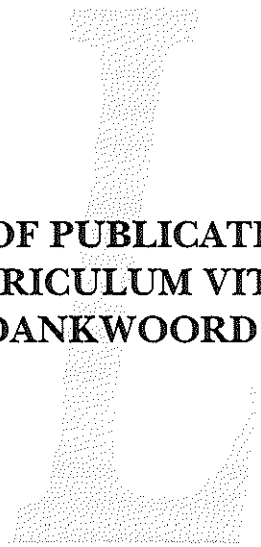


Figure 5.3



LIST OF PUBLICATIONS
CURRICULUM VITAE
DANKWOORD

LIST OF PUBLICATIONS

(*Publications and manuscripts based on the studies described in this thesis)

Naus NC, Luyten GPM, Klaver CCW, van Drunen E, Verhoeven ACA, Mooy CM, Mulder P, Paridaens ADA, Slater RM, de Klein A. Concurrent loss of chromosomes 1p and 3 predicts a decreased disease free survival in eye melanoma patients, submitted.*

NAUS NC, VAN DRUNEN E, SLATER RM, VERHOEVEN ACA, MOOY CM, PARIDAENS ADA, LUYTEN GPM, DE KLEIN A.Detection ofgenetic prognostic markers in uveal melanoma biopsies using fluorescent in situ hybridization, submitted*

LUYTEN GPM, NAUS NC, MOOY CM, VAN DRUNEN E, SLATER RM, DE KLEIN A. FISH analysis on fine needle aspiration biopsies in 3 cases of suspect uveal melanoma, submitted.*

NAUS NC, VERHOEVEN ACA, MUIJTJENS MJM, ALIREDJO R, LUYTEN GPM, DE KLEIN A.Methylation is the major cause of *p16* ^{INK4a} inactivation in uveal melanoma cell lines, submitted.*

NAUS NC, VAN DRUNEN E, DE KLEIN A, LUYTEN GP, PARIDAENS DA, ALERS JC, KSANDER BR, BEVERLOO HB, SLATER RM. Characterization of complex chromosomal abnormalities in uveal melanoma by fluorescence in situ hybridization, spectral karyotyping, and comparative genomic hybridization. Genes Chromosomes Cancer. 2001 Mar;30(3):267-273.*

NAUS NC, ZUIDERVAART W, RAYMAN N, SLATER R, VAN DRUNEN E, KSANDER B, LUYTEN GP, DE KLEIN A.
Mutation analysis of the PTEN gene in uveal melanoma cell lines. Int J Cancer. 2000 Jul 1;87(1):151-3.*

MOOY CM, NAUS NC, DE KLEIN A, VAN DEN BOSCH WA, PARIDAENS DA. Orbital chondrosarcoma developing in a patient with Paget disease. Am J Ophthalmol. 1999 May;127(5):619-21.

BLUYSSEN HA, NAUS NC, VAN OS RI, JASPERS I, HOEIJMAKERS JH, DE KLEIN A. Human and mouse homologs of the Schizosaccharomyces pombe rad17+ cell cycle checkpoint control gene. Genomics. 1999 Jan 15;55(2):219-28.

BLUYSSEN HA, VAN OS RI, NAUS NC, JASPERS I, HOEIJMAKERS JH, DE KLEIN A. A human and mouse homolog of the Schizosaccharomyces pombe rad1+ cell cycle checkpoint control gene. Genomics. 1998 Dec 1;54(2):331-7.

TANG NE, LUYTEN GP, MOOY CM, NAUS NC, DE JONG PT, LUIDER TM. HNK-1 antigens on uveal and cutaneous melanoma cell lines. Melanoma Res. 1996 Dec;6(6):411-8.

LUYTEN GP, NAUS NC, MOOY CM, HAGEMEIJER A, KAN-MITCHELL J, VAN DRUNEN E, VUZEVSKI V, DE JONG PT, LUIDER TM. Establishment and characterization of primary and metastatic uveal melanoma cell lines. Int J Cancer. 1996 May 3;66(3):380-7.*

NAUS NC, LUYTEN GP, STIJNEN T, DE JONG PT. Astigmatism and visual recovery after phacoemulsification and conventional extracapsular cataract extraction. Doc Ophthalmol. 1995;90(1):53-9.

CURRICULUM VITAE

Nicole Corine Naus werd geboren op 10 februari 1971 te Oss. Op vierjarige leeftijd, niet lang na haar verhuizing naar "oos sjoen Limburg", besloot zij arts te worden.

In 1989 behaalde zij het VWO diploma aan het Bouwens van der Boye College in Panningen, waardoor zij eindelijk Geneeskunde kon gaan studeren aan de Erasmus Universiteit Rotterdam.

Van 1991 tot en met 1994 werkte Nicole, naast haar studie, in het studententeam van de afdeling Psychiatrie (5 Noord).

Haar eerste oogheelkundige en tevens afstudeer-onderzoek vond plaats in 1993 onder supervisie van Gre Luyten. Nicole onderzocht het verschil in visus en astigmatisme tussen patienten geopereerd met phacoemulsificatie en extracapsulaire cataract extractie.
Aansluitend werkte Nicole tot haar afstuderen (mei 1994) in het laboratorium van de afdeling klinische genetica/oogheelkunde. Daar keek ze naar de betrokkenheid van het nm-23 gen in oogmelanomen.

Enthousiast geworden voor de oogheelkunde besloot Nicole naar haar artsexamen (juni 1996) op het onderzoek naar genetische afwijkingen in oogmelanomen te promoveren.

Tijdens haar promotie bij de afdeling genetica (Prof. D. Bootsma, Dr. A. de Klein en Dr. G.P.M. Luyten) werkte Nicole twee maanden bij de afdeling Ophthalmology & Orthoptics van het Royal Hallamshire Hospital in Sheffield (Prof. I. Rennie, Dr. K. Sisley).

Vanaf februari 2001 is Nicole in opleiding tot oogarts in het Academisch Ziekenhuis Rotterdam.

DANKWOORD

Er bestaat geen grotere zegen dan weinig aan je hoofd te hebben;
er is niets erger dan je voortdurend zorgen te moeten maken. Slechts degenen die boven zorgen zijn uitgestegen,kennen de gelukzaligheid van een probleemloos bestaan. Alleen degenen die hun geest tot rust hebben gebracht, weten hoe erg het is veel zorgen te hebben.
Huanchu Daoren - Tao van Eenvoud, omstreeks 1600

De zorgen over het schrijven van een proefschrift zijn nu voorbij. Zonder hulp van vele anderen was dat echter niet mogelijk geweest. In deze paragraaf bedank ik allen die een grote of kleine steen hebben bijgedragen aan de totstandkoming van mijn boekje.

Allereerst bedank ik natuurlijk mijn beide co-promotoren.
Annelies, dank voor je kritische blik en oeverloos vertrouwen in het onderzoek. Deze werkten voor mij erg inspirerend. Bedankt dat je de dagelijkse begeleiding voor je rekening wilde nemen en voor de open deur als ik weer eens iets niet begreep. Ik hoop dat we ook in de toekomst elkaar kunnen blijven aanvullen.
Gre, jij hebt mij al vroeg enthousiast gemaakt voor het melanomen onderzoek. Ik dank je voor de mogelijkheid om dit promotie onderzoek te starten, dat begon als een uit de hand gelopen afstudeeronderzoek. Jouw klinische invalshoek heeft enorm bijgedragen tot de brug die we hebben kunnen slaan tussen lab en ziekenhuis.

Mijn promotor, *Prof. dr. D. Bootsma*, bedank ik voor de betrokkenheid en stimulans tot het halen van deadlines, die er uiteindelijk toe hebben geleid dat het boekje op tijd af is.
De kleine commissie, *Prof. dr. A. Geurts van Kessel, Prof. dr. P.T.V.M. de Jong* en *Prof. dr. G. van Rij* dank ik voor de snelle beoordeling van mijn proefschrift. *Professor Van Rij* wil ik tevens bedanken voor de mogelijkheid mijn opleiding tot oogarts in Rotterdam voort te zetten.
De leden van de grote commissie, *Prof. dr. J.W. Oosterhuis, Prof. dr. R. Kanaar*, dank ik voor het zitting nemen in de commissie.

Een belangrijk deel van het onderzoek heeft zich afgespeeld op de afdeling celbiologie & genetica, op lab 734.
In de tussentijd is er veel veranderd. Mensen zijn weg gegaan en anderen zijn gekomen. Ik bedank iedereen voor de hulp bij allerlei experimenten, waar ik als "domme" arts geen ervaring mee had, maar vooral voor de gezelligheid.
Met name wil ik de "vaste kern" *Alex, Iris, Yvette, Rosalinde* en *Manja*, bedanken voor de bijdrage en interesse.
Manja, je was niet alleen een enorme vraagbaak op het lab en heb je me veel technieken bijgebracht. Bovenal dank ik je voor je vriendschap en gezelligheid. Jouw

opbeurende woorden, vooral in het begin van mijn AIO periode, hebben ertoe geleid dat er nu een mooi boekje voor je ligt. Ik wens jou en Wim heel veel succes en plezier toe in Amerika en "dat ge bedank zijt dat widde".
Ook de studenten *Wieke, Nazik, Riena* en *Anneke*, die aan dit onderzoek hebben meegewerkt, dank ik voor hun inzet. Ik vond het erg leuk met jullie te werken.
Emine, ik ben blij dat ik een voorzet heb kunnen geven voor jouw project. Maak er iets moois van!

Een andere afdeling waar ik heel wat uren heb doorgebracht is de klinische genetica, lab 816.
Dr. R. Slater, beste *Ros*, dank voor het minutieus nakijken van de vreselijke tabellen met karyotyperingen en voor de taalkundige correcties. Hierdoor is dit boekje een goed leesbaar geheel geworden. Dank je dat je als expert zitting wilde nemen in de commissie. Heel veel geluk aan de andere kant van de oceaan.
Prof. dr. A. Hagemeijer, u stond aan de wieg van de cytogenetisch analyse van de oogmelanomen. Dank hiervoor. Ik hoop dat wij in de toekomst onze samenwerking kunnen uitbreiden.
Berna, je staat niet alleen op afdeling, maar ook daarbuiten altijd klaar voor moral support. Dank voor je gezelligheid en heel veel succes in je nieuwe functie.
Ellen en Marjan, dank voor het karyotyperen van het tumormateriaal.
Ook alle andere *dames van de 8ste*, dank voor jullie interesse en de kopjes thee.

Ik zou geen resultaten kunnen hebben verzameld zonder tumormateriaal. Dat had ik vooral aan *Dr. C.M. Mooy, Neeltje*, te danken. Wij hebben samen in heel wat ogen geprikt en gesneden. Je deur stond altijd open voor vragen of om even wat coupes te bekijken. De resultaten mogen er zijn. Dank je voor alle hulp.
Liesbeth, Nel en *Mike Kliffen*, stonden altijd klaar als er weer eens onverwacht "een oog binnenkwam". Bedankt!
Dr. D. Paridaens, beste *Dion*, jou bedank ik voor het insturen van alle oogmelanomen en voor het bijwonen van en de inbreng op de werkbesprekingen.
Janneke Alers dank ik voor haar bijdrage aan de CGH experimenten. Ik heb veel van je geleerd en wens je veel succes bij je verdere carrière.

De kamer waar ik, zeker het laatste half jaar, veel tijd heb doorgebracht is kamer Ee1610, oogheelkunde.
Antoinette, ruim vier jaar lang zijn wij elkaars kamergenoot geweest. Bedankt voor je gezelligheid, de kopjes koffie en de kilo's snoep die we naar binnen werkten, vooral tijdens jouw zwangere maanden. Ik hoop je snel als collega AGIO te kunnen begroeten. Succes bij de laatste loodjes.
Ingrid en *Jan-Roelof*, ik vond de dagen dat jullie ook op de 16e zaten altijd erg gezellig. Dank daarvoor.
Ook alle andere medewerkers van de afdeling oogheelkunde bedank ik voor hun interesse. *Kees, Corrie* en *Monique* bedank ik voor het prikken van alle bloedjes van de oogmelanoom patiënten. Hierbij gaat mijn dank natuurlijk ook uit naar de patiënten zelf, die bereid waren bij elke controle vijf buizen bloed in te leveren.

Mijn nieuwe collega AGIO's *Marjolein*, *Roger*, *Jaqueline*, *Raan* (sorry Raan, weddenschap verloren) en *Caroline*, bedankt voor de vrijheid die jullie mij in de afgelopen maand hebben gegeven en voor de belangstelling voor de vorderingen. *Caroline*, bedankt voor alle vruchtbare discussies over correlaties, confounders en hogere statistiek.
"Hoi Nicole met Nicole" van Basten, jouw regelvaardigheid en organisatorisch talent bewonder ik zeer (de gratificatie is bijna binnen). Dank voor al je tijd.

Dr. Karen Sisley and *Prof. Ian Rennie*, thanks for giving me the opportunity to work at your lab in Sheffield for two months. I enjoyed our time in Jerusalem and Philadelphia very much and I hope to see you soon in Amsterdam. *Karen* and *Chris*, thanks for lending your house to me.

Mijn paranimfen, *Ernst* en *Hennie*, ik ben vereerd dat jullie mij ter zijde willen staan. *Ernst*, dank je voor je vriendschap, je vertrouwen in een goede afloop van dit boekje en het gefilosofeer over opleidingen en onderzoek doen. Ik hoop dat we snel tijd vinden om, samen met Sjaanke en Michiel weer eens een weekendje Hamelen te doen.
Hennie, ik ben erg blij dat je onze oogmelanomen club bent komen versterken. Dank voor al je hulp bij de laatste loodjes. Ik weet zeker dat we een goed team zullen zijn.

De Bergpolderstraat, ja dat is natuurlijk een verhaal apart. *Martine*, *Aart*, *Jeroen* en *Renkse*, jullie zijn echt geweldig! Altijd tijd voor een kopje koffie (thee mag ook), een wijntje of pannenkoeken. Als ik weer eens veel te lang gewerkt had, was er altijd wel iemand die Guus al binnen had gelaten of eten had gegeven. Dank voor deze sociale controle.

Saskia en *Renske*, al jaren vormen wij een drie-eenheid. Jullie vriendschap en vertrouwen in mij, betekenen heel veel voor me. Nu dit boekje af is, heb ik eindelijk weer eens tijd voor de hammam of een avond beppen. Ik zal jullie hulp nooit vergeten.

Natuurlijk bedank ik ook alle andere *vrienden* en *(schoon)familie* voor hun belangstelling.

Pa en ma, door jullie ben ik zoals ik ben. Jullie hebben mij geleerd door te zetten, ook als het even niet gaat zoals je wilt. Jullie betrokkenheid en nimmer aflatend vertrouwen, raakt mij diep. Dit boekje is voor jullie.

Mijn allerliefste Michiel, dank je voor alle steun, suggesties, hulp, betrokkenheid en nog veel meer. Eigenlijk wil ik maar een ding tegen je zeggen en dat is: ja, ik wil.