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University of Glasgow

Research Thesis for the Degree of Doctor of Medicine

PROGNOSTIC ASSESSMENT OF
CHOROIDAL MELANOMA BASED ON
INTERPHASE CYTOGENETICS AND
PATHOLOGICAL FEATURES

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March 2007

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SUMMARY

It is now well recognised that in uveal melanoma and specifically choroidal melanoma, monosomy 3 has a stronger association with death caused by metastases than clinical and histological parameters. However, the identification of the presence or absence of monosomy 3 and its correlation with morphological heterogeneity within tissue sections has not been assessed. Furthermore, the role of monosomy 3 in predicting time to death is not fully understood. All these aspects have been investigated in more detail in this thesis.

In the introduction to this thesis previously published work concerning new and well-established prognostic factors in choroidal melanoma is reviewed. Diagnostic modalities including cytogenetic studies and treatment of this malignant neoplasm and its metastatic disease are also discussed.

In the first study of this thesis the technique of chromosome *in situ* hybridisation (CISH) was adapted to allow its application to archival choroidal melanoma, which was both formalin and gluteraldehyde fixed. The advantage of this technique compared with other techniques available is that it is applicable to archival material allowing the direct correlation of chromosomal changes with histology. CISH was shown to be an effective method for assessing monosomy 3 in these archival paraffin embedded tissue sections. The efficiency of the CISH technique was compared with fluorescence *in situ* hybridisation (FISH). The main additional advantage of FISH is that it is possible to assess the copy number of several chromosomes at one time.

However, a consistent signal could not be obtained with FISH in archival tissue thus the CISH technique was adopted for further study of archival choroidal melanoma.

The CISH technique was then used to assess the presence or absence of monosomy 3 in an archival series of melanoma examining tumours from individuals that had died from metastatic melanoma and comparing them with individuals that had survived. The results confirmed the work of previous studies that monosomy 3 is an important predictor of death in choroidal melanoma. However, this study also showed that disomy 3 does not guarantee survival since a small group of tumours displaying disomy 3 resulted in metastatic death. The reasons for this may be related to the inclusion criteria used in this investigation since this was a retrospective study. Furthermore, with the CISH technique used in this study the probes were centromere specific and regional losses of chromosome 3 might not have been detected. However, regional losses seem to occur only in a minority of cases. Finally, and probably more likely, there are additional alterations that allow invasion and metastases in choroidal melanoma. This study also showed that monosomy 3, in some cases, could also be predicted from histology, particularly by the presence of epithelioid cells and vascular loops. One advantage of this archival series was the long follow up available such that late deaths from metastatic melanoma were represented (the longest time to death for metastatic melanoma was 14 years) as well as those that succumbed to metastases at around 3 years. Additionally this study showed that monosomy 3 does not influence time until death as there was no significant difference in this interval between metastatic melanomas, with and without monosomy 3.

Another advantage of CISH is that it is possible to assess chromosome copy number in conjunction with cell morphology. This aspect of the technique was assessed in the final study of this thesis. A group of choroidal melanomas with well-defined populations of epithelioid and spindle cells were identified from the original study group. Counting was undertaken within these defined morphological populations and monosomy 3 was heterogeneous in a subpopulation of these choroidal melanomas occurring in epithelioid but not spindle shaped cells. This has implications for cytogenetic screening for monosomy 3 since the genetic information obtained from small samples may not be representative. Therefore, a sample composed purely of spindle cells might carry less prognostic significance than one containing both spindle and epithelioid cells.

In conclusion, CISH has proven a valuable additional technique allowing retrospective studies of monosomy 3 in choroidal melanoma therefore including larger numbers than most prospective studies and a longer follow-up. Utilising CISH has provided important additional information concerning the significance of monosomy 3 in choroidal melanoma.

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Declaration

None of the work in this thesis has appeared in any other submitted thesis to this or any other University. I personally performed all the laboratory work described in this thesis. Dr Roberts also reviewed the histopathological features.

The following papers and abstracts have been accepted for publication and contain material included in this thesis:

Papers:

1 Sandinha T, Farquharson M, McKay I, Roberts F. Correlation of heterogeneity for chromosome 3 copy number with cell type in choroidal melanoma of mixed-cell type. *Invest. Ophthalmol. Vis. Sci.* 2006 December; 47(12):5177-80

2 Sandinha MT, Farquharson M, McKay I, Roberts F. Monosomy 3 predicts death but not time until death in choroidal melanoma. *Invest. Ophthalmol. Vis. Sci.* 2005 October; 46 (10):3497-501

3 Sandinha MT, Farquharson M, Roberts F. Identification of monosomy 3 in choroidal melanoma by chromosome *in situ* hybridisation. *Br J Ophthalmol.* 2004 December;88 (12):1527-32.

Abstracts:

1 Sandinha MT, Farquharson M, Roberts F. Assessment of clonal heterogeneity in choroidal melanoma by chromosome *in situ* hybridization for chromosome 3. *Ophthalmic Research*, 2005, 37S1, 4353.

2 Sandinha MT, M Farquharson, I McKay, F Roberts. Survival of patients with choroidal melanoma: correlation with histopathological features and monosomy 3. *Ophthalmic Research*, 2004, 36S1, 2243.

3 MT Sandinha, M Farquharson, I McKay, F Roberts. Survival of patients with choroidal melanoma: correlation with histopathological features and monosomy 3. *Invest. Ophthalmol. Vis. Sci.* 2004 45: E-Abstract 1212.

5 MT Sandinha, M Farquharson, F Roberts. Identification of monosomy 3 in choroidal melanoma by chromosome *in situ* hybridization. *Invest. Ophthalmol. Vis. Sci.* 2003 44: E-Abstract 1548.

Aspects of this work have also been presented at meetings of the following societies:

European Association for Vision and Eye Research (EVER), Portugal, October, 2005

Scottish Ophthalmological Club (SOC), Edinburgh, February, 2005

European Association for Vision and Eye Research (EVER), Portugal, September
2004

Association for Research in Vision and Ophthalmology (ARVO), Florida, April 2004

Scottish Ophthalmological Club (SOC), Renfrewshire, October 2003

Oxford Ophthalmological Congress, Oxford, July 2003

Association for Research in Vision and Ophthalmology (ARVO), Florida, May 2003

1 INTRODUCTION

1.1 Epidemiology, Environmental and Host Risk Factors for Choroidal Melanoma

Choroidal melanoma is part of the melanomas of the uveal tract and is the most common primary intraocular malignancy in adults, with an overall estimated incidence of six cases per one million subjects per year in the western world (Teikari and Raivio, 1985, Swerdlow, 1983, Singh and Topham, 2003, Mooy and De Jong, 1996, Jensen, 1963, Gislason *et al.*, 1985, Bergman *et al.*, 2002). The reported incidence rate of uveal melanoma has ranged from 0.3 to 9.1 cases per million and these differences are attributed to variations in inclusion and methods used in the different studies (Table 1.1).

Table 1.1 Mean age-adjusted incidence of uveal melanoma in different countries (adapted from Singh & Topham, 2003)

REGION	INCIDENCE PER 1000000	REFERENCES
Norway	9.0	Mork, 1961
Denmark	7.4	Jensen, 1963
England (UK)	6.4	Swerdlow, 1983
Iceland	6.0	Gislason <i>et al.</i> , 1985
Finland	7.6	Teikari & Raivio, 1985
Japan	0.3	Kaneko, 1993
Sweden	9.1	Bergman <i>et al.</i> , 2001
United States	4.3	Singh & Topham, 2003

Large studies indicate a slight male predominance but the reason for this is unknown (Singh and Topham, 2003, Bergman *et al.*, 2002). Unlike UV-induced cutaneous melanoma which has shown a marked increase in incidence in white populations during the past decade (Bergman *et al.*, 2006), the incidence of choroidal melanoma has not increased (Singh and Topham, 2003, Bergman *et al.*, 2002). However the incidence of choroidal melanoma does vary between different groups studied and appear to be influenced by race, geographical location and a genetic predisposition (Bergman *et al.*, 2002). For example, the incidence in whites is eight times that in blacks and it occurs most frequently in elderly white-skinned individuals (Egan *et al.*, 1988). Geographically, the incidence of ocular melanoma is also higher in northern countries compared with southern countries. This may be because northern populations tend to have a fair complexion with light iris colour and these features are considered to be strong risk factors for choroidal melanoma (Bergman *et al.*, 2002). Family history of uveal melanoma is rare (0.6% of all uveal melanoma cases) and so far seventy three families with uveal melanoma (including ciliary body and choroidal melanomas) have been reported in the literature (Kodjikian *et al.*, 2003). Furthermore, the mode of inheritance still requires to be established in these familial cases. Patients with a family history of melanoma have also been reported to have a 4-fold greater risk for second primary malignant neoplasms. (Singh *et al.*, 1996). Choroidal naevus is one of the best documented predisposing lesions for choroidal melanoma. Although the risk of choroidal melanoma in individuals with choroidal naevus is low, this risk seems to be much greater than the risk in individuals without choroidal naevus. It has been reported that 1 in 5000 people with choroidal naevus will develop a melanoma each year. (Egan *et al.*, 1988). Predisposing factors for choroidal melanoma also include congenital ocular melanocytosis (naevus of Ota)

(Vclazquez and Jones, 1983, Egan *et al.*, 1988), neurofibromatosis type 1 (Antle *et al.*, 1990) and familial atypical mole and melanoma (FAM-M) syndrome (previously called dysplastic naevus syndrome) (Singh *et al.*, 1996, Egan *et al.*, 1988).

1.2 Prognostic Indicators of Choroidal Melanoma

1.2.1 Background

Choroidal melanoma is characterised by an unpredictable clinical course with an overall 5- and 10-year survival of 74 % and 55%, respectively (Seddon *et al.*, 1983). There are various clinical and pathological features which are predictors of survival but these are unreliable in individual cases. Death is usually due to liver metastases (Diener-West *et al.*, 1992, Mooy and De Jong, 1996) and the median survival time for a patient with liver metastases is only 7 months (Kath *et al.*, 1993). The peak mortality from metastatic disease occurs within 3 years of diagnosis but metastases can occur late up to 36 years later (Newton, 1965, McLean *et al.*, 1982).

1.2.2 Clinical Prognostic Parameters

Age

Choroidal melanoma is very rare in childhood and the incidence increases with age reaching a peak in late middle age (Egan *et al.*, 1988). Kaplan Meier survival curves comparing younger with older patients show that long-term survival rates do not differ significantly (McLean, 1995). This suggests that the cure rates for the two

groups are similar. Therefore the differences in survival may be due to more rapid death in older patients with metastatic melanoma (McLean *et al.*, 2004).

Tumour size

One of the most consistent prognostic indicators for metastases of choroidal melanoma is tumour size (Gamel *et al.*, 1992, McLean *et al.*, 1982, Coleman *et al.*, 1993, Damato *et al.*, 1996). The clinical tumour size may be defined as small (3mm or less in thickness and smaller than 10mm diameter), medium (between 3 and 5 mm in thickness and/or between 10 and 15 mm in diameter), or large (greater than 5 mm in thickness and/or greater than 15mm in diameter) (Shields *et al.*, 1991). McLean and associates (McLean *et al.*, 1982) also found that the largest tumour dimension correlated better with survival than the thickness of the tumour. Multivariate analysis in different studies found the largest tumour dimension to be a significant independent variable (Seddon *et al.*, 1983, Coleman *et al.*, 1993, Affeldt *et al.*, 1980). In the study by Seddon *et al.* larger tumours were more likely to be anteriorly located and occur in patients with a history of glaucoma (Seddon *et al.*, 1983). Overall, larger tumours carry a worse prognosis but small tumours have also been shown to cause death by metastases (Shields *et al.*, 1995a) (Figure 1.1). In a study by Diener-West *et al.*, the 5-year mortality rates following enucleation were 16% for small tumours (largest tumour diameter-LTD- <10mm), 32% for medium (LTD 10-15mm), and 53% for large (LTD>15mm) tumours (Diener-West *et al.*, 1992).

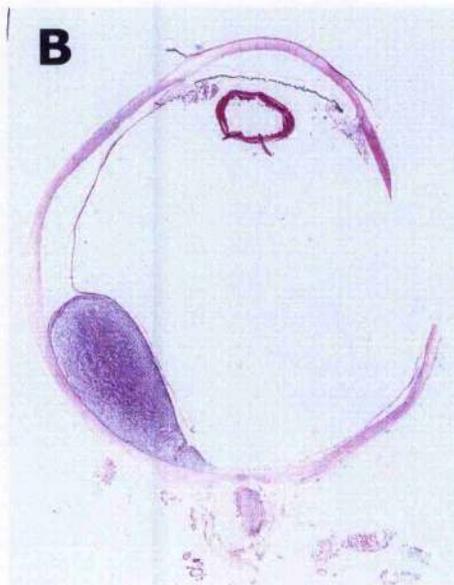
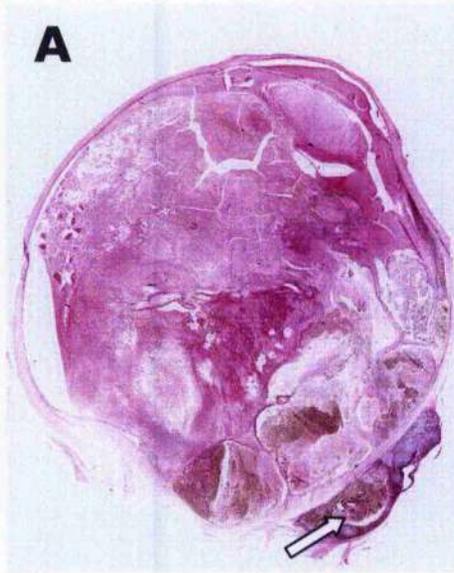


Figure 1.1 Choroidal melanoma gross pathology. A: large tumour. B: small tumour

The large tumour (A) has also presented with extraocular extension (arrow).

Tumour location

When a choroidal melanoma that invades the ciliary body is diagnosed, the original uveal location where the tumour initiated may be difficult to resolve but is usually considered choroidal if the tumour is located predominantly in the choroid. The location of the anterior margin of the tumour was considered an important independent predictor of outcome in the study by Seddon *et al.* (Seddon *et al.*, 1983). In the same study, tumours were coded as follows: posterior to the equator, anterior to the equator without involvement of the ciliary body, and anterior to the equator involving the ciliary body; choroidal tumours involving the ciliary body had the worst prognosis, followed by those choroidal tumours not involving the ciliary body but anterior to the equator. Packard similarly showed a poorer survival for anteriorly placed choroidal tumours (Packard, 1980). Choroidal tumours extending to the ciliary body tend to be larger than posterior ones (posterior to the equator), but tumour size alone does not explain the association with poorer survival (Seddon *et al.*, 1983). For posteriorly located tumours, those located adjacent to the optic disc have a worse prognosis, related to more frequent extrascleral extension (Affeldt *et al.*, 1980).

1.2.3 Histopathological Prognostic Parameters

Cell type

Cell type appears to be one of the most consistent prognostic factors. In the most commonly used modified Callender classification of uveal melanomas, the morphology of the cells is classified as spindle, epithelioid, or mixed (spindle and

epithelioid) (McLean *et al.*, 1983) (Figure 1.2). Pure spindle cell uveal melanoma has the best prognosis, pure epithelioid cell melanoma the worst, and mixed cell melanoma an intermediate prognosis (McLean *et al.*, 1982). Seddon *et al.* attempted to define the amount of epithelioid cells required to infer a poor prognosis and concluded that more than two epithelioid cells present per high-power microscopic field (HPF) was sufficient (Seddon *et al.*, 1983). In this study, the number of epithelioid cells/HPF was interrelated with other variables but on multivariate analysis it was an independent risk factor. Thus, tumours with a higher number of epithelioid cells were also larger, more anteriorly located and had more mitotic figures. Cell type has also been recognised as an independent factor in other series (Seddon *et al.*, 1987, Moshari *et al.*, 2001, McLean *et al.*, 1977, Damato *et al.*, 1996, Coleman *et al.*, 1993, Affeldt *et al.*, 1980). Occasionally balloon cells, similar to those in cutaneous melanoma may be identified in some uveal melanomas. Their nature and importance is controversial. It has been suggested that they may represent spindle melanoma cells that have undergone extensive cytoplasmic lipid degradation; however this was not confirmed by electron microscopy (EM) (Jakobiec *et al.*, 1979). An EM study of 3 patients with balloon cell melanoma of the choroid revealed premelanosomes and complex melanosomes in the cytoplasm of balloon cells without evidence of significant lipid (Rodrigues and Shields, 1976). Lane *et al.* found a higher number of balloon cells in irradiated tumours (Lane *et al.*, 1997) and Jakobiec *et al.* found that melanomas with prominent balloon cells had a better prognosis (Jakobiec *et al.*, 1979). Conversely Khalil concluded that the presence of balloon cells does not alter the prognosis (Khalil, 1983).

Nuclear and nucleolar size

Although the reproducibility of the modified Callender cell type classification (McLean *et al.*, 1983) has been shown, several studies have indicated that objective measurements such as nuclear and nucleolar area from randomly selected cells are better predictors of patient outcome than Callender's cell type (Gamel *et al.*, 1992, McLean and Gamel, 1988, Gragoudas *et al.*, 1987). In particular, the mean of ten largest nucleoli and the standard deviation of nucleolar area have been shown to be prognostically significant (Gragoudas *et al.*, 1987, Gamel *et al.*, 1992). These techniques are however labour intensive and the selection of cells for measurement is still subjective.

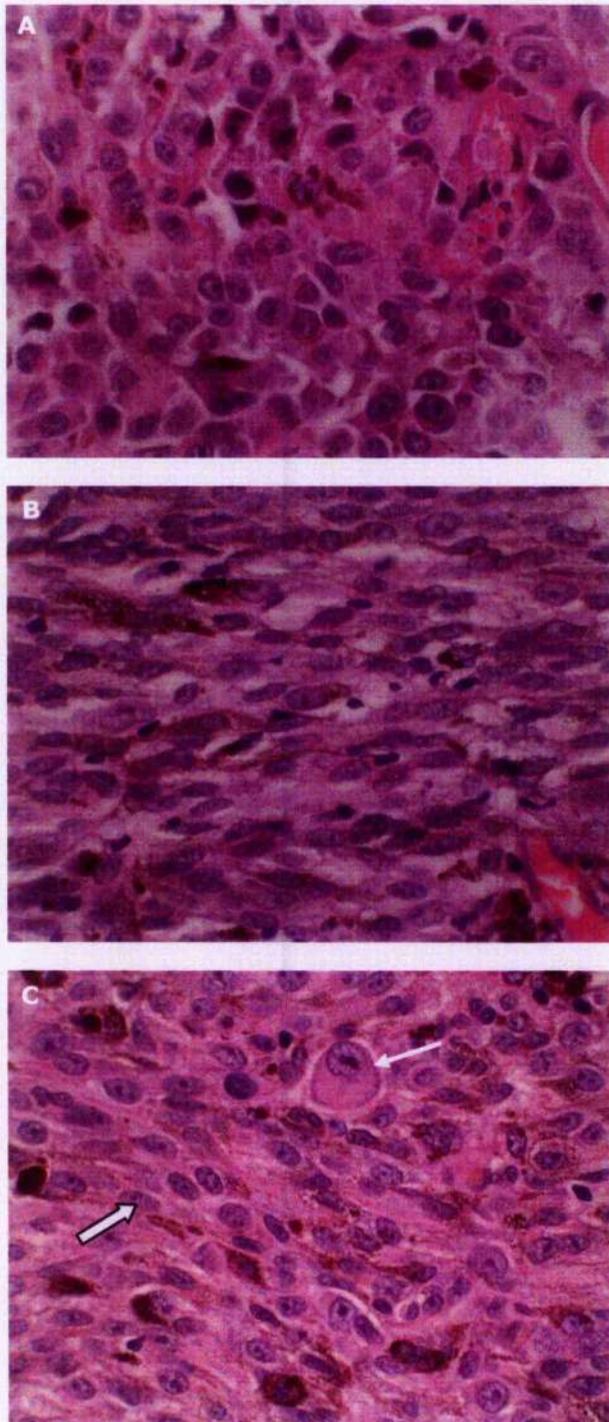


Figure 1.2 Three different cell type tumours.

H&E stained sections (x 400) illustrating the three main choroidal melanoma cell types. A: Epithelioid cell type tumour. B: Spindle cell type tumour. C: Mixed cell type tumour with epithelioid cells (small arrow) and spindle cells (large arrow).

Microvascular patterns and microvascular density (MVD)

The prognostic significance of microvessels in uveal melanoma has been assessed in a number of studies that have included quantitative variables such as microvascular density (MVD) (Mäkitie *et al.*, 1999a, Lane *et al.*, 1997, Foss *et al.*, 1997, Foss, 1996) and qualitative ones such as microvascular patterns that are used to assess the arrangement of the microvessels (Folberg *et al.*, 1992, Folberg *et al.*, 1993, Foss *et al.*, 1997, Kivelä *et al.*, 2004, Mäkitie *et al.*, 1999h, McLean *et al.*, 1997).

Microvascular patterns

Microvascular patterns are three dimensional architectural arrangements of microvessels and extravascular matrix in uveal melanoma and were introduced by Folberg *et al.* in 1992 when they presented nine morphological types of microvessel architecture (Folberg *et al.*, 1992). The patterns can be identified by periodic acid Schiff stain (PAS) without hematoxylin counterstain (Folberg *et al.*, 1992). PAS also stains melanoma cells, but the use of green filter enhances contrast of PAS-positive structures helping in recognition of microvessels (Folberg *et al.*, 1992). The incidence of vascular loops in uveal melanoma is reported to be approximately 60% (Folberg *et al.*, 1993, Foss *et al.*, 1997, Kivelä *et al.*, 2004). According to the original classification (Folberg *et al.*, 1992) the normal pattern consists of normal uncompressed choroidal vessels. The silent pattern consists of no apparent tumour vessels. The straight pattern comprises randomly oriented straight vessels that are not linked with each other. The parallel pattern included straight vessels that are aligned parallel to each another. The parallel with cross-link pattern contains vessels of

parallel pattern that are also linked to each other. The arcs and arcs with branching patterns are incomplete loops. The loop pattern consists of vessels that form a completely enclosed loop (Figure 1.3). The network pattern is composed of at least three back-to-back closed loops. By definition, if networks are present, loops are present (Folberg *et al.*, 1992). In a follow up study of 234 patients Folberg *et al.* showed that patients who survived over 10 years had tumours that less frequently displayed the following patterns: parallels with cross-linking, loops and networks (Folberg *et al.*, 1993). In the same study, using multivariate analysis, the presence of networks and parallel vessels with cross-links were independent prognostic factors. Parallel with cross-linking, loops and networks have also shown a strong association with other potential prognostic factors such as epithelioid cells and tumour-infiltrating lymphocytes (Folberg *et al.*, 1993). Other studies have shown that patients with tumours containing networks and parallel vessels with cross-linking that occupy 2% of cross-sectional have a significantly worse prognosis than those patients with tumours containing a smaller percentage of these patterns (Mehaffey *et al.*, 1997). Further studies have shown that tumour cells of malignant uveal and metastatic cutaneous melanoma are able to form microvascular patterns, in particular microvascular loops and networks *in vitro* without presence of endothelial cells (Folberg *et al.*, 2000). This concept of vessel formation without endothelial cells is called “vasculogenic mimicry” (Maniotis *et al.*, 1999).

The results of these experiments suggest that the generation of these channel like structures is associated with remodelling of extracellular matrix with overexpression of genes that regulate relevant molecules such as collagen VI (Maniotis *et al.*, 1999, Daniels *et al.*, 1996).

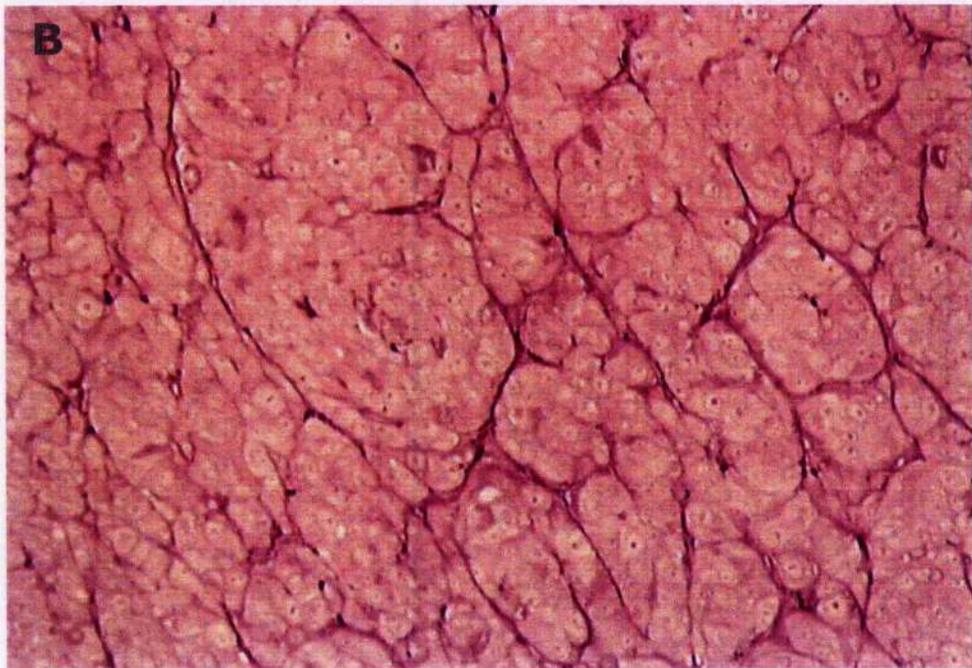
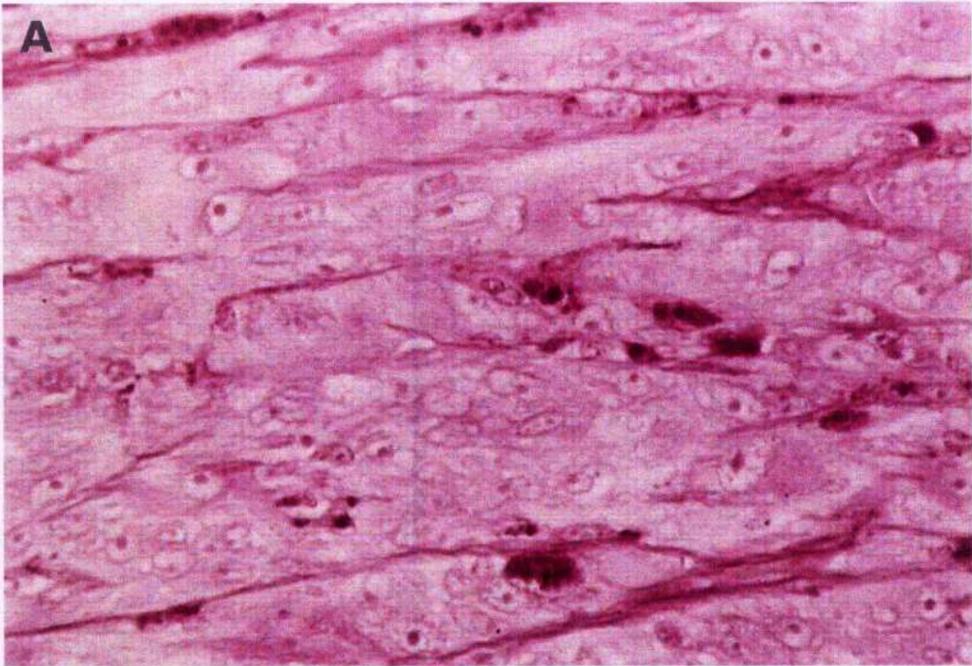


Figure 1.3 Microvascular loops. A&B: PAS-stained choroidal melanoma showing loops (x250)

Whilst the majority of researchers have shown that vascular loops carry a poor prognosis, opinions differ as to the nature and function of the vascular loops. Folberg *et al.* defined them as periodic acid-Schiff-positive patterns representing blood vessels (Folberg *et al.*, 1993), but according to Foss *et al.* they are formed from connective tissue (Foss *et al.*, 1997). However both groups associated vascular loops with a poor prognosis. A few researchers have disputed this and found no such associations (Seregard *et al.*, 1998, Lane *et al.*, 1997).

Microvascular density (MVD)

Microvessel density (MVD) reflects the proliferation of capillary endothelium within the tumours (Foss *et al.*, 1997) and high MVD has been shown to be an important prognostic factor for a number of cancers (Mäkitie *et al.*, 1999a). Studies have reported that high MVD is associated with a shortened survival of patients with uveal melanoma and is higher in tumours with microvascular loops and networks (Mäkitie *et al.*, 1999a, Foss *et al.*, 1997). Conversely, Lane *et al.* failed to document any survival difference between tumours with different microvessel counts (Lane *et al.*, 1997).

Necrosis

The cause of necrosis in melanomas is still unclear but tends to occur in large tumours that have outgrown their blood supply. Immunity may also play a role (Moshari *et al.*, 2001). Around 7% of choroidal and ciliary body melanomas show

total necrosis. Several investigators have studied the effect of both partial and total necrosis on survival and concluded that tumour necrosis was not an independent prognostic factor (Moshari *et al.*, 2001, de la Cruz *et al.*, 1990, COMS, 1998 a). The COMS study (COMS, 1998 a) and de la Cruz *et al.* (de la Cruz *et al.*, 1990) graded the necrotic tumours in 4 categories depending on the area affected by necrosis. In the COMS study (COMS, 1998 a), necrosis was associated with large tumour size and epithelioid cell type.

Intermediate filaments

Interaction between tumour cells and surrounding stroma are considered essential for tumour progression and dissemination. Hendrix *et al.* performed immunohistochemistry for vimentin and cytokeratin in uveal melanoma. Vimentin is an intermediate filament common to mesenchymal tissues but also present in uveal melanoma whereas carcinomas typically express keratin, which is an epithelial marker (Hendrix *et al.*, 1998). They defined cells that coexpressed both vimentin and cytokeratin (CK) as “interconverted” and suggested that this indicated metastatic potential (Hendrix *et al.*, 1998). However in a study of 52 uveal melanomas Fuchs *et al.* showed that whilst all cases were positive for vimentin, 48.1% showed immunoreactivity for different subtypes of CK (Fuchs *et al.*, 1992). Although metastases were more common when the primary tumour was immunoreactive for CKs, this was related to the fact that CKs positivity was more frequent in mixed and epithelioid cell melanomas (Fuchs *et al.*, 1992).

Cell-matrix interaction

Cell adhesion molecules are membrane receptors involved in various cellular processes, including tumorigenesis. Of these, loss of intercellular cell adhesion molecule-1 has been linked to the development of metastases within the first 5 years of diagnosis (Anastassiou *et al.*, 2000). Conversely, Woodward *et al.* showed that invasive melanoma cell lines expressing α 1- and α 4-integrins displayed better adhesion to extracellular matrix substrates and endothelial cells than noninvasive cell lines that did not display those integrins (Woodward *et al.*, 2005).

Matrix metalloproteinases (MMPs) are secreted by tumour cells and play a role in degrading the extracellular matrix (Väisänen *et al.*, 1999). Expression of matrix metalloproteinase-2 (MMP-2) has been linked with decreased uveal melanoma survival (Väisänen *et al.*, 1999).

The expression of epidermal growth factor receptor (EGFR) and insulin-like growth factor-1 receptor (IGF-1R) in uveal melanoma cell lines have also been shown to correlate with metastatic potential (All-Ericsson *et al.*, 2002, Hurks *et al.*, 2000). In an increasing number of malignant cell types, IGF-1R has been shown to be essential for tumorigenesis as well as for the establishment and maintenance of a transformed phenotype (All-Ericsson *et al.*, 2002). All-Ericsson *et al.* studied the expression of IGF-1R in 36 uveal melanomas. This was present in 94.4% of cases and high levels of expression were associated with a decreased survival (All-Ericsson *et al.*, 2002).

Markers of cell proliferation

Mitotic rate in uveal melanomas is remarkably unpredictable but appears to be of prognostic value in several studies (Folberg *et al.*, 1992, McLean *et al.*, 1977). McLean *et al.* observed a correlation between tumour mitotic activity and mortality (McLean *et al.*, 1977). Mitotic activity was determined by counting the number of mitotic figures in 40 high power fields (HPFs). However more recently it has been suggested that the use of undefined HPFs introduces subjective variability and makes comparisons between centres difficult and as such it has been recommended that mitotic counts are expressed per mm² (Mudhar *et al.*, 2004).

Cells that are in the proliferative phase of the cell cycle can be characterised with antibodies to proteins such as the proliferating cell nuclear antigen (PCNA) and Ki-67. Seregard *et al.* reported that a high count of PCNA-positive cells was associated with an increased risk of metastases (Seregard *et al.*, 1996) but conversely Karlsson *et al.* did not find any association between (PCNA) positivity and survival (Karlsson *et al.*, 1996). In this latter study, a high level of Ki-67 positivity was associated with large epithelioid cell tumours and therefore a shorter survival.

Tumour pigmentation

Heavy tumour pigmentation has been associated with a decreased survival in several univariate studies (Seddon *et al.*, 1983, McLean *et al.*, 1977) but different classifications of the amount of pigmentation make comparisons between these studies difficult. However the relationship between heavy tumour pigmentation and

two other prognostic indicators such as epithelioid cell type and large tumour size suggests that the prognostic significance of tumour pigmentation may be secondary to other tumour characteristics (COMS, 1998 a).

Retinal detachment

Exudative retinal detachment (RD) is detected clinically in up to 75% of eyes with malignant uveal melanoma, and it is the most common abnormality that accompanies this tumour (Kivelä *et al.*, 2001). Exudative RD in conservatively managed eyes with uveal melanoma has also been associated with the rapidity of growth and the extent of necrosis of the tumour (Kivelä *et al.*, 2001). Therefore RD is an established marker of clinical disease activity (Shields *et al.*, 1995a) but the influence on survival remains controversial (Coleman *et al.*, 1993, Shields *et al.*, 1995a). Kivelä *et al* have showed that in a study of 142 cases of uveal melanoma, associated RD was not an independent prognostic factor; however tumour size and vascular loops were strong predictors of exudative RD in that study. (Kivelä *et al.*, 2001).

1.2.4 Clinicopathological Prognostic Parameters

Extraocular extension

The fibrous layer of the eye, the sclera, provides a barrier to the growth of intraocular melanoma but intraocular melanoma can invade the sclera through the emissaries channels including the vortex veins and the ciliary arteries and nerves (McLean *et al.*, 2004). The reported incidence of extrascleral extension of uveal melanomas

ranges from 10-15% (Affeldt *et al.*, 1980, Shamma and Blodi, 1977). Large intraocular tumours are more likely to be accompanied by extrascleral extension of the primary (Shamma and Blodi, 1977) but small melanomas invading outside the eye have been reported and may produce an extraocular mass bigger than the intraocular portion of the tumour (Sassani *et al.*, 1985, Coleman *et al.*, 1993). Peripapillary choroidal melanomas frequently invade the optic nerve head but only rarely extend significantly posterior to the lamina cribrosa (McLean *et al.*, 2004). It remains unclear whether there is a difference in prognosis between extraocular spread via the vortex veins compared with direct infiltration through the sclera (Mudhar *et al.*, 2004). Extrascleral extension has been reported to be higher in pathological series because both microscopic and nodular forms of transcleral tumour extension are included compared with clinical series that consider only lesions visible pre- or intraoperatively (Augsburger *et al.*, 2004b, De Potter *et al.*, 1996) (Figure 1.4).

In the COMS study (COMS, 1998 a), extraocular extension was an unfavourable prognostic sign and the presence of extraocular extension was observed in about 8% of enucleated eyes. In a study by Seddon *et al.* the 10 year mortality was doubled to about 75% compared with cases with no extraocular extension (Seddon *et al.*, 1983). In the study by Affeldt *et al.* (Affeldt *et al.*, 1980), although patients demonstrating extrascleral extension carried an overall poor prognosis, approximately 1/3 survived for 20 years without metastases or died of non-tumour-related causes.

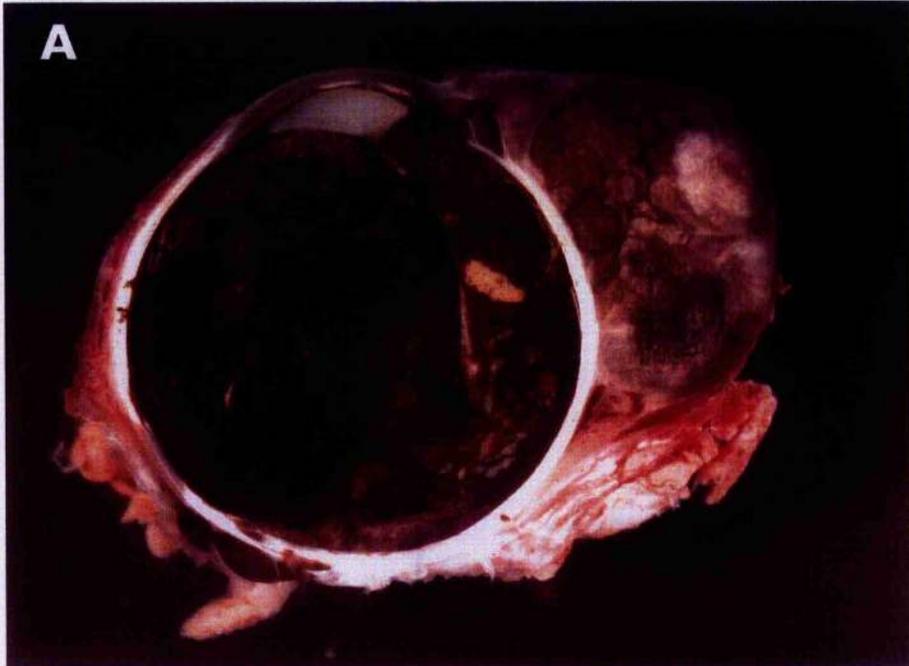


Figure 1.4 Macroscopic specimens after extended enucleation secondary to choroidal melanoma. A: tumour showing orbital mass adherent to outer aspect of scleral layer; B: tumour showing optic nerve and scleral invasion.

1.2.5 Immune responses in choroidal melanoma

The role of the immune system in the development of metastases is unclear and it remains uncertain whether tumour infiltrating lymphocytes (TILs) play a significant role in tumour related immunity. Lymphocytic infiltration in the COMS study was not an independent prognostic factor but in this study spindle cell type tumours had the least inflammation and epithelioid cell tumours the greatest degree of inflammation (COMS, 1998 a). About 5-12% of uveal melanomas show evidence of TILs; these are mostly T cells and their presence may reflect the degree of tumour antigen expression (Durie *et al.*, 1990). In another study, from the AFIP (de la Cruz *et al.*, 1990) the presence of more than 100 TILs per 20 HPFs was associated with a decreased survival; the survival rate at 15 years of patients with tumours containing a high lymphocytic count was 36.7% compared with 69.6% for the patients with a low lymphocytic count. An immunohistochemical analysis of TIL revealed that T cells predominated in 73.8% of the tumours; B cells were observed less often (in 14% of patients) (Whelchel *et al.*, 1993). In this study T-lymphocytic infiltration was associated with metastases. The number of TILs has been correlated with HLA expression by the uveal melanoma (de Waard-Siebinga *et al.*, 1996) and an association between high HLA class I antigen expression in uveal melanoma lesions and poor prognosis has been suggested (Ericsson *et al.*, 2001, Blom *et al.*, 1997). It has also been suggested that changes in expression of the class I molecules can influence the development of metastases through a direct effect on the susceptibility of tumour cells lysis by natural killer (NK) cells. Therefore, uveal melanoma cells with a low HLA Class I expression would be killed by NK cells before they reach the liver (Blom *et al.*, 1997).

1.2.6 DNA aneuploidy

DNA quantification can be carried out by flow cytometry to determine the nuclear DNA content (ploidy) and proliferative activity of tumour cells (Mooy and De Jong, 1996). Tumour cells may have a noncomplementary amount of DNA (aneuploid) and this is considered to reflect genetic instability and impaired cell cycle control (Mooy and De Jong, 1996). DNA aneuploidy has been associated with the epithelioid cell type (Mooy *et al.*, 1995a).

1.2.7 Oncogene activation

C-myc is an oncogene involved in cell proliferation and located on chromosome 8; abnormalities in this gene have been linked to uveal melanoma (Saraiva *et al.*, 2004). A high expression of the c-myc oncogene is known to be associated with poor survival in cutaneous melanoma (Tulley *et al.*, 2003). Conversely in uveal melanoma, a low expression of c-myc is associated with higher mortality (Chana *et al.*, 1998). Interferon has been used as an adjuvant agent in uveal melanoma and there is some evidence suggesting that c-myc has an important role in the action of interferon alpha. Overexpression of the c-myc gene, and the subsequent failure to downregulate its expression seems to be associated with interferon resistance. (Tulley *et al.*, 2003).

1.2.8 Programmed cell death

Uveal melanomas can express p53, a tumour suppressor gene whose protein product induces apoptosis and cell-cycle arrest; abnormalities of this gene are present in many cancers. (Saraiva *et al.*, 2004). Expression of Bcl-2, a proto-oncogene that inhibits programmed cell death has shown no prognostic significance in uveal melanoma (Mooy *et al.*, 1995b). In a study by Coupland *et al* high levels of p53 expression were associated with the presence of metastases at 5 years (Coupland *et al.*, 2000). Somatostatin can induce arrest of cell growth and/or apoptosis by binding a membranc receptor. Ardjomand *et al* found high levels of somatostatin receptor-2 to be significantly associated with longer survival (Ardjomand *et al.*, 2003)

1.2.9 Chromosomal abnormalities

Cytogenetic analysis of uveal melanoma has identified several nonrandom chromosomal aberrations, that appear to correlate better with prognosis than clinical and pathological features (White *et al.*, 1998, Vajdic *et al.*, 2003, Speicher *et al.*, 1994, Sisley *et al.*, 1997, Scholes *et al.*, 2003, Prescher *et al.*, 1996, Prescher *et al.*, 1990, Aalto *et al.*, 2001) (Table 1.2). Among the most important genetic changes observed are loss of an entire chromosome 3 homologue (monosomy 3). This has been shown to be present in around 50% of choroidal melanomas (White *et al.*, 1998, Sisley *et al.*, 1997, Prescher *et al.*, 1996, Gordon *et al.*, 1994). Monosomy 3 has been shown to be a significant predictor of poor prognosis in several studies of uveal melanoma (White *et al.*, 1998, Sisley *et al.*, 1997, Sisley *et al.*, 2000, Prescher *et al.*, 1996, Kilic *et al.*, 2005, Aalto *et al.*, 2001) and it is also often associated with an

increase in chromosome 8q. (Sisley *et al.*, 1990, Sisley *et al.*, 1992, Prescher *et al.*, 1995). Amplification of 8q in conjunction with monosomy 3 correlates with reduced survival and is usually associated with a ciliary body location (Sisley *et al.*, 1997, Sisley *et al.*, 1992).

In some of the above studies (Kilic *et al.*, 2005, Aalto *et al.*, 2001), loss of 1p (region p36) was a predictor of an unfavourable outcome and was more frequent in large melanomas (Sisley *et al.*, 2000). In a study of 120 uveal melanomas, 89% of the metastasised tumours with loss of 1p36 also had monosomy 3 (Kilic *et al.*, 2005); among other numerical changes in chromosomes 1,3, 6 and 8, only the concurrent loss of the short arm of chromosome 1 and all of chromosome 3 was an independent predictor of decreased disease-free survival (Kilic *et al.*, 2005).

The results observed by Kilic *et al* in their more recent study of 74 primary uveal melanomas (Kilic *et al.*, 2006) have also confirmed the findings of other studies that stated that monosomy of chromosome 3 seems to be an early event, occurring before alterations of chromosome 8,1 and 6 (White *et al.*, 1998, Sisley *et al.*, 1997, Prescher *et al.*, 1996). In some of the above studies changes in chromosome 6 were present as losses of 6q and gains of 6p (White *et al.*, 1998, Kilic *et al.*, 2006, Kilic *et al.*, 2005, Aalto *et al.*, 2001). Gain of 6p was associated with spindle cell type (Kilic *et al.*, 2005) and was reported to be higher (but not reaching statistical significance) in non-metastasising primary tumours (Aalto *et al.*, 2001). Conversely, losses of 6q were significantly higher in the metastasising group compared with the non-metastasising tumours (Aalto *et al.*, 2001).

Table 1.2 Chromosomal changes in choroidal melanomas

CHROMOSOME	ABNORMALITY	REFERENCES	
Chr. 1	1p loss	Kilic <i>et al.</i> , 2006; Aalto <i>et al.</i> , 2001 Sisley <i>et al.</i> , 1990	
	1q gain	Kilic <i>et al.</i> , 2006; Aalto <i>et al.</i> , 2001	
Chr 2	2p loss	Kilic <i>et al.</i> , 2006	
	Chr 2 gain	Gordon <i>et al.</i> , 1994	
	2q gain	Kilic <i>et al.</i> , 2006	
Chr 3	3p loss	Kilic <i>et al.</i> , 2006; Aalto <i>et al.</i> , 2001 Horsman & White, 1993 Vajdic <i>et al.</i> , 2003 Presher <i>et al.</i> , 1990; Gordon <i>et al.</i> , 1994 Speicher <i>et al.</i> , 1994; Sisley <i>et al.</i> , 2000	
	3q loss	The same as for 3 p loss	
	Chr 6	6p gain	Kilic <i>et al.</i> , 2006; Aalto <i>et al.</i> , 2001 Sisley <i>et al.</i> , 1990; Speicher <i>et al.</i> , 1994 Presher <i>et al.</i> , 1990; Vajdic <i>et al.</i> , 2003
		6q loss	Kilic <i>et al.</i> , 2006; Aalto <i>et al.</i> , 2001 Sisley <i>et al.</i> , 1990; Speicher <i>et al.</i> , 1994 Presher <i>et al.</i> , 1990; Vajdic <i>et al.</i> , 2003 Horsman & White, 1993
Chr 7	6p loss	Kilic <i>et al.</i> , 2006	
	7q gain	Kilic <i>et al.</i> , 2006; Sisley <i>et al.</i> , 1990 Gordon <i>et al.</i> , 1994	
Chr 8	8p gain	Kilic <i>et al.</i> , 2006; Aalto <i>et al.</i> , 2001	
	8p loss	Kilic <i>et al.</i> , 2006	
	8q gain	Kilic <i>et al.</i> , 2006; Aalto <i>et al.</i> , 2001 Horsman & White, 1993 Vajdic <i>et al.</i> , 2003 Presher <i>et al.</i> , 1990; Speicher <i>et al.</i> , 1994	
Chr 9	9p gain	Kilic <i>et al.</i> , 2006; Gordon <i>et al.</i> , 1994	
	9p loss	Kilic <i>et al.</i> , 2006; Speicher <i>et al.</i> , 1994	

Table 1.2 continued

CHROMOSOME	ABNORMALITY	REFERENCE
Chr 10	10p loss	Vajdic <i>et al.</i> , 2003
	10q loss	Vajdic <i>et al.</i> , 2003
Chr 11	11q gain	Kilic <i>et al.</i> , 2006
Chr 13	13q loss	Aalto <i>et al.</i> , 2001
	13 q gain	Gordon <i>et al.</i> , 1994
Chr 15	15p loss	Kilic <i>et al.</i> , 2006
	15q loss	Kilic <i>et al.</i> , 2006
Chr 16	16q loss	Kilic <i>et al.</i> , 2006
		Vajdic <i>et al.</i> , 2003
	16p gain	Aalto <i>et al.</i> , 2001
Chr 20	20q gain	Aalto <i>et al.</i> , 2001
Chr 21	21p loss	Kilic <i>et al.</i> , 2006
Chr 22	22p loss	Kilic <i>et al.</i> , 2006
	22q gain	Aalto <i>et al.</i> , 2001

Loss of chromosome 3 may also be associated with isochromosome formation of 1q, 6p,8q,16p,20q and 22q but the mechanism is not known (Aalto *et al.*, 2001) and the relationship with prognosis remains unclear. It is also apparent from the different studies that the chromosomal changes in uveal melanoma are also distinct from those associated with cutaneous melanoma. For example, involvement of chromosome 6 is less common in uveal melanoma, whereas loss of chromosome 3 is infrequent in cutaneous melanoma (Horsman and White, 1993).

The association of monosomy 3 with histopathological parameters has been the subject of several investigations. Some studies have shown a correlation with cell type (specifically the presence of epithelioid cells) (White *et al.*, 1996, Scholes *et al.*, 2003, Onken *et al.*, 2004, Kilic *et al.*, 2006). In addition, Scholes *et al* and Kilic *et al* found a correlation between monosomy 3 and intratumoural vascular loops, large tumour size and ciliary body involvement (Scholes *et al.*, 2003, Kilic *et al.*, 2006). However other studies did not report any significant association between histopathological parameters such as large size tumours (Sisley *et al.*, 1997) or vascular loops and the presence of epithelioid cells (Sisley *et al.*, 1997, Prescher *et al.*, 1996). These differences between studies may have arisen because of differences in the study populations.

The presence of monosomy 3 in metastasising uveal melanoma suggests a requirement for inactivation of multiple tumour suppressor genes (TSGs) located on both the short and long arms of chromosome 3 (White *et al.*, 1998, Prescher *et al.*, 1996). Previous studies have shown that in the majority of cases chromosome 3 loss of heterozygosity involves an entire chromosome homologue but in a small number

of tumours only regional losses on chromosome 3 are identified (Tschentscher *et al.*, 2001, Prescher *et al.*, 1996, Sisley *et al.*, 1997, White *et al.*, 1998, Scholes *et al.*, 2001, Speicher *et al.*, 1994). Tschentscher *et al.* studied 333 uveal melanomas and found partial deletions in 15 tumours, suggesting that TSGs might be the target of the deletions (Tschentscher *et al.*, 2001). In this study 8 tumours had a partial deletion of the long arm at 3q24-q26 and in 5 tumours the partial deletion was in the short arm at 3p25. Scholes *et al.* tried to identify tumour-suppressor loci that may contribute to the pathogenesis of uveal melanoma (Scholes *et al.*, 2001). Microsatellite analysis were undertaken and their data support a role for retinoblastoma (RB) loci inactivation but also raise the possibility of the involvement of additional loci close to RB and p16/cyclin-dependent kinase inhibitor 2 (CDKN2A) (Scholes *et al.*, 2001). However none of the major tumour suppressor genes, including RB, seem to be mutated in uveal melanomas with any significant frequency, as evidenced by the normal expression of the RB protein in most of these tumours (Harbour, 2006).

Gene expression profiling studies have shown that uveal melanomas with and without monosomy 3 represent two distinct entities suggesting that they were not recognised before because they are not distinguishable by clinicopathological features (Tschentscher *et al.*, 2003). Further gene expression profiling studies by Onken *et al.* defined two classes of uveal melanoma (class 1 low-grade and class 2 high-grade) and has found an association between class 2 and epithelioid cells and class 1 and spindle cells (Onken *et al.*, 2004). Genes that discriminate class 1 from class 2 include clusters of down-regulated genes on chromosome 3 and up-regulated genes on chromosome 8q (Onken *et al.*, 2004) but no specific cancer genes have

been linked to these loci (Harbour, 2006). It is still to be recognised whether class 1 tumours evolve into class 2 tumours or whether the two classes develop along distinct pathways (Harbour, 2006).

Methods of assessment of chromosomal abnormalities

The techniques used for cytogenetic analysis of uveal melanoma include standard cytogenetics (Horsman and White, 1993, Sisley *et al.*, 1990), comparative genomic hybridisation (CGH) (Aalto *et al.*, 2001, Tsechentscher *et al.*, 2001), microsatellite analysis (Scholes *et al.*, 2001, Scholes *et al.*, 2003, Parrella *et al.*, 1999) and FISH (McNamara *et al.*, 1997, Naus *et al.*, 2002, Patel *et al.*, 2001).

In standard cytogenetics, fresh tissue is required and disaggregated tumour cells are cultured and chromosomes analysed in metaphase. However the cells which grow in culture may not be representative of the total tumour population (Speicher *et al.*, 1994). Comparative genomic hybridisation and PCR-based microsatellite analysis have been undertaken on both fresh (Scholes *et al.*, 2001, Scholes *et al.*, 2003) and archival paraffin-embedded tissue (Parrella *et al.*, 1999). Both these techniques involve the extraction of DNA from the tissue samples. In CGH differentially labelled tumour and normal DNA samples are simultaneously hybridised to normal metaphase chromosomes. Regions of gains or losses within the tumour DNA can be identified by an increased or decreased colour ratio of the two fluorochromes used for the detection of hybridised DNA sequences along these reference chromosomes.

In microsatellite analysis paired primers are used to amplify microsatellite markers on chromosomes of interest. Both these techniques are performed using DNA

isolated directly from many tumour cells without culture and theoretically should more accurately represent the tumour cell population *in vivo*. However tissue samples can be contaminated with non-tumour DNA that may be amplified during PCR.

FISH has been used to assess cytogenetic abnormalities in uveal melanomas (McNamara *et al.*, 1997, Naus *et al.*, 2002, Patel *et al.*, 2001) and by utilising different fluorochromes several chromosomes or chromosomal regions can be assessed simultaneously. The studies described however have utilised only fresh tissue and stained slides prepared from disaggregated tumour cells. FISH analysis for other chromosome regions has been performed on tissue sections of choroidal melanoma (Walker *et al.*, 2002) but in the study by Patel *et al.* (Patel *et al.*, 2001) the authors commented that gluteraldehyde fixed tissue is not suitable for FISH analysis.

Using these techniques for cytogenetic analysis, there is also the potential risk of unknowingly examining non-representative tumour populations because these methods do not maintain tissue morphology. Therefore, techniques where tissue morphology is maintained would be advantageous. In addition techniques which could be applied to paraffin embedded tissue would allow studies to be undertaken on archival material with longterm follow up.

Chromosome in situ hybridisation (CISH)

In situ hybridisation (ISH) is a technique that allows identification of specific nucleic acid sequences within individual cells, either in tissue sections or in whole cell preparations. It is based on the complementary binding of a nucleotide probe to a specific target sequence of DNA or RNA in the cell (McNicol and Farquharson, 1997). The added probe is labelled with a reporter molecule and sites of binding are visualized by their location. Initially, radioactive labelling limited the application to research areas. Fluorescent markers are the most commonly applied reporter molecules in diagnostic work (FISH techniques) but other signal-generating systems exist such as enzymes and metals. The major advantages of using enzyme-mediated detection systems in ISH techniques are the permanence of signal and the use of cheaper and simpler light microscopes in comparison to fluorescent microscopes. The availability of different systems will enable the simultaneous detection of different nucleic acid sequences, also generating a more permanent signal (Leitch *et al.*, 1994). The use of labelled chromosome-specific DNA probes in combination with the ISH technique enables the detection of numerical and structural chromosome aberrations in both metaphase spreads and interphase nuclei (Poddighe *et al.*, 1992). This technique, chromosome *in situ* hybridisation, if applied to choroidal melanoma will offer several advantages over currently available techniques. Firstly, tissue morphology is maintained and this ensures that the nuclei assessed are indeed tumour cells. Secondly, it takes account of tumour heterogeneity and allows the correlation of genotype with phenotype. Finally, it will be possible to study archival tissue therefore providing adequate numbers for study and longterm follow up.

1.3 Diagnostic Modalities

1.3.1 Ocular examination and colour photography

Ophthalmoscopic examination of choroidal melanoma by binocular indirect ophthalmoscopy almost always establishes the diagnosis. Colour photography is also indispensable for follow up of indeterminate melanocytic lesions and after conservative treatment of melanoma.

1.3.2 Fluorescein Angiography

“Double circulation” consisting of choroidal and tumour vessels that are leaky in late phases of a fluorescein fundus angiography is suggestive of uveal melanoma (Shields *et al.*, 1991) but the use of fluorescein angiography is limited since no pathognomonic pattern exists (Lederer and Edelstein, 2004).

1.3.3 Ultrasonography

Ultrasonography has greatly helped ophthalmologists in the management of choroidal melanomas and it is performed in almost all patients with choroidal masses. Ultrasound examination is particularly useful for measuring tumour dimensions when planning treatment and when assessing the response to treatment. With A-scan, a melanoma shows a high initial spike and low to medium reflectivity with decreasing amplitude followed by a prominent spike corresponding to the

sclera. B scan typically shows a choroidal mass with acoustic hollowness and choroidal excavation (Shields *et al.*, 1991).

1.3.4 Magnetic resonance imaging (MRI) and computerized tomography

MRI and computerized tomography are not performed routinely since these rarely provide information that is not obtained more conveniently using ultrasonography. However MRI is required for radiation treatment planning in some centers (Damato, 2006).

1.3.5 Biopsy

Choroidal melanoma can be accurately diagnosed without the use of invasive techniques in around 98% of cases (Bechrakis *et al.*, 2002). However for those tumours that can not be diagnosed clinically and where a therapeutic decision will be made depending on the diagnosis various intraocular biopsy methods have been proposed (Shields *et al.*, 1991). Intraocular biopsy is most commonly performed transvitreally by fine-needle aspiration (FNA) although the transscleral technique is still in use in some centers (Bechrakis *et al.*, 2002). However there has been some concern that FNA could induce tumour seeding along the needle tract (Karcioglu *et al.*, 1985) and that material obtained might not be representative of the entire tumour (Folberg *et al.*, 1985). Sen *et al.* have undertaken transretinal biopsy of choroidal tumours using a 25-gauge vitrector (Sen *et al.*, 2006). Although the study group was small (14 cases) this technique appears to provide a larger sample than FNA and it is a shorter procedure than 20-gauge biopsy. No adverse outcomes were identified in this small series of patients.

1.3.6 Cytogenetic Studies

At present, cytogenetic studies are carried out if a tumour sample is obtained for diagnostic reasons or after local resection or enucleation. In the future, the results of cytogenetic studies may influence treatment and as such it may be desirable to sample small tumours in order to prevent metastases (Damato, 2006). Genetic tumour classification will also help to identify patients who might benefit from adjuvant therapy.

1.4 Treatment

The main objective of treatment in choroidal melanomas is to avoid the development of a painful and unsightly eye, either by enucleation or by eye-conserving therapies. Malignant choroidal melanomas were treated for decades by enucleation but sight conservative treatments have been encouraged since there is no convincing difference in survival between radical surgery of the eye and eye conserving therapies (Char *et al.*, 1988, Foulds *et al.*, 1987, Seddon *et al.*, 1990). Largest tumour diameter influences retention of the globe (Foulds *et al.*, 1987) and tumour height determines the type of radiation and dose in plaque brachytherapy treatments (Damato, 2004). However it is generally believed that intraocular melanoma has already disseminated at the time when treatment of the primary tumour is given.

1.4.1 Enucleation

An earlier theory stated that enucleation at a very early stage was most likely to prevent metastases particularly for small melanomas in order to prevent the initial dissemination of tumour cells (Zimmerman *et al.*, 1980, Manschot and van Peperzeel, 1980). However, later it was suggested that surgical manipulation during enucleation disseminated tumour cells into the vascular system, thereby increasing melanoma-related mortality (Zimmerman and McLean, 1979). This hypothesis was based on the observation of the pattern of mortality which peaked at 2-3 years after enucleation and stabilised to the pre-enucleation level by the 7th year after enucleation and also on the assumption that 1% of patients with malignant melanoma would die each year. Thus, in order to prevent cellular dissemination and metastases, various adjuvant treatments were proposed, such as pre-enucleation radiation, but this did not improve survival (Char *et al.*, 1988). This hypothesis was controversial. Siegel *et al.* reanalysed the survival data appraised in the Zimmerman study and found no evidence to suggest that surgery promoted or accelerated the development of metastatic disease (Siegel *et al.*, 1979). Other studies have also failed to show improvement in survival after enucleation (Augsburger *et al.*, 2004a, Foulds *et al.*, 1987, Seddon *et al.*, 1990) and therefore in recent years, alternative treatments to enucleation have been used more frequently to manage posterior uveal melanoma, with the aim of preserving vision. These include radiotherapy, local resection and transpupillary thermotherapy (Shields *et al.*, 1991). A non-intervention approach for small tumours was also adopted. Enucleation is however still required in some cases; for example, for small and medium-sized choroidal melanomas documented to be growing and invading the optic nerve, tumours too extensive at presentation or for

the relief of pain due to neovascular glaucoma, which is most common after brachytherapy of large tumours (Damato, 2004, Shields *et al.*, 1991).

1.4.2 Eye conserving therapies

Observation

It is common for small melanocytic lesions of the choroid to be observed without treatment until any growth is documented (COMS, 1997). Although it remains unclear whether treatment of small tumours prevents metastases, it is important for the clinician to recognise those tumours at high risk for growth and treat early (Shields *et al.*, 2002). The factors that predict aggressive behaviour of a small choroidal tumour include thickness greater than 2 mm, subretinal fluid, symptoms, orange pigment and margin at the optic disc (Shields *et al.*, 2000). Observation only is also indicated for elderly or debilitated patients as long as the eye is comfortable (Damato, 2004).

Radiotherapy

Radiotherapy given either with episcleral radioactive plaques sutured to the sclera over the tumour (brachytherapy) or with accelerated protons or helium ions often saves functional vision in the eye with uveal melanoma particularly when the tumour is small (Gragoudas *et al.*, 1987, Shields *et al.*, 1991). Stereotactic radiotherapy has similar indications to proton beam radiotherapy and delivers fine beams of photons to the tumour from many directions (Damato, 2006).

Radiotherapy induces widespread DNA damage, which kills many tumour cells and prevents surviving cells from proliferating (Harbour *et al.*, 2003). Not all tumour cells are sterilised by irradiation and some cells may recover (Rousseau, 2004). The growth potential of such tumour cells is however unpredictable (Schilling *et al.*, 1997).

The radioactive sources more commonly used are cobalt⁶⁰, iodine¹²⁵, palladium¹⁰³ or ruthenium¹⁰⁶. Iodine¹²⁵ and ruthenium¹⁰⁶ are more commonly used in North America and Europe, respectively (Finger, 1997). Ruthenium plaques which emit β radiation have a more limited range and are the first choice of therapy for choroidal tumours up to 5mm thick and not extending close to the optic disc (i.e.< 3 mm) whereas iodine plaques which emit gamma radiation can treat tumours up to 8-10mm thick (Damato, 2004). With plaque radiotherapy the aim is to deliver at least 80 Gy to the tumour apex (Shields *et al.*, 1991).

Compared with brachytherapy, proton beam radiotherapy is more expensive, time consuming and more likely to cause discomfort. This form of treatment is usually reserved for small, posterior choroidal tumours less than 6 mm thick and extending close to the optic disc and for large choroidal tumours (tumours more than 5 mm thick), as an alternative to trans-scleral local resection (Damato, 2006). As with brachytherapy, local tumour recurrence after proton beam radiotherapy is associated with increased mortality but the explanation for this is still unclear (Egan *et al.*, 1998).

Depending on the site and dose of radiotherapy complications of radiation retinopathy, cataract and neovascularisation can occur (Damato, 2006). These complications could potentially be treated by intraocular administration of steroids and antiangiogenic agents (Damato, 2006).

Phototherapy

Thermotherapy given through a dilated pupil (transpupillary thermotherapy-TTT) consists of the delivery of a long-duration, long-wavelength (810nm) diode laser through a large spot size (Oosterhuis *et al.*, 1995). It has been shown to be an effective treatment for selected small choroidal melanomas and also for larger ones in combination with brachytherapy (Shields *et al.*, 2002, De Potter *et al.*, 1996). In transpupillary thermotherapy there is low lateral spread of heat into adjacent tissues; therefore it may be especially useful for tumours in the macular area and adjacent to the disc to retain central vision (Oosterhuis *et al.*, 1995). However concerns have been raised regarding the long-term tumour control with primary TTT (Harbour *et al.*, 2003, Shields *et al.*, 2002) since this method does not seem to completely eradicate intrascleral tumour cells (Keunen *et al.*, 1999), that may be present in up to 55.7% of uveal melanomas (COMS, 1998 a). Therefore, at present, the main role for TTT seems to be as an adjunct to plaque radiotherapy (Shields *et al.*, 2002).

Local resection

The two techniques of local resection for choroidal melanomas include trans-scleral local resection and endoresection. Melanomas which meet the criteria for trans-scleral local resection can also be managed by radiotherapy in most cases; however relative indications for the former include tumours located more anteriorly, with a smaller base and a greater thickness (tumours exceeding 5-6 mm in thickness), since such tumours require a much higher radiation dose (Shields *et al.*, 1991). Local resection is not commonly performed because it is difficult and requires careful patients selection due to the profound hypotensive anaesthesia necessary for this procedure. Trans-retinal resection or endoresection of choroidal melanoma remains controversial because of the risk of seeding tumour cells; endoresection can however be performed for juxtapapillary tumours up to 10mm in diameter if the patient requires good vision in the affected eye (Damato, 2004).

Orbital exenteration

Exenteration of the orbital contents has been considered an acceptable method to treat advanced cases of uveal melanomas with extraocular extension into the orbit (Shammas and Blodi, 1977). Due to improvement of diagnostic techniques and earlier recognition it has become uncommon for patients to initially present with extensive extraocular involvement requiring orbital exenteration. (Shields *et al.*, 1991). Although there is justification for orbital exenteration in such cases, controversy continues regarding both the indications for its use and its success in improving patient prognosis. Shammas and Blodi (Shammas and Blodi, 1977) found

that early exenteration for extraocular extension was curative, with 87% of the patients surviving 5 years or more but Kersten *et al.* reported that this form of treatment was palliative and prognosis was not altered in those who had an exenteration (Kersten *et al.*, 1985). Data based on mathematical studies of tumour doubling time and related metastases also suggest that metastases of choroidal melanoma can potentially occur early in the course of the disease, before treatment for primary tumour is given (Eskelin *et al.*, 2000).

1.5 Metastatic disease

1.5.1 Prediction and mortality rates

The prognosis for uveal melanoma is difficult to predict and despite advances in diagnosis and management of uveal melanoma, the overall mortality remains high (50%) due to a propensity for the tumour to metastasise to the liver (Diener-West *et al.*, 1992, Mooy and De Jong, 1996). According to the COMS Group, the metastases develop in the liver in 93% of cases (COMS, 2001). Despite treatment advances the survival rate for choroidal melanoma still remains 72% at 5 years, 60% at 10 years and 55% at 15 years (Rousseau, 2004). The median survival time for a patient with liver metastases is only 7 months (Kath *et al.*, 1993). The peak mortality from metastatic disease occurs within 3 years of diagnosis but the clinical course is unpredictable since metastases may present up to 36 years later (McLean *et al.*, 1982, Newton, 1965).

1.5.2 Screening for metastases

The liver is involved in up to 95% of patients with metastatic disease, with about 50% of patients also developing extrahepatic metastases, most often in the lungs, bone, skin and brain (Eskelin *et al.*, 1999). The reason why the liver is almost always involved is unknown. Foss suggested that it could be explained in terms of expression of adhesion molecules but the liver is most frequently involved by many malignancies (Foss, 1996). Lindegaard *et al.* found metastases to the central nervous system (CNS), kidney, heart, or adrenal glands was only in patients with optic nerve invasion, suggesting that this feature may result in a different metastatic pattern (Lindegaard *et al.*, 2006). Since there are no lymphatics within the eye, involvement of regional nodes is very rare, occurring only when there is extraocular spread.

To date, the procedures and time intervals which constitute an optimal follow up have not been established and differences exist between different centres (Hicks *et al.*, 1998, Eskelin *et al.*, 2000). Although it is accepted that biochemical liver function tests are insensitive (Diener-West *et al.*, 2004), Eskelin *et al.* have shown that liver ultrasonography and biochemical liver function tests can detect metastases before the onset of symptoms in about 59 % of patients when performed annually and in more than 95% if performed every 6 months (Eskelin *et al.*, 1999). However Hicks *et al.* concluded that all single liver function tests had poor sensitivity for metastatic disease and recommended routine liver ultrasonography at both presentation and follow up (Hicks *et al.*, 1998). Another area of common tumour involvement is the lungs and in one series (Kath *et al.*, 1993) the percentage of pulmonary involvement was 46%. These authors suggested that a chest radiograph should be performed at least every 6 months. However it has also been suggested that

chest radiography is of no additional value since patients with pulmonary metastases usually already have hepatic metastases (Damato, 2004).

1.5.3 Treatment of systemic metastases

Regimens/trials for chemotherapy

Currently there is no effective treatment for metastatic uveal melanoma. Various chemotherapeutic regimes have failed to show significant survival benefit in patients with extensive disease (Kath *et al.*, 1993). A review of the M.D. Anderson Cancer Center experience compared the results of different modalities available to treat liver metastases including systemic chemotherapies, hepatic intra-arterial chemotherapies and chemoembolisation of liver metastases (Bedikian *et al.*, 1995). Of the three modalities of therapy only chemoembolisation using cisplatin-based regimens produced a meaningful response rate. However toxicity and complication rates with these cisplatin regimens are high and therefore they have not become a standard treatment (Rousseau, 2004). Pyrhönen *et al.* had a modest response in treating metastatic uveal melanoma in hepatic and extrahepatic sites with a combination of bleomycin, vincristine, lomustine and dacarbazine (the BOLD regimen) plus interferon- α regimen (Pyrhönen *et al.*, 2002). Laboratory tests suggest that gemcitabine and treosulphan might be effective but clinical studies are necessary (Neale *et al.*, 2001).

Immunotherapy

Immunotherapy with interleukin-2 or interferon- α and - γ has not shown consistent activity in metastatic uveal melanoma. In contrast, *in vitro* data has shown an effect of interferon- α and - γ on primary human uveal melanoma cell lines (Wöll *et al.*, 1999).

Gene therapy

Studies in mice were undertaken by Alizadeh *et al* to determine whether transfer of interferon- beta-(IFN- β) gene will protect against liver metastases arising from intraocular melanomas (Alizadeh *et al.*, 2003). The results suggested gene transfer as a possible strategy for controlling liver metastases arising from intraocular melanomas. This study was based on the knowledge that adenovirus vectors have a predilection for transferring genes to the liver after IV injection, also that IFN- β is a potent activator of natural killer (NK) cells and that the liver has the highest concentration of NK cells in the body (Alizadeh *et al.*, 2003).

Ma *et al.* also developed a mouse model to test whether plasminogen activator inhibitor type I (PAI-1) affected the development of liver metastases (Ma *et al.*, 1997). Plasminogen activator (PA) is known to facilitate tumour metastases by promoting invasion of tissue barriers but PAI-1 can inhibit PA bound to the cellular receptor and also block tumour cell binding to the extracellular matrix. In these studies intraocular injection of adenovirus expressing PAI-1 resulted in reduction of liver metastases by 50% (Ma *et al.*, 1997). These results support the disruption of PA

function through gene transfer as a therapeutic strategy for preventing metastases and prolonging host survival.

Surgery for metastases

Surgical excision of an isolated hepatic metastases can sometimes prolong life significantly (Fournier *et al.*, 1984). Other results suggest that the combination of chemotherapy and surgery may have useful activity. For example, Rajpaland *et al.* treated 11 patients who developed metastatic disease with chemotherapy and surgery and 9 remained tumour-free for at least 12 years (Rajpal *et al.*, 1983). In a prospective study, Salmon *et al.* showed that those patients in whom curative resection was possible, the mean survival was significantly better (22 months) than the overall survival of 9 months for patients who had biopsies or tumour reductions with partial surgery and chemotherapy (Salmon *et al.*, 1998).

Targeted therapies

New prognostic factors/molecular targets, such as specific membrane receptor inhibitors are being evaluated to provide the biologic basis for the development of new treatments. In vitro assays have shown that treatment with IGF-1R blocking antibody reduces the expression of the receptor at the cell surface (All-Ericsson *et al.*, 2002). In this study, All-Ericsson *et al.* investigated the immunohistochemical expression of IGF 1R in 36 uveal melanomas and found a correlation between high levels of expression of IGF-1R and decreased survival rates. As shown in this study

and in others (Girnita *et al.*, 2000, Dricu *et al.*, 1997, All-Ericsson *et al.*, 2002), experimental treatment with α -IR3 (IGF-1R blocking antibody) or tunicamycin (inhibitor of IGF-1R N-linked glycosylation) diminishes the expression of the receptor at the cell surface and also decreases cell viability and DNA synthesis.

The proto-oncogene *c-kit* encodes a transmembrane receptor-type protein tyrosine kinase (kit) whose ligand is a stem cell factor (SCF, also known as steel factor, kit ligand, and mast cell growth factor) (Mouriaux *et al.*, 2003). *C-kit* is expressed and is important for the development of normal cells such as cutaneous melanocytes, mast cells and hematopoietic stem cells but it has also been detected in a wide variety of human malignancies such as gastrointestinal stromal tumours and more recently in uveal melanomas, suggesting that growth and survival of uveal melanoma cells is dependent on *c-kit* activation (Mouriaux *et al.*, 2003, All-Ericsson *et al.*, 2004). Furthermore, the KIT pathway can be blocked with the tyrosine kinase inhibitor imatinib mesylate (ST1571); this was initially approved for the treatment of chronic myeloid leukaemia (Druker *et al.*, 2001) but it has also been shown to be effective in KIT-positive gastrointestinal stromal tumours (van Oosterom *et al.*, 2001). All-Ericsson *et al.* suggested that this drug could also have a possible therapeutic benefit in uveal melanoma through its inhibitory effects on *c-kit* phosphorylation and cell proliferation (All-Ericsson *et al.*, 2004). Imatinib has also been suggested a potential treatment for patients with ocular melanoma expressing *c-KIT* (Fiorentini *et al.*, 2003).

Angiostatin is a degradation product of plasminogen and is produced in the presence of a primary tumour. It also inhibits the growth and development of metastases

although the exact mechanism remains uncertain (Apte *et al.*, 2001). Apte *et al.* showed that uveal melanoma cell lines produced angiostatin *in vitro* and that removing a source of angiostatin by enucleating a melanoma-containing eye could exacerbate metastatic disease (Apte *et al.*, 2001). Exogenous angiostatin may therefore have potential therapeutic implications in the management of patients with primary intraocular melanomas.

1.6 Counselling the Patient with a Choroidal Melanoma

Patients with choroidal melanomas, even if the eye and vision are maintained often suffer a lot of distress because of the unpredictable overall outcome. As none of the different treatments available offer a survival advantage, the treatment of choice should be guided by the effects on quality of life (Foss *et al.*, 2000). This fact should be highlighted to all new patients to avoid unnecessary distress if later on they develop metastatic disease. Therefore, the main aim in the management of patients is to restore quality of life. To determine whether quality of life differs between patients with choroidal melanoma treated by enucleation and those treated by radiation therapy, Cruickshanks *et al.* studied 147 patients (65 treated by enucleation and 82 treated by radiation therapy) and concluded that treatment for choroidal melanoma does not seem to be associated with a large difference in quality of life in long-term follow up (Cruickshanks *et al.*, 1999).

A modified questionnaire described by Foss *et al.* (Foss *et al.*, 2000) known as MOOD (measure of outcome in ocular disease) includes a 21 item questionnaire which assesses the patient's assessment in terms of visual function and the impact of treatment. The MOOD proved to be well accepted by the patients and its validity was

supported by high correlations between the vision and impact scales. MOOD total scores were significantly higher for patients who reported being very satisfied compared with those who were not. Such tools may help to identify problems encountered after treatment of choroidal melanoma and in guiding follow-up.

Cytogenetic studies may be an important additional tool for counselling these patients. Although patients with melanoma containing two copies of chromosome 3 have a better prognosis for survival some may still die from metastases. By identifying tumours that are monosomic for chromosome 3 patients at high risk of metastases-related death can be identified for close follow up. Patients with clinical metastatic disease unresponsive to treatment may prefer to be informed of their poor prognosis so that they can make appropriate arrangements while they are well (Scholes *et al.*, 2003).

1.7 Aims and Objectives

The aim of my work is to develop the CISH technique to identify monosomy 3 in archival tissue. To date the majority of studies have been prospective and the ability to study archival tissue has the advantage of large specimen numbers and long follow up. A further aim is to compare the efficiency of the CISH technique with FISH when assessing monosomy 3 in archival tissue sections of choroidal melanomas. Having developed the technique for archival tissue a further aim is to assess the time until death in patients with and without monosomy 3. The final aim will be to assess whether monosomy 3 corresponds with morphological subclones.

**2 IDENTIFICATION OF MONOSOMY 3 IN
CHOROIDAL MELANOMA BY
CHROMOSOME *IN SITU*
HYBRIDISATION**

2.1 Background

The majority of cytogenetic studies on uveal melanoma have required fresh tissue for short term culture, fluorescence in situ hybridisation or DNA extraction. DNA may also be extracted from paraffin embedded tissue for analysis (Aalto *et al.*, 2001, Scholes *et al.*, 2001, Sisley *et al.*, 2000, White *et al.*, 1998, Naus *et al.*, 2002, Patel *et al.*, 2001, Scholes *et al.*, 2003, Prescher *et al.*, 1996, Sisley *et al.*, 1997). None of the techniques described, however has identified the presence or absence of monosomy 3 within tissue sections.

In this study I have used the alternative approach of interphase cytogenetics where the use of chromosome-specific DNA probes in combination with the ISH technique allows the detection of numerical and structural chromosome aberrations in both metaphase and interphase nuclei (Poddighe *et al.*, 1992).

2.2 Specific Objective

To develop the technique of CISH to assess chromosome 3 copy number in tissue sections from formalin or gluteraldehyde fixed archival choroidal melanomas.

2.3 Materials and Methods

2.3.1 Study population

Fifty six archival specimens of choroidal malignant melanoma were obtained from the Western Infirmary Pathology files. The specimens included local resection and enucleation specimens; surgery was performed between 1973 and 1992. All tissues had been previously fixed in gluteraldehyde or formalin and embedded in paraffin wax. Twelve of the 56 cases had been formalin fixed (FF). The remaining tumours had all been fixed in gluteraldehyde (GF). The FF and GF specimens were of similar ages. In 14 enucleation specimens (3 FF and 11 GF) normal retina was used as an internal control. In addition, eight cases of normal human skin (all gluteraldehyde fixed) were included as an external control. Full ethical approval in accordance with local policy was obtained for the use of these tissue samples.

2.3.2 Chromosome *in situ* hybridisation

Pretreatment of Slides

Sections, 4µm thick, were mounted on aminopropyltriethoxysilane coated glass slides. Before use, the slides were baked at 65°C for at least 4 hours. The tissue sections were dewaxed in 100% xylene and rehydrated in graded ethanol to water. For heavily pigmented tumours, the slides after being dewaxed were placed in 5% hydrogen peroxide in TBS (tris buffered saline) overnight at room temperature and then washed in water. All sections were microwaved in Tris-EDTA (4.5mM Tris, 1mM EDTA, pH8) at full pressure for 5 min. After rapid cooling the sections were

then digested with pepsin (0.4% pepsin in 0.2M hydrochloric acid) for 30 min at 37°C and post-fixed for 10 min in tissue fixative (Streck Laboratories Inc., Omaha, NE, USA). Finally, sections were dehydrated in 100% ethanol and air-dried.

DNA Probes

Chromosome-specific repetitive sequence probes for chromosome 3 (D3Z1) and chromosome 18 (D18Z1) were purchased from Q-Bio gene (Illkirch, France). Both are alpha satellite chromosomes which specifically hybridises to the highly repeated alphoid DNA located at the centromere of human chromosomes 3 and 18. Chromosome 18 was used as a control chromosome, as it appears to rarely show abnormalities in uveal melanoma. Both commercial probes were ready labelled with digoxigenin. Probes were diluted 1:10 in a hybridisation mix consisting of 70% formamide, two times the standard concentration of standard saline citrate (SSC) (1xSSC is 0.15M sodium chloride and 0.015M sodium citrate, pH7), 500µg/ml salmon sperm DNA, and 10% dextran sulphate.

In situ hybridisation

The probe in the hybridisation mix and DNA in the tissue sections were co-denatured together using the Omnislide modular system (ThermoLife Sciences, Hampshire, UK) at 80°C for 5 min. The tissue sections were then incubated with the probe at 37°C overnight. After hybridisation, slides were washed twice in 2 x SSC at room temperature for 5 min and then in 1xSSC at 70°C for 5 min. Prior to

immunohistochemical detection of hybridised probe the slides were washed in 4xSSCT (4xSSC, 0.05% Tween-20) and blocked for 30 min at room temperature in 4 x SSCT, 10% blocking reagent (Roche, USA). The slides were then incubated with anti-digoxigenin alkaline phosphatase (AP) Fab fragments (Roche, USA) 1:500 dilution in 4xSSCT, 10% blocking reagent for 30 min at room temperature. Slides were washed in 4xSSCT for 5min, and then rinsed in distilled water. The slides were then incubated in NBT/BCIP solution (0.4mM nitroblue-tetrazolium (NBT), 0.38mM 5-bromo-4chloro-3-indolyphosphate (BCIP), 1.25M levamisole in 100mM HCl pH9.5, 100mM NaCl, 50mM magnesium chloride) overnight. The sections were rinsed in tap water and counterstained with haematoxylin. Sites of binding were identified as blue-black dots.

Quantification of hybridisation signals

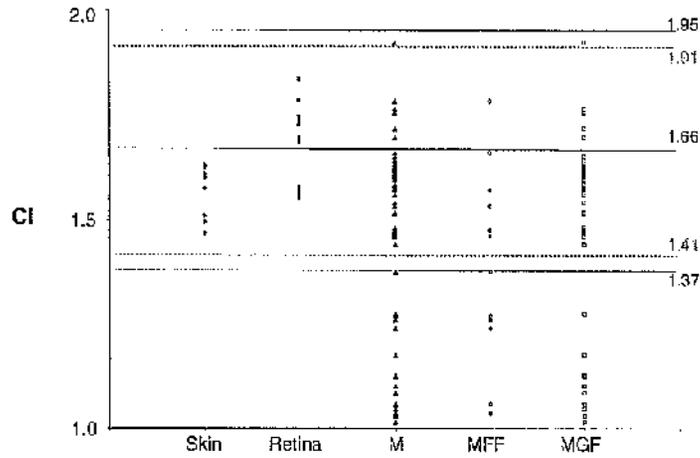
Chromosome-specific centromeric probes were hybridised to sections of choroidal melanoma. In order to obtain control values, centromeric copy numbers for the 2 chromosomes were assessed using retina where present in the tissue sections and separate sections of normal skin. The evaluation and interpretation of LSH signals were carried out as previously described (Murphy *et al.*, 1995, Hopman *et al.*, 1992). Firstly, tissue sections were examined by light microscopy using an oil immersion lens (magnification x 1000) and an eyepiece graticule to prevent recounting of nuclei. Overlapping nuclei and minor hybridisation signals were not analysed and only nuclei with the histological appearance of tumour cells were evaluated. Poor quality hybridisations were excluded. For each section the number of signal spots per

nucleus was recorded for 200 nuclei and the assessor was masked for the outcome of the patient.

The hybridisation data were analysed in two ways to assess the degree of chromosome imbalance for each sample and each chromosome. Firstly, the chromosome index (CI), which gives an average chromosome copy number, was calculated by dividing the total number of hybridisation spots counted by the total number of nuclei counted (Dhingra *et al.*, 1994). CIs are shown in figure 2.1. For chromosome 3 the mean CI for retina and skin was 1.65 and 1.51 respectively. For chromosome 18 the mean CI for retina and skin was 1.63 and 1.50. A tumour was defined as monosomic for chromosome 3 if its CI was less than 3 standard deviations (3SD) from the mean (*i.e.* less than 1.37) (Russel *et al.*, 1999).

The second method used to identify monosomy was the signal distribution (SD), which can potentially detect relatively small populations with chromosomal numerical imbalances. To define the signal distribution the percentage of the nuclei counted with one, two or more than two hybridisation sites was calculated. A tumour was described as monosomic for chromosome 3 if the percentage of nuclei with one hybridisation site was greater than 60% of the nuclei counted (Russel *et al.*, 1999).

A Chr 3



B Chr 18

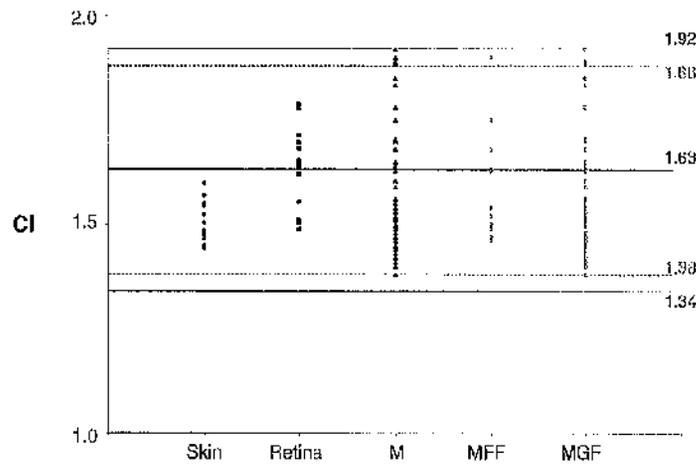


Figure 2.1 Chromosome index for chromosome 3 and 18

Scattergram showing distribution of chromosome index (CI) for chromosome 3 (A) and 18 (B) in skin (solid diamond), retina (solid square), malignant melanoma (M, solid triangle); formalin fixed (FF, open circle), glutaraldehyde fixed (GF, open square). The lines denote the mean CI (thick solid line), SD3 (thin solid line) or 2.58D (broken line) for retina. Values falling 3SD below the mean were classified as chromosome loss.

The criteria for both signal distribution and CI were based on published estimates and previous experience of the technique and take into account nuclear truncation (Murphy *et al.*, 1995, Hopman *et al.*, 1992, Dhingra *et al.*, 1994). Nuclear truncation is an important aspect of interphase cytogenetic analysis since the way the section is cut will influence CI. Hence, the chromosomal content of the nuclei included in the section will increase as a section thickness increases but as demonstrated before CI is not significantly influenced by a change in section thickness from 4 to 8 μm . Tumours had to show chromosome loss by both CI and signal distribution to be regarded as monosomic.

2.3.3 Statistical analysis

Differences in chromosomal indices between tumour and retina, GF and FF cases and older and newer tissues were compared using the two-sided Mann-Whitney test with a *priori* level of statistical significance set at $p < 0.05$.

2.4 Results

2.4.1 *In situ* hybridisation

In situ hybridisation for chromosome 3 and 18 was successfully performed on 52 choroidal melanomas. Four cases were excluded because of heavy tumour pigmentation and large areas of necrosis. There was no apparent visual difference in hybridisation reactions in FF tumours when compared with GF tumours. Examples

of choroidal melanomas and normal retina hybridised with chromosome 3 and 18 are shown in figure 2.2.

2.4.2 Assessment of chromosome index

The CI for chromosome 3 and 18 in choroidal melanoma and controls is shown in figure 2.1. There was no significant difference between the CI for FF tissues when compared with the corresponding GF tissues. There was no significant differences between the CI for tumours removed more than 20 years ago when compared with those removed less than 10 years ago. For choroidal tumours the CIs for chromosome 18 were not significantly different compared with that of normal retina and all lay within 2.75 standard deviations of the mean CI for normal retina. For 15 tumours the CIs for chromosome 3 were significantly different compared with normal retina ($p=0.0013$) and had a CI more than 3 standard deviations from the mean. For the rest of the tumours the CIs for chromosome 3 were not significantly different compared with that of normal retina and all lay within 2 standard deviations of the mean.

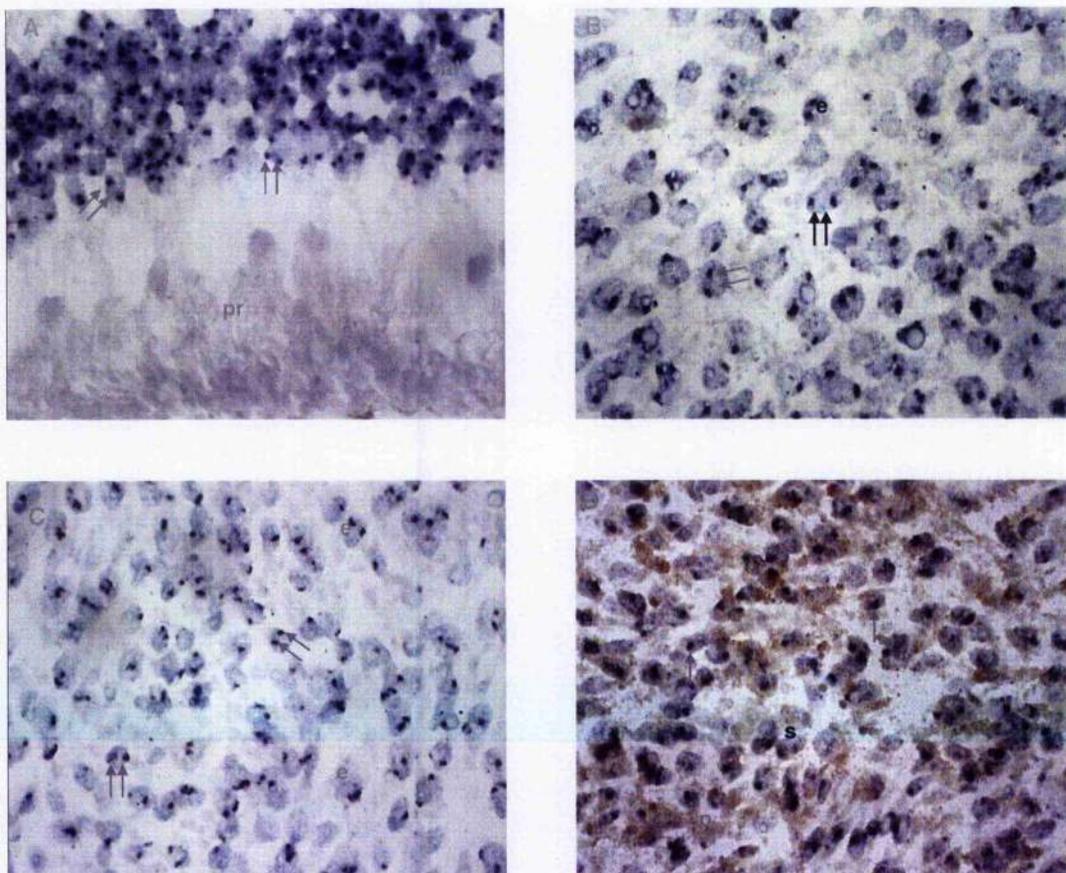


Figure 2.1 Chromosome *in situ* hybridisation.

A: Gluteraldehyde fixed normal retina, hybridised with chromosome 18, showing two copies (double arrow) in most cells of the outer nuclear layer (ONL). The photoreceptors (pr) are towards the bottom of the picture. B: Gluteraldehyde fixed, metastasising epithelioid (e) melanoma, hybridised with chromosome 18, showing two copies (double arrow) in most cells. C: Formalin fixed, non-metastasising epithelioid (e) melanoma, hybridised with chromosome 3, showing two copies (double arrow) in most cells. D: Gluteraldehyde fixed, metastasising spindle (s) melanoma with moderate pigmentation (p), hybridised with chromosome 3, showing one copy in most cells (single arrow) (magnification x 1000)

2.4.3 Assessment of signal distribution

For chromosome 18 the mean signal distribution for 2 or more hybridisation sites per nucleus was 64% for retina, 62% for skin and 57.5% for the choroidal tumours (FF 57%; GF 58%). Therefore, by SD all the samples were balanced for chromosome 18. For chromosome 3 the mean signal distribution for 2 or more hybridisation sites per nucleus was 63% for retina, 54% for skin and 43% for the choroidal tumours (FF 41.5%; GF 44%). By SD all the cases of normal retina and skin were balanced for chromosome 3. However by SD 15 cases of choroidal melanoma had greater than 60% of nuclei with only one hybridisation site and were therefore defined as monosomic for chromosome 3 by this parameter (see table 2.1).

2.4.4 Assessment of monosomy 3

Tumours had to show chromosome loss by both CI and signal distribution to be regarded as monosomic and therefore the data for CI and SD were combined to assess monosomy 3. The results are summarised in table 2.2. Using both criteria 15 of the 52 cases of choroidal melanoma were defined as monosomic for chromosome 3. In all cases both parameters agreed and there were no cases where either the CI or SD indicated monosomy but the other parameter did not.

Chr 3	No of cases with over 40, 50, 60 or 70% of tumour cell nuclei containing only one hybridisation site			
	40%	50%	60%	70%
Skin, n=8	8	8	1	0
Retina, n=14	6	0	0	0
Melanomas, n=52	45	25	15	14
FF, n=12	11	8	6	5
GF, n=40	34	17	9	9

FF, formalin fixed; GF, glutaraldehyde fixed

Table 2.1 Signal distribution for chromosome 3 showing the percentage of nuclei with only one hybridisation site

	Chromosome 3		Chromosome 18	
	Loss	Balanced	Loss	Balanced
Melanomas, n=52	15	37	0	52

Table 2.2 Tumours showing evidence of chromosomal changes by signal distribution and chromosome index.

2.5 Discussion

This is the first study in which interphase cytogenetics has been applied to define the presence or absence of monosomy 3 in tissue sections of paraffin-embedded choroidal melanomas. The main advantages of CISH, used in this study, over other techniques are that it allows the direct assessment of chromosomal gains and losses in interphase and metaphase nuclei within a tissue section. This ensures that the nuclei assessed are indeed tumour cells and that the chromosomal changes within the tumour cells can be directly compared with adjacent normal tissue. Cytogenetic studies on archival tumour specimens are important because of the availability of large series of fixed tumours that permit retrospective study of malignant progression, correlation with long clinical follow up and analysis of rare tumours. CISH was performed on archival tissue that was either formalin or gluteraldehyde fixed and I have successfully performed CISH on gluteraldehyde fixed tissue samples up to 30 years old.

In my laboratory CISH has previously been used to assess chromosomal gains and losses in other formalin fixed tumours such as adrenocortical tumours (Russel *et al.*, 1999). In these tissues good hybridisation signals could be obtained when slides were simply pre-digested with pepsin for up to one hour. This method of tissue preparation worked for formalin fixed choroidal melanomas but signal could not be obtained with gluteraldehyde fixed tissues. However by introducing a more rigorous pre-treatment of microwaving under pressure in Tris-EDTA buffer, prior to pepsin digestion, good hybridisation signals were obtained. Interestingly, this additional step did not affect tissue preservation of formalin fixed cases and there were no significant differences between results obtained for CI and SD between

gluteraldehyde and formalin fixed cases. This pre-treatment was therefore adopted for all cases in this study. The criteria for scoring ISH signals, although previously established in other studies (Murphy *et al.*, 1995, Hopman *et al.*, 1991, Hopman *et al.*, 1992, Emmerich *et al.*, 1989), was checked by two observers using control sections and repeatedly assessing different fields. The numbers of nuclei per field were between 30 and 50 and 200 nuclei was an acceptable scoring criteria to identify the main chromosomal fractions. Initially 20 slides were also independently assessed by my supervisor, Dr F. Roberts, and at any time during the study, when tumours presented with poorer staining intensity or more difficult morphology. At a later stage, and without knowledge of the initial result, I repeated the assessment for a random sample of 30 specimens. Inter- and intra- observer variation in counting was consistently less than 10%.

Using this technique monosomy 3 was identified in 15 of 52 cases (28.8%) of choroidal melanoma; the other 37 tumours were balanced for chromosome 3. The 52 cases of choroidal melanoma were all balanced for chromosome 18. This chromosome was selected as a control as it rarely shows abnormalities in uveal melanoma. Using these criteria the results obtained for CI and SD agreed in all cases of monosomy 3. The cut-off points were selected in accordance with this laboratory's previous experience of the technique. A normal chromosome complement was defined as 3 standard deviations from the mean CI. Other researchers have used a value of 2.58 standard deviations (Bulten *et al.*, 1998). If this value was applied to this study one case would not have been defined as monosomic by CI. However for SD a strict cut off point of 60% of cells showing fewer than two hybridisation sites to define chromosomal loss was used compared with 40% or less used in other studies

(Visscher *et al.*, 1996, Sneige *et al.*, 1996). In Table 2.1 the number of cells with 40, 50 and 70% of nuclei with only one hybridisation site is displayed. It is clear that in this study a cut off point of less than 60% would identify many normal tissues as monosomic. The identification of cells with only one hybridisation site in normal tissue reflects the effects of nuclear truncation and ultimately will be affected by the thickness of sections used, which may vary between studies. In this study 4 μm sections were used as this gave the best hybridisation signal. Using the higher cut off point of 70% one case would have been excluded. This is the same case that would have been excluded by defining a cut off point for CI 2.58 standard deviations from the mean. This does not significantly alter the results but indicates that the cut off points may have to be re-assessed as more cases are studied.

The principal drawback of this technique is the effect of nuclear slicing on the number of signal spots, with the number of spots varying from zero to the actual copy number of the specific chromosome in that nucleus (nuclear slices in a thicker section will have a more complete chromosomal representation, whereas a smaller number will be present in a thinner section) (Dhingra *et al.*, 1992). However with 4 μm thick sections as used in this study, the CI does not change significantly compared to other thicknesses and also it gave the best hybridisation signal (Dhingra *et al.*, 1994). A second drawback to this technique is that it is difficult to detect a change in copy number that affects only a small fraction of cells, missing for example the presence of monosomy. However CI calculation and the distribution of signal spots prove to be complementary to each other in analysing ISH on tissue sections. The CI gives an average chromosome copy number and is therefore better suited to describe clonal changes within a tumour whereas the analysis of signal

distribution can potentially detect relatively small populations of cells with numerical imbalances in chromosomes (Dhingra *et al.*, 1994). Another confounding problem results from variations in hybridisation efficiency that can occur from one experiment to another. Strict adherence to the criteria for signal counting can further help eliminate false-positive signals caused by nonspecific binding (Hopman *et al.*, 1988, Murphy *et al.*, 1995). Non-specific probe binding can also be excluded by the incorporation of appropriate controls. In enucleation specimens normal retina was used as an internal control. In addition, normal human skin was included as an external control.

In previous studies, other techniques rather than *in situ* hybridisation were used for the assessment of non-random chromosomal abnormalities such as standard cytogenetics (Sisley *et al.*, 1997, Sisley *et al.*, 1990, Horsman and White, 1993), CGH (Tsechentscher *et al.*, 2001, Prescher *et al.*, 1996, Aalto *et al.*, 2001), microsatellite analysis (Parrella *et al.*, 1999, Scholes *et al.*, 2001, Scholes *et al.*, 2003) and FISH (McNamara *et al.*, 1997, Naus *et al.*, 2002, Patel *et al.*, 2001). The studies described however have utilised only fresh tissue and stained slides prepared from disaggregated tumour cells. CISH avoids the requirement for fresh tissue and the potential examination of non-representative tumour populations or non-tumour DNA. The preservation of tissue architecture during the process of CISH is the principal advantage of this method; chromosome heterogeneity can then be detected within the tumour with correlation of areas of specific chromosomal imbalances with the histological findings. This will help with the identification of subclones within a tumour and provide information on the role of monosomy 3 in tumour development.

Direct comparisons are not possible between the results of interphase cytogenetic studies and techniques that involve DNA extraction such as microsatellite analysis and CGH (Ried, 1998). Unlike these techniques the main disadvantage of the CISH technique described is that fine mapping of changes cannot be achieved with alpha repeat centromeric probes. Microsatellite analysis and CGH have shown that in the majority of cases chromosome 3 loss of heterozygosity involves an entire chromosome homologue however in a small number of tumours regional losses on chromosome 3 have been identified and such regional losses cannot be detected with this technique as it stands (Scholes *et al.*, 2001, Speicher *et al.*, 1994). However probes for other chromosome regions are available and the technique could be adapted to detect regional losses. The technique could also be adapted to detect other chromosomes known to show gains or losses in uveal melanoma such as 8q (Sisley *et al.*, 1997) and 6q (Aalto *et al.*, 2001), which are significantly associated with poor overall survival. It is also not possible to characterise specific tumour suppressor genes that may be involved. Nonetheless CISH represents a valuable additional technique that will allow the study of monosomy 3 in tissue sections allowing the correlation of genotype with phenotype. With CISH it is possible to screen a large archival series and define a group of tumours for more detailed study using alternative cytogenetic techniques. Finally the technique can easily be applied to routine pathology specimens without special treatment and could therefore identify high-risk patients who may benefit from close monitoring in whom effective adjuvant treatment might particularly be indicated.

**3 DETECTION OF MONOSOMY 3 IN
ARCHIVAL CHOROICAL MELANOMA
USING FLUORESCENCE *IN SITU*
HYBRIDISATION. A COMPARISON WITH
CHROMOSOME *IN SITU*
HYBRIDISATION**

3.1 Background

Fluorescence *in situ* hybridisation allows the interphase cytogenetic analysis of either fresh or archival tumour tissue. One of the main advantages of FISH compared with CISH is that multiple genetic changes can be examined in one preparation by the use of probes labelled with spectrally distinct fluorescent dyes (Leitch *et al.*, 1994). In patients with uveal melanoma FISH has been used on samples of disaggregated tumour cells to identify loss of chromosome 3 and extra copies of 8q. However these investigators have encountered several problems with the FISH technique including weak hybridisation signals in paraffin embedded tissue samples and masking of the hybridisation signal by autofluorescence. Patel *et al* have also commented that GF tissue is not suitable for FISH analysis (Patel *et al.*, 2001).

It is generally accepted that the key to successful FISH on archival tissue is the use of efficient pretreatments that will expose the target genes and allow the penetration of the probes (Chin *et al.*, 2003). The requirement for sufficiently rigorous pretreatments to expose target genes but without destroying tissue morphology may explain some of the difficulties encountered in previous studies.

3.2 Specific Objectives

1. To develop the technique of FISH to assess the presence of chromosome 3 in archival tissue sections of choroidal melanoma.
2. To compare the hybridisation signal obtained using FISH with that of CISH.

3.3 Materials and Methods

3.3.1 Study population

Twenty archival specimens of choroidal malignant melanoma were selected from the original series. Cases that had been both successful and unsuccessful with CISH were considered. These included cases that had been unsuccessful due to heavy tumour pigmentation. The specimens included 7 local resections and 13 enucleations. Surgery was performed between 1974 and 2002. Three of the 20 cases had been FF. The remaining tumours had all been GF. In 13 enucleation specimens (ten GF and three FF normal retina was used as an internal control (as for CISH). Lymph node tissue was used as an external control.

3.3.2 Fluorescent *in situ* hybridisation

In developing the technique the effect of 2 different pre-treatments and both a direct and indirect treatment were assessed. The numbers subjected to each different stage are shown in Figure 3.1.

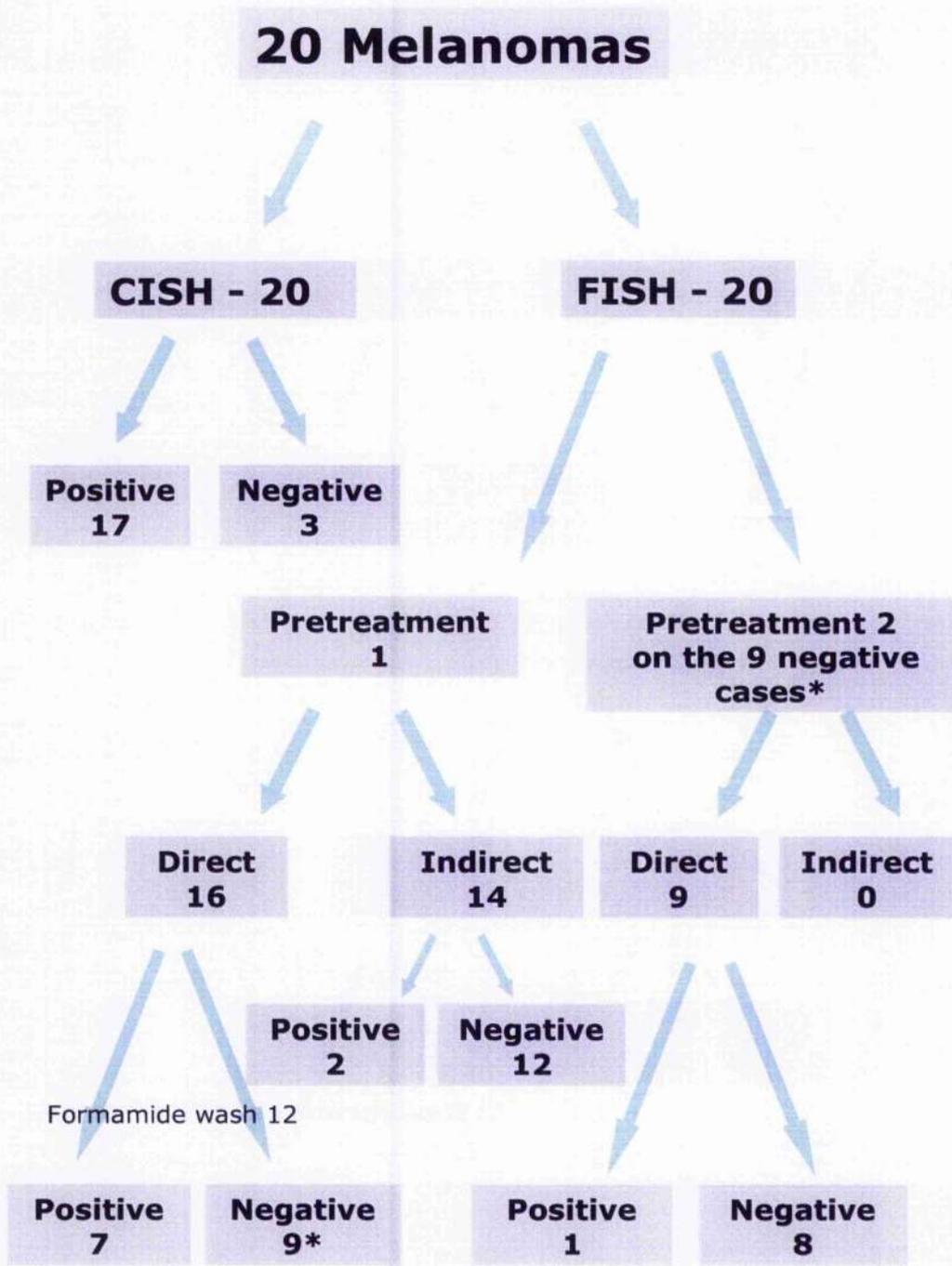


Figure 3.1 A flow chart describing the different stages when attempting to apply FISH in paraffin wax embedded tissue sections.

(* = pretreatment 2 in negative results subgroup)

Pretreatment of Slides

Sections of 4 μm were pretreated by one of two different methods. Firstly all 20 cases were pretreated as for CISH (pretreatment 1), as described in chapter 2. Briefly, the sections were pretreated by microwaving followed by digestion in pepsin (0.4% pepsin in 0.2M hydrochloric acid) for 30 minutes at 37°C. Nine of the original 20 cases were also subjected to an alternative pre-treatment (pre-treatment 2) using the reagents and protocol in a commercial kit, kit II (Abbott, Berkshire, UK). The pretreatment solution in this kit consisted of sodium thiocyanate (NaSCN). The paraffin pretreatment reagent kit II had been used before in my laboratory on rhabdomyosarcoma and has been shown to be superior to the in-house method of microwaving under pressure. Briefly, paraffin-embedded tissues were dewaxed through xylene and alcohols and allowed to air dry. Slides underwent immersion in pre-treatment sodium thiocyanate (NaSCN) solution at 80°C for 10 minutes, washed in water then digestion in proteinase K in 0.2 M HCl at 37°C for 20 minutes, followed by fixation in 10% buffered formalin.

DNA Probes

Two different probes were assessed. The first probes assessed were biotin labelled centromeric probes to chromosomes 3 and 18 (Cambio, Cambridge UK). For these probes hybridisation sites were detected using streptavidin labelled with a fluorescent label, alexa fluor 594 (Invitrogen, Paisley UK). These indirect probes were applied separately to 14 cases (all GF) initially pretreated with pretreatment 1. The second probes assessed were alpha satellite probes to chromosome 3 (3p11.1-q11.1) and chromosome 18 (18p11.1-q11.1) both directly labelled with spectrum

orange (Abbott). These probes were applied separately to 16 cases (13GF, 3FF) initially subjected to pre-treatment 1 (including 11 cases already assessed by the indirectly labelled probes) and to the subset of 9 cases (all GF) subjected to pretreatment 2 (Figure 3.1).

In situ Hybridisation

The appropriately diluted probe was applied to the prepared section using the Omnislide modular system (ThermoLife Sciences, Hampshire, UK) at 80°C for 5 minutes and then allowed to hybridise overnight at 37°C. Post hybridisation washes for the indirectly labelled probes consisted of one five minute wash in 2xSSC at 37°C followed by two five minute washes in 60% formamide/2xSSC and two five minute washes in 2xSSC. For the directly labelled probes, the hybridisation washes were one two minute wash in 2xSSC/0.3% Igepal (Sigma) at 72°C followed by one minute wash in 2xSSC 0.1% Igepal at room temperature. In repeat experiments formamide washes were applied to reduce green background for the direct labelled probe in 12 cases. These formamide washes consisted of three ten minute washes in 50% formamide/2xSSC at 42°C followed by one ten minute wash in 2xSSC at room temperature and a one five minute wash in 2xSSC/0.1% Igepal at room temperature. After the washes, every slide was then air dried in the dark and mounted using DAPI in vectashield mounting medium (Vector labs, Peterborough, UK). The coverslips were sealed with nail polish and the slides stored at 4°C prior to analysis. Hybridisation signals were assessed using an Olympus fluorescent microscope and images captured using image analysis software (Cytovision Applied Imaging, Newcastle, UK).

Identification of hybridisation signal

The quality of FISH signals was analysed by assessing the relative fluorescent signal intensity of the probe and the amount of autofluorescence background. FISH was considered successful if strong hybridisation signals could be detected with a low level of background autofluorescence.

3.3.3 Chromosome *in situ* hybridisation

CISH was performed as previously described in chapter 2. Poor quality hybridisation signals were excluded.

3.4 Results

3.4.1 FISH for CHR 3 and CHR 18

Overall FISH was successful in 8 of 20 (40%) paraffin-embedded choroidal melanomas. Using the indirect biotin labelled probe FISH was successful in only 1 of 14 cases with an additional case showing weak signal. The successful case was GF. Using the directly labelled probe following pre-treatment 1, seven of the 16 specimens showed good hybridisation signal. Of the 11 cases previously assessed using the indirect probe 4 cases had a strong signal, only two of which had previously shown signal (one strong, one weak). After the pretreatment 2 was applied to the remaining 9 negative cases only one case showed a useful signal with the direct probe. Formamide washes did not reduce background signal.

In the cases with good hybridisation signal, there was no significant difference between the signal for FF tissues when compared with the corresponding GF tissues (figure 3.2). Out of these successful cases, it would have been possible to count at least 100 cells. For the internal controls only one case showed good signal in the retina (figure 3.3) but the tumour in this case did not show any signal. Detectable signal was observed in the lymph node in all cases.

3.4.2 CISH FOR CHR 3 and CHR 18

In situ hybridisation for chromosome 3 and 18 was successfully performed on 17 of the 20 choroidal melanomas. Three cases were excluded because of heavy tumour pigmentation; these cases did not show any useful hybridisation signal even after prebleaching with 5% hydrogen peroxide according to the method described in chapter 2. In the positive cases, as with the FISH technique, there was no significant difference between the signal for FF tissues when compared with the corresponding GF tissues. All the controls showed good signal with CISH for both chromosomes.

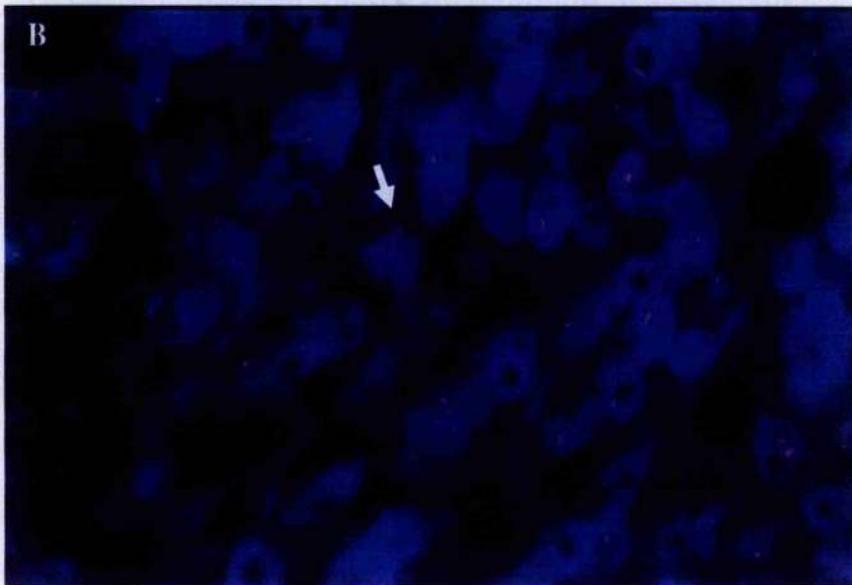
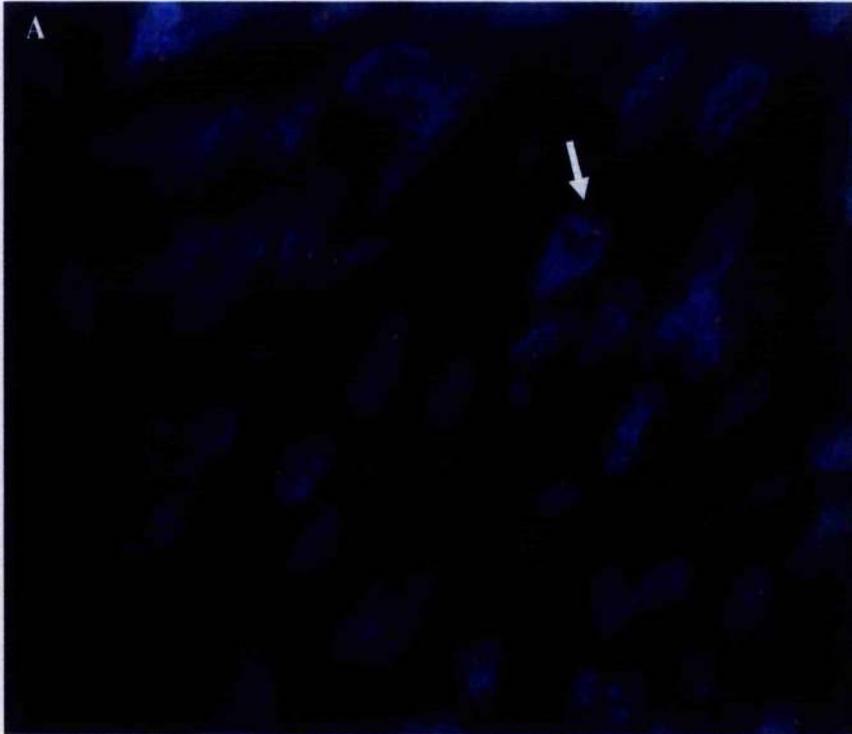


Figure 3.2 Fluorescence *in situ* hybridisation.

A: Gluteraldehyde fixed choroidal melanoma, hybridised with chromosome 3, showing one copy (arrow) in most cells. B: Formalin fixed choroidal melanoma, hybridised with chromosome 3, showing one copy (arrow) in most cells.

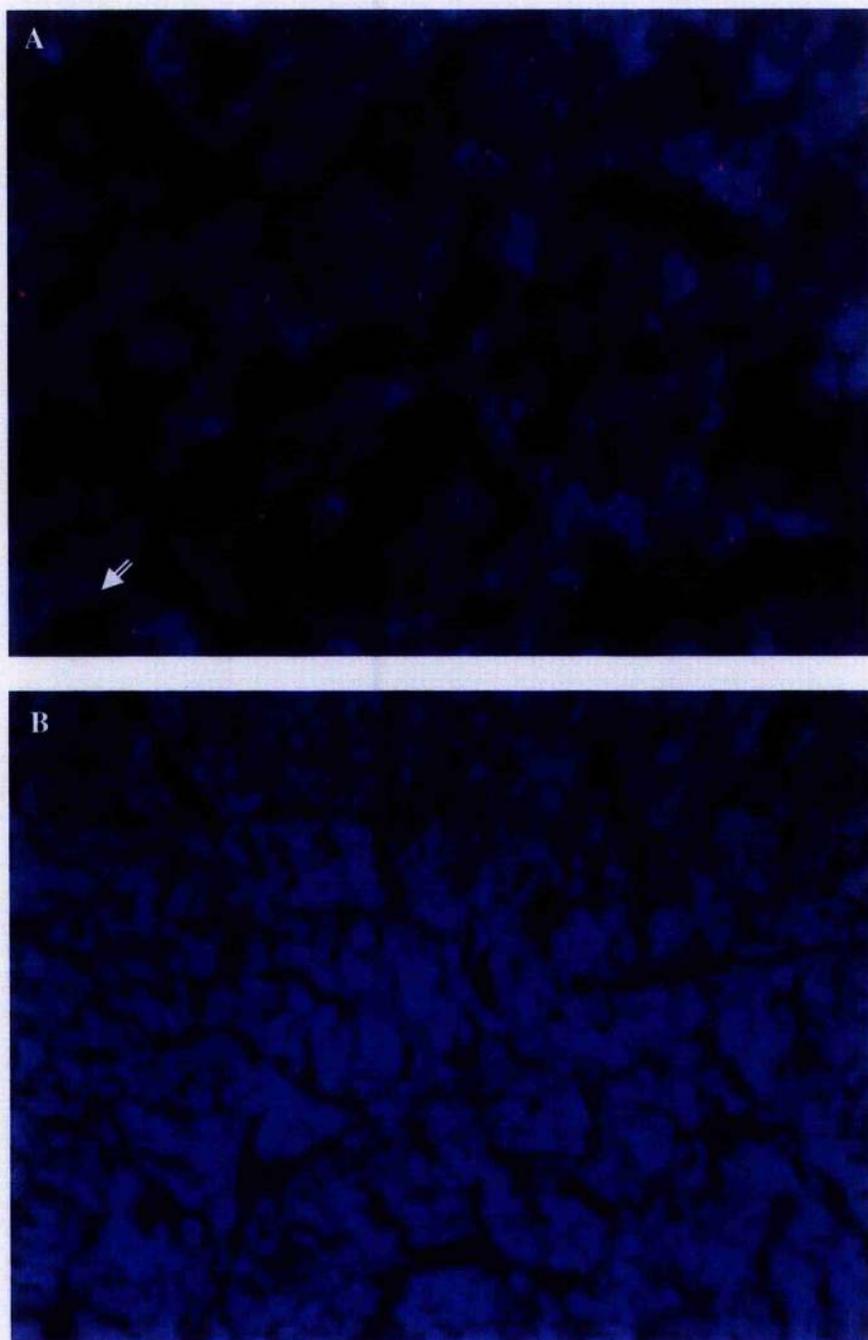


Figure 3.3 Fluorescence *in situ* hybridisation.

A: Gluteraldehyde fixed retina (internal control) showing two copies of chromosome 3 (arrow) in most cells. B: Same specimen after FISH, with negative signal for chromosome 3 in most tumour cells.

3.5 Discussion

Fluorescence *in situ* hybridisation has increased in importance over the past decade as a powerful method for detecting DNA target sequences in cells from a wide variety of sources, including formalin fixed paraffin wax embedded tumour samples (McKay *et al.*, 1997). Analysis of interphase nuclei from archival paraffin blocks eliminates the need for fresh tissue, allowing comparison of chromosomal anomalies with other prognostic factors.

Although the use of FISH in cytogenetics has improved the knowledge of chromosomal abnormalities in various pathological conditions, FISH has limitations when applied to archival paraffin embedded tissue samples. One limitation is the masking of relatively weak hybridisation signals by high levels of background autofluorescence (McKay *et al.*, 1997). The background staining depends in part on the tissue endogenous enzyme activity and if this is not blocked it can lead to unacceptable background labelling.

Formamide has been used to regulate stringency (an indicator of the approximate percentage of nucleotides that are correctly matched in the probe) and therefore reduce background staining but when formamide washes were incorporated into the protocol used in this study, they did not improve the quality of the hybridisation signals. Similarly the additional step utilised with the directly labelled probes has been shown to reduce background. Furthermore the directly labelled probes were easier to detect. In this study the directly labelled probes resulted in better hybridisation signals compared with the indirectly labelled probes.

Another limitation in using FISH is related to the fixative used, which limits probe penetration. Most paraffin wax embedded tissues are nowadays fixed in formalin which results in the formation of methylene bridges between amino groups that reduce the penetration of nucleic acid probes (Chin *et al.*, 2003). Gluteraldehyde cross-links proteins more extensively, preventing probe penetration and requiring stronger unmasking procedures (Uehara *et al.*, 1993). Despite the different methods attempted in order to improve the quality of the signal, including commercial kits designed for use on fixed material obtaining consistent good quality signal in GF tissues remains a challenge.

FISH is therefore technically demanding when applied to archival material, particularly when fixed in gluteraldehyde. This technique has been applied to fresh tissue and slides prepared from disaggregated tumour cells usually fixed in formalin. However applying FISH on fresh material and disaggregated cells results in loss of histological architecture and potential sample contamination with non-tumour cells (Qian *et al.*, 1996). FISH analysis of paraffin-embedded specimens has been applied to other solid tumours in several different studies (Tanner *et al.*, 2000, Qian *et al.*, 1996, Matsuta *et al.*, 1994, Chin *et al.*, 2003) but has not been reported in any previous study on sections of paraffin-embedded choroidal melanomas.

This study has shown that although FISH can be applied to paraffin embedded choroidal melanoma including GF specimens the results are inconsistent and appear to offer few advantages over CISH in the study of archival tissue. Following the results of this study the CISH technique has been used in the remaining studies described in this thesis.

**4 MONOSOMY 3 PREDICTS DEATH BUT
NOT TIME TO DEATH IN CHOROIDAL
MELANOMA**

4.1 Background

The overall mortality from uveal melanoma is approximately 50% due to the development of liver metastases. These metastases are not usually evident when the primary tumour is treated and much current research has been conducted to identify reliable prognostic markers in primary uveal melanoma for early detection of high-risk patients whom prophylactic treatment would be justified. Specifically, monosomy 3 has been recognised to have a stronger association with a poor prognosis than do clinical and histological parameters and is in some series associated with a reduction in the 5-year survival time from almost 100% to only 30% (Prescher *et al.*, 1996). It is generally accepted that the peak mortality from metastatic disease from choroidal melanoma occurs within three years of diagnosis (Diener-West *et al.*, 1992). However the clinical course of patients with uveal melanoma is unpredictable and a significant number of deaths still occur after 5, 10 and 15 years. The occurrence of very late metastases up to 36 years after initial surgery has also been described (Newton, 1965). To date the role of monosomy 3 in predicting time until death has not been assessed.

4.2 Specific Objectives

- 1 To confirm the prognostic value of monosomy 3 in predicting death caused by metastases
2. To assess whether the presence of monosomy 3 can be predicted from tumour histology.

3. To assess whether monosomy 3 is related to time until death caused by metastases and whether life expectancy can be predicted in patients after surgical excision of a melanoma displaying monosomy 3.

4.3 Materials and Methods

4.3.1 Study population

A total of 111 samples were studied; these were archival specimens from patients diagnosed with choroidal melanoma and obtained from Glasgow Western Infirmary Pathology files. They included sixty enucleations, 50 local resections (LRs) and 1 exenteration. The patients were divided into two groups: group 1 (patients who had died from metastatic disease) and group 2 (patients alive or who had died from other causes after a minimum follow up period of 1 year-mean 16.85 years; range 1 to 30 years). There were 71 cases in group 1, metastasising melanoma (MM), and 40 cases in group 2, non-metastasising melanoma (NMM). Follow up time was recorded from date of treatment to death or to last follow up. The surgery was performed between 1974 and 1992 in patients with MM and between 1973 and 1991 in patients with NMM. Patients with MM were identified either from the cancer registry or case notes as having proven liver metastases either by imaging, biopsy or post mortem examination. Patients with NMM were both alive and well or had a cause of death other than metastatic melanoma and no evidence of metastatic disease at last follow up. All tissues had been previously fixed in gluteraldehyde or formalin and embedded in paraffin wax.

4.3.2 Clinical features

Clinical details were obtained from cases notes and the cancer registry. The details sought included age, sex, treatment of tumor, survival status and cause of death. The time to death was calculated from the date of local resection (LR) or enucleation. Survival status was determined up to August 2003. After obtaining these clinical details all samples were anonymised.

4.3.3 Histological features

Sections (4 μm) were cut and stained with haematoxylin and eosin (H&E) and PAS without counterstain for light microscopy. The following histopathological parameters were assessed; cell type, extent of necrosis, maximum tumour dimension, extraocular extension, closed vascular loops, pigmentation, mitotic activity, retinal detachment and lymphocytic infiltration. The cell type was assessed according to the modified Callender classification (spindle, mixed, epithelioid) from a H&E stained section (McLean *et al.*, 1983). The presence of other cell types including balloon cells was also recorded. Pigmentation was described as amelanotic to low, intermediate, and strong pigmentation as defined in the COMS study (COMS, 1998 a). The largest tumour dimension was recorded and the tumour classified as small, if less than 11mm; medium, 11 to 15 mm; and larger, greater than 15 mm. The extent of invasion was assessed as follows; rupture of Bruch's membrane, intrascleral channels (ISI), extraocular extension (EOX), optic nerve and orbital (noted at surgery). Information on ocular invasion was also obtained from the original pathology report.

Closed vascular loops were assessed in PAS stained sections with the aid of a green filter as described by Folberg *et al.* (Folberg *et al.*, 1993) and Foss *et al.* (Foss *et al.*, 1997). The number of mitotic figures per 40 high power field was counted. The extent of retinal detachment was graded into one of three categories: 1. no RD; 2. focal RD: detachment adjacent to tumour (detachment of neurosensory retina over and around the melanoma) or on the same side of tumour but not adjacent and 3. total RD: detachment of neurosensory retina with three to four quadrants of the retina involved. The exenteration specimen and the cases of local excision could not be assessed for RD. Semi-quantitative assessment of associated lymphocytic infiltration was as follows: none or minimal if there were none or only very few foci of inflammation; moderate if multiple foci were present and heavy if almost the entire tumour is involved.

4.3.4 Chromosome *in situ* hybridisation

The number of copies of chromosome 3 was assessed by CISH as described in chapter 2. Chromosome 18 was again used as a control chromosome. In 20 enucleation specimens normal retina was used as an internal control. In addition, 19 cases of normal human skin (all GF) were included as an external control.

4.3.5 Statistical analysis

The relationship between metastasising melanoma and age, mitotic activity, tumour size and pigmentation was assessed by the Wilcoxon's rank sum test. For the other

prognostic variables (monosomy 3, sex, cell type, intrascleral and extraocular spread, lymphocytic infiltration, presence of vascular loops, necrosis and RD), associations were assessed by Fisher's exact test. The p value was corrected for the multiplicity of tests by the Holm's method (Holm, 1979, Aickin and Gensler, 1996). The Holm's method ensures that there is no false conclusion, compensating for the multiplicity of statistical tests. Multivariate analysis of several risk factors was based on a stepwise discriminant analysis, as implemented in the SPSS software package (SPSS Inc., Chicago, Illinois 60606). Survival time for a patient after surgical excision of a melanoma with monosomy 3 was compared with those balanced for chromosome 3 using the two-sample t-test. Life expectancy for patients with tumours displaying monosomy 3 was predicted using a survival curve corrected for the patient's age.

4.4 Results

4.4.1 Clinical and histological features associated with metastasising melanoma

The mean age of patients with MM was 57.8 years and 52.8 years for the NMM group (range 18-80 and 18-78, respectively). Patients with MM were slightly older than those with NMM but this difference was not statistically significant ($p = 0.16$ by the Wilcoxon Rank Sum Test). The male/female distribution was similar in both groups; group 1 (MM 1/1.03) and group 2 (NMM 1.11/1). There was no significant difference in the form of treatment between the two groups; group one included 37 enucleations, 33 local resections and one exenteration and group two, 23 enucleations and 17 local resections. The clinical-pathological features of MM and NMM melanoma are summarised in table 4.1 and the results of the univariate

statistical analysis are shown in table 4.2. Using the more stringent Holm-adjusted p value the factors that were significantly associated with metastases were mitotic activity, lymphocytic infiltration, presence of epithelioid cells, absence of balloon cells, presence of vascular loops, intrascleral or extraocular extension, tumour size and monosomy 3 (Table 4.2).

Clinical or histological factor	Statistical method used to test for association with metastases	p value	Holm-adjusted p value
Age	Wilcoxon's rank sum test	0.16	0.48
Sex	Fisher's exact test	0.769	1.00
Retinal detachment	Fisher's exact test	0.076	0.304
Presence of cystic spaces	Fisher's exact test	0.85	1.000
Pigmentation of tumour	Wilcoxon's rank sum test	0.042	0.210
Mitoses	Wilcoxon's rank sum test	< 0.001	< 0.01
Lymphocytes in tumour	Fisher's exact test	0.000004	0.00005
Epithelioid cells	Fisher's exact test	< 10 ⁻¹⁰	< 1.3 × 10 ⁻⁹
Balloon cells	Fisher's exact test	0.003	0.024
Loops	Fisher's exact test	0.0002	0.0022
Necrosis	Fisher's exact test	0.021	0.126
Invasion of surrounding tissue	Fisher's exact test	0.00647	0.0453
Size of tumour	Wilcoxon's rank sum test	< 0.001	< 0.01
Missing chromosome 3	Fisher's exact test	5.38 × 10 ⁻¹⁴	7.5 × 10 ⁻¹³

Table 4.1 Clinical and histological factors tested for association with metastases.

	<i>Metastasising Melanoma</i> <i>n=71</i>			<i>Non-metastasising Melanoma</i> <i>n=40</i>		
<i>Age(mean)</i>	57.8 years			52.8 years		
<i>Gender</i>	Male 36		Female 35	Male 19		Female 21
<i>Treatment</i>	Enucleation 37	LR 33	Exenteration 1	Enucleation 23		LR 17
<i>Chromosome 3</i>	1 Copy 47		2 Copies 24	1 copy 0		2 Copies 40
<i>Chromosome18</i>	1 Copy 0		2 Copies 71	1 copy 0		2 Copies 40
<i>Cell Type</i>	Epithelioid present 57		Spindle 14	Epithelioid present 7		Spindle 33
<i>Balloon Cells</i>	Yes 7		No 64	Yes 13		No 27
<i>Necrosis</i>	Yes 23		No 48	Yes 3		No 37
<i>Pigment</i>	None-low 36	Moderate 25	Heavy 8	Low-low 27	Moderate 12	Heavy 1
<i>Lymphocytes</i>	None-low 59	Moderate 8	Heavy 4	None-low 38	Moderate 2	Heavy 0
<i>Invasion</i>	No 32	ISI 21	EOX 18	No 23	ISI 14	EOX 2
<i>Mitoses</i>	<5 53	≥5 15	>10 3	<5 38	≥5 1	>10 1
<i>Loops</i>	Yes 39		No 32	Yes 8		No 32
<i>Size</i>	<11mm 8	11-15mm 16	≥15mm 47	<11mm 10	11-15mm 15	≥15mm 15
<i>Retina Detachment</i>	No 0	Focal 11	Total 26	No 1	Focal 6	Total 9

Table 4.2 Clinical and histological parameters of metastasising and non-metastasising choroidal melanomas.

Multivariate analysis was then performed to determine which of these interrelated variables were independently related to outcome. The following factors (in their order of satisfying the criterion for addition to the model) were significantly associated ($p < 0.0001$) with metastasising melanoma: monosomy 3, presence of epithelioid cells, lymphocytic infiltration and tumour size $> 15\text{mm}$. From a knowledge of these four factors, 89% of the tumours could be correctly predicted to be from group 1 (MM) or group 2 (NMM).

4.4.2 Association of monosomy 3 with metastasising melanoma

Monosomy 3 was detected in 47 of the 71 cases of MM (66.1%) and was significantly associated with metastases-related death ($p = 0.0001$). The remaining 24 MM and all 40 NMM had two copies of chromosome 3 and therefore defined as balanced.

4.4.3 Prediction of monosomy 3 from clinical and histological features

Multivariate discriminant analysis of the histological features of both MM and NMM indicated that the presence of epithelioid cells and vascular loops were statistically significant predictors of monosomy 3, when considered together. Both satisfied the confidence criterion $p < 0.0001$. The discriminant function derived gave the correct prediction of chromosome number in 70% of cases.

4.4.4 Prediction of survival time in tumours with monosomy 3

Among the 71 patients in group 1 (MM), 47 displayed monosomy 3, with a mean survival time of 4.31 years \pm 2.83 years (SD). The remaining 24 cases with 2 copies of chromosome 3 had a mean survival time of 4.21 \pm 3.34 years. There was no significant difference in time until death of those with tumours with monosomy 3 compared with those with 2 copies of chromosome 3 in this group ($p = 0.90$) (Figure 4.1).

4.4.5 Prediction of life expectancy of patients after excision of a melanoma displaying monosomy 3

All 47 patients with melanomas displaying monosomy 3 had died. The mean survival time was 4.3 years (median, 3.5). The survival curve, corrected for the patient's age at the time of surgery and based on the assumption of a linear relationship, suggests that very long term survival with monosomy 3 is rare (Figure 4.2).

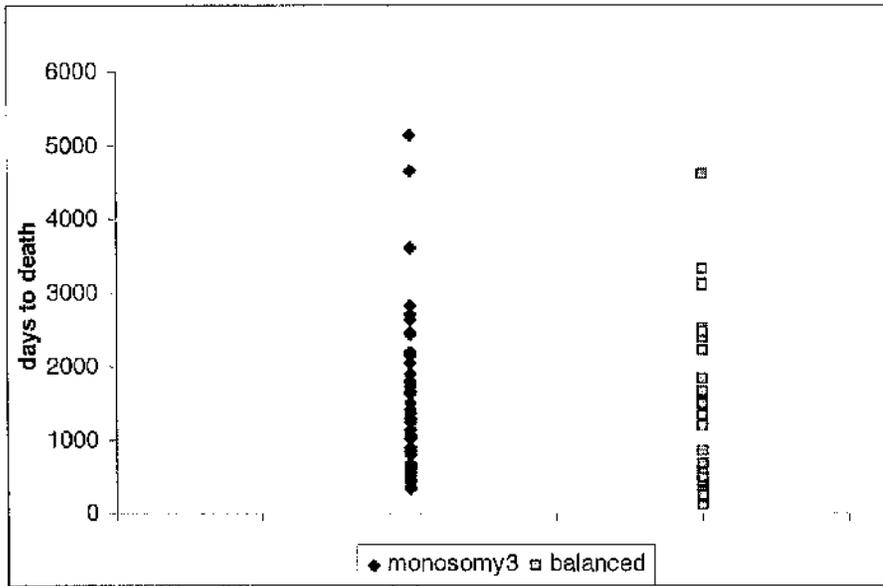


Figure 4.1 Time from surgery to death of patients with metastasising melanoma.

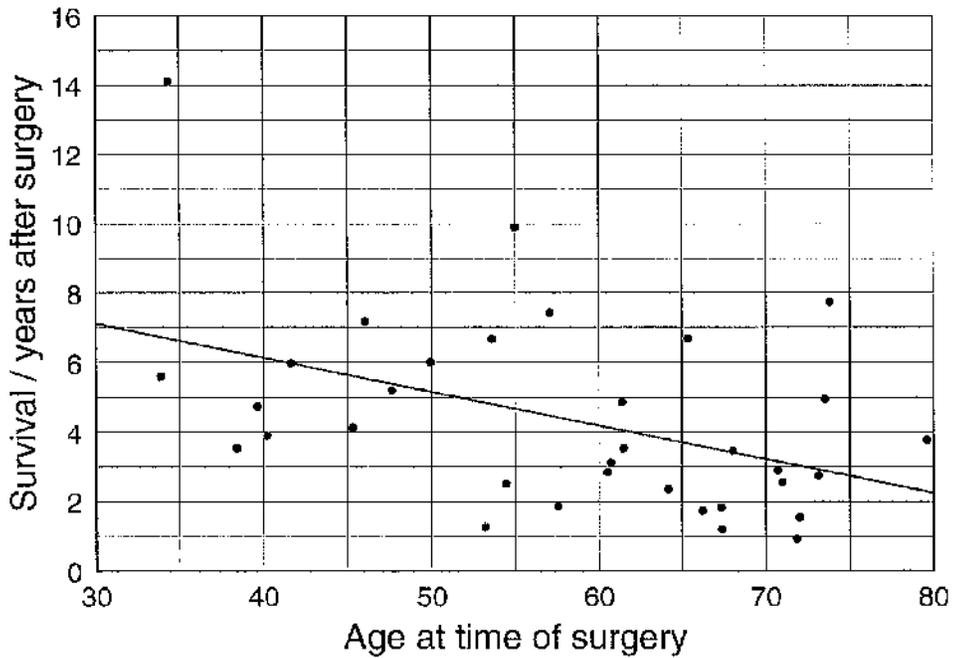


Figure 4.2 Survival plot corrected for the patient's age at time of surgery.

4.5 Discussion

Monosomy 3 has been shown by many investigators to be associated with death from metastases after treatment of uveal melanoma (White *et al.*, 1998, Sisley *et al.*, 1997, Sisley *et al.*, 2000, Scholes *et al.*, 2001, Scholes *et al.*, 2003, Prescher *et al.*, 1996, Patel *et al.*, 2001, Naus *et al.*, 2002, Aalto *et al.*, 2001). Using the technique of chromosome *in situ* hybridisation described in chapter 2, and applying the same cut off points to this larger series, the findings of these previous studies has been confirmed. In this study monosomy 3 was identified in 66.1% of melanomas that caused death from metastases. Previous studies have identified monosomy 3 in 50 to 73% of choroidal melanomas (Aalto *et al.*, 2001, Scholes *et al.*, 2001, Patel *et al.*, 2001, Scholes *et al.*, 2003, Prescher *et al.*, 1996, Sisley *et al.*, 1997). It is not possible however to directly compare these values, as unlike some of these studies the data set was selected on the basis of death or survival from uveal melanoma and is therefore not considered to be as representative of all cases of choroidal melanoma.

Although monosomy 3 was confirmed as a significant predictor of metastases-related death, a second smaller but significant group of patients who died of metastatic melanoma even though their tumours contained 2 copies of chromosome 3 was identified. There are several possible explanations for this. First, this is a retrospective study that includes samples from the 1970s. Samples may therefore have been included from patients who would now be treated without surgery. Without tissue samples, patients with similar tumours could not be included in other studies. These differences may also be related to the CISH technique used in this investigation, since the probes are centromere specific, and regional losses of chromosome 3 would not be detected. However in previous studies in which other

techniques were used, including microsatellite analysis and comparative genomic hybridisation, investigators found that in most cases the entire copy of chromosome 3 is lost and that regional loss occurs in only a minority. (Tschentscher *et al.*, 2001, Prescher *et al.*, 1996, Sisley *et al.*, 1997, White *et al.*, 1998, Scholes *et al.*, 2001). Furthermore, the prognostic significance of these regional losses is unclear. Scholes *et al.* (Scholes *et al.*, 2003) identified loss of heterozygosity of chromosome 3 in 60 cases; of these, 6 cases showed only regional losses. Five of these six patients were alive at the end of the 4-year study period, suggesting that these partial deletions may not carry the prognostic significance of complete loss of heterozygosity. It is also possible that this technique has failed to identify a small subclone of cells, within the tumor, displaying monosomy 3. However the presence of subclones of cells is more likely to be identified using CISH compared with standard cytogenetics, as a whole histological section of the tumour is studied and not just selected cells that have been successfully cultured and identified in a metaphase spread. Finally, and probably most likely, there may be other alterations that are important in the development of metastases in choroidal melanoma. These may include other as yet unidentified cytogenetic abnormalities or may be related to alterations in tumour biology that allow invasion and metastases. The metastatic cascade involves a series of events including detachment of tumour cells from each other followed by both attachment to and degradation of the extracellular matrix, thus allowing migration of tumour cells. As mentioned above, an increase in the risk of metastases has been shown with loss of expression of intercellular cell adhesion molecule-1 (Anastassiou *et al.*, 2000) and conversely with expression of $\alpha 1$ - and $\alpha 4$ -integrins (Woodward *et al.*, 2005). MMPs may also play a role in degrading the extracellular matrix, particularly with increased expression of matrix metalloproteinase-2 (Väisänen *et al.*, 1999). The

molecular mechanisms that lead to the metastatic phenotype in choroidal melanoma are not fully understood but may be related to isolated point mutations in relevant genes or their promoters, so that they would not be detected in chromosomal studies.

In this retrospective study, there were 71 patients who died from metastases, with time from surgery to death ranging from 4 months to 14 years; two thirds of these had tumours displaying monosomy 3 and a mean survival time of 4.31 years; the remaining third were apparently balanced for chromosome 3 and had a mean survival time of 4.21 years. There was no significant difference in time to death between the two groups (Figure 4.1). This study has also shown that survival beyond 10 years is possible but rarely occurs in patients with a tumour displaying monosomy 3. Because some of the patients were identified from a database in which they were only included after death, it is possible that very long-term survivors may be underrepresented in this data, with consequent underestimation of survival time. However using a survival curve to predict life expectancy of these patients suggests that very long-term survival with monosomy 3 is probably rare, and as such this bias will be small.

In this study the presence of monosomy 3 could be partly predicted from tumour histology. Specifically, the presence of epithelioid cells ($p < 0.001$) and closed vascular loops ($p < 0.001$) were significantly associated with monosomy 3 when each characteristic was considered individually. When these two histological characteristics were included in a multivariate predictive model, the predictions were correct in 70% of cases. Scholes *et al.* (Scholes *et al.*, 2001) also showed a statistical association between monosomy 3, closed vascular loops and epithelioid cells

however using a forward stepwise logistic regression model they showed that monosomy 3 could only be reliably predicted in patients with large epithelioid tumors (Scholes *et al.*, 2003). In this study large tumor size (>15mm) was not a significant predictor for monosomy 3. The distribution of tumor sizes was similar in this study to that of Scholes *et al.* (Scholes *et al.*, 2003) and it is therefore unlikely to represent sample selection. Other researchers have shown no association of monosomy 3 with histopathological features (Prescher *et al.*, 1996, Sisley *et al.*, 1997, White *et al.*, 1998).

In this study, in the univariate model analysis, infiltration of the tumour by lymphocytes was also significantly associated with metastases ($p = 0.000004$ by Fisher's exact test), which is in keeping with previous research (de la Cruz *et al.*, 1990). In the multivariate analysis, the presence of lymphocytic infiltration ranked fourth as a predictor for survival, when compared with presence of monosomy 3, epithelioid cell type and large size tumours. Conversely, lymphocytic infiltration in the COMS study did not appear to be an independent prognostic factor. This study also showed that spindle cell type tumours had the least inflammation and epithelioid cell tumours the greatest degree of inflammation (COMS, 1998 a). In this series, considering that cell type and tumour size were dominant factors in patient survival, it would be reasonable to assume that the majority of the poor prognosis for tumours with lymphocytic infiltration should be the result of associations with cell type and tumour size.

According to Kivelä *et al.* tumour size and microvascular loops are strong predictors of exudative RD (Kivelä *et al.*, 2001). In this study, RD was not convincingly related

to any of the other tumour characteristics; specifically tumour size and vascular loops. This study was also limited by the fact that in more than 50% of the cases retina wasn't available because LRs were carried out; for this reason RD was excluded from the stepwise analysis to predict monosomy 3 from the histology.

In conclusion, this study confirms that monosomy 3 in choroidal melanoma is a significant predictor of metastases related death. However death from metastatic melanoma occurs in a significant number of cases that do not display monosomy 3, suggesting that other molecular events are important in the metastatic cascade in these patients. Furthermore, this emphasises a cautious approach to the use of cytogenetic studies for patient counselling. In this study the presence of monosomy 3 could be correctly predicted in up to 70% of cases by the presence of an epithelioid cell component and vascular loops, but it was not related to tumour size. In patients with metastases, there was no difference in time until death in those with tumours displaying monosomy 3 compared with those tumours that had two copies of chromosome 3. However as with all MMs, life expectancy in patients with tumours displaying monosomy 3 is generally short.

**5 CORRELATION OF HETEROGENEITY
FOR CHROMOSOME 3 COPY NUMBER
WITH CELL TYPE IN CHOROICAL
MELANOMA OF MIXED CELL TYPE**

5.1 Background

The majority of previous cytogenetic studies have considered uveal melanoma as a homogeneous entity utilizing DNA extracted from whole tumours or performing classical cytogenetics performed on selected samples of tumour cells. However morphological heterogeneity is well recognised in uveal melanoma. The majority of tumours are composed of variable proportions of epithelioid and spindle-shaped cells (COMS, 1998 a). There have been few studies of cytogenetic heterogeneity in uveal melanoma. Monosomy 3 has been associated with a reduction in the 5-year survival from 100% to 30% due to metastatic death (Prescher *et al.*, 1996). Therefore, cytogenetic heterogeneity carries significant clinical implications for the sampling of tumours for cytogenetic studies.

5.2 Specific Objectives

1. To evaluate the presence of cytogenetic heterogeneity for chromosome 3 in choroidal melanomas with morphologically distinct spindle and epithelioid cell types using the technique of chromosome *in situ* hybridisation on archival paraffin embedded tissue.

5.3 Materials and Methods

5.3.1 Study population

Sixty four cases of choroidal malignant melanoma of mixed cell type (modified Callender system) (McLean *et al.*, 1983) were identified from the eye pathology files, Western Infirmary, Glasgow, between the years 1975 and 2002. From these 64 tumours the cases where the populations of spindle and epithelioid cells were relatively discrete were identified. All tissues had been previously fixed in gluteraldehyde or formalin and embedded in paraffin wax.

5.3.2 Survival status

The survival status of the patients and cause of death were obtained from cases notes and the cancer registry. The time to death was calculated from the date of treatment, and survival status was determined up to August 2003.

5.3.3 Chromosome *in situ* hybridisation

The number of copies of chromosome 3 was assessed by CISH using chromosome-specific centromeric probes, as previously described. Chromosome 18 was the control chromosome. Copy number of chromosome 3 was assessed in both spindle and epithelioid areas. A minimum of 200 nuclei were counted in both spindle and epithelioid areas. Chromosome number was assessed using both chromosome index and signal distribution as described above. Briefly, CI gives an average chromosome

copy number and is calculated by dividing the number of hybridisation sites by the number of nuclei counted. Chromosome loss is defined as a CI less than 3 standard deviations from the mean for retina (normal tissue). The SD is defined as the percentage of nuclei with only one hybridisation site. A signal distribution of more than 60% was the cut off point used to define chromosome loss. The tumour regions had to show chromosome loss by both CI and SD to be regarded as monosomic. The cut-off points were selected in accordance with our laboratory's previous experience of the technique.

5.3.4 Statistical Analysis

The relationship of pattern of monosomy 3 distribution with tumour diameter, age and survival was tested by pairwise t-test and pairwise rank sum test.

5.4 Results

5.4.1 Study population

Twenty-two suitable cases were identified from the original group of 64 mixed choroidal melanomas. Nineteen of the cases were from patients who had died from metastatic melanoma and 3 were from patients alive or dead from other causes.

5.4.2 Chromosome 3 copy number in spindle and epithelioid areas

The tumours separated into 3 groups based on the copy number of chromosome 3 in spindle and epithelioid areas. Ten (45%) melanomas displayed two copies of chromosome 3 in both spindle and epithelioid cells and were defined as balanced for chromosome 3 (BB). This included the 3 cases where the patients were alive or dead from causes other than metastases. Five (23%) displayed monosomy 3 in both spindle and epithelioid cell areas (M3M3). In the remaining 7 cases (32%) the epithelioid areas displayed monosomy 3 but the spindle cell areas contained 2 copies of chromosome 3 (M3B) (Figure 5.1).

5.4.3 Statistical analysis

The pattern of monosomy 3 (BB, M3M3, M3B) was not significantly related to clinical features of the patients such as tumour size, age at surgery or time to death.

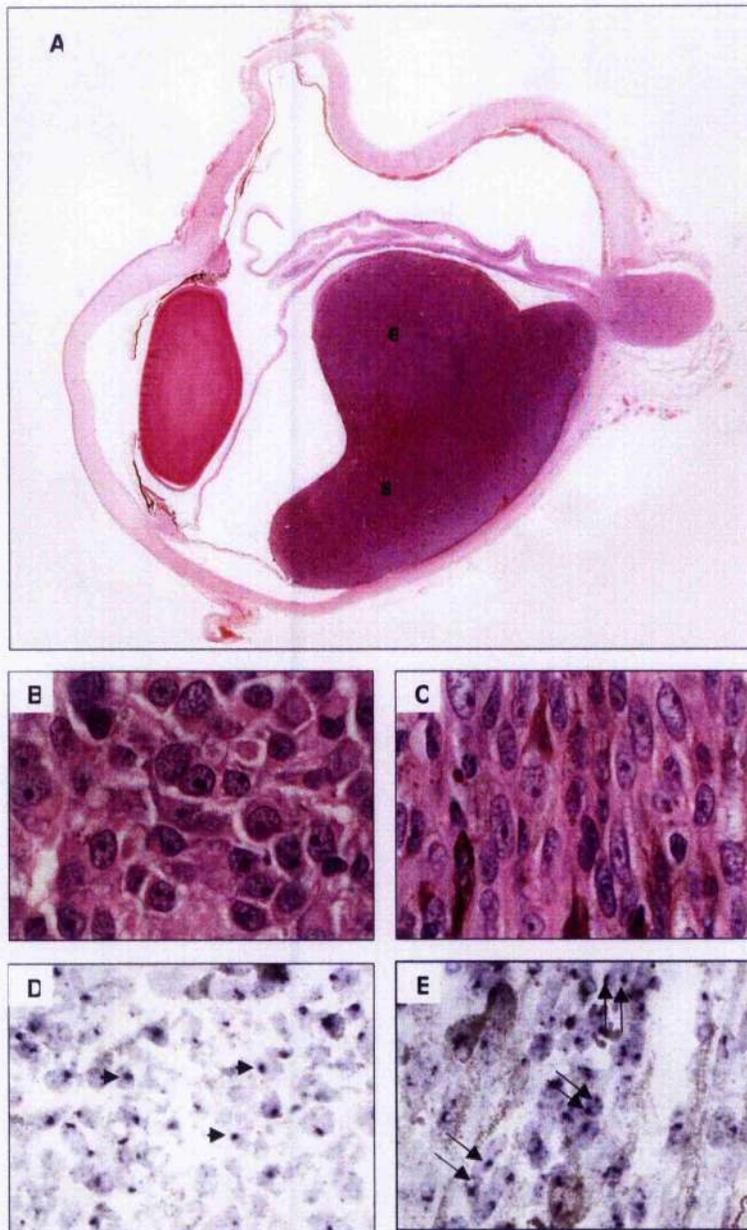


Figure 5.1 Heterogeneity for chromosome 3 in a mixed cell tumour.

A: Large choroidal melanoma with poorly pigmented epithelioid nodule (e) and more heavily pigmented spindle cell area (s) (H & E,x1). B & C: High power view from the epithelioid and spindle areas, respectively (both H & E, x400). D: Chromosome *in situ* hybridisation for chromosome 3 in epithelioid cells. The round cell morphology is still evident and the majority of cells show only one hybridisation site (single arrows) (Chromosome 3, x400), E: Chromosome *in situ* hybridisation for chromosome 3 in spindle cells. The spindle cell morphology is still evident and the majority of cells show two hybridisation sites (double arrows) (chromosome 3, x400).

5.5 Discussion

Monosomy 3 in choroidal melanoma is a significant predictor of metastases related death and has been associated with a reduction in the 5-year survival from 100% to 30% (Prescher *et al.*, 1996). Although monosomy 3 is an important predictor of metastatic death we have previously shown that there is also a small but significant number of people with metastasising melanoma whose tumours are balanced for chromosome 3. One possible explanation for our cases of metastasising melanoma without monosomy 3 is the presence of genetic heterogeneity. Morphological heterogeneity of choroidal melanoma is well recognised with the majority of cases being of mixed cell type (Mooy and De Jong, 1996, COMS, 1998 a). The objective of this study was to evaluate corresponding loss of heterozygosity of chromosome 3 in areas of spindle and epithelioid cell type using CISH. In 23% of all melanomas, monosomy 3 was identified in both spindle and epithelioid cell areas whereas in 32% it was present only in the epithelioid cell areas. Forty five percent of melanomas were balanced in both spindle and epithelioid areas. This latter group included 7 cases of metastasising melanoma. Therefore, cytogenetic heterogeneity for chromosome 3 does not explain metastases in cases balanced for chromosome 3 in both spindle and epithelioid areas. White *et al.* have previously reported a case of clonal heterogeneity in a uveal melanoma. In this case the tumour was found to have distinct pigmented and non-pigmented areas on gross examination (White *et al.*, 1998). Tissue samples were collected from both of these areas for standard cytogenetics. Histological examination revealed small regular epithelioid cells in the pigmented area and large pleomorphic epithelioid cells in the non-pigmented area. Standard cytogenetic analysis showed 2 copies of chromosome 3 in the pigmented area compared with monosomy 3 in the non-pigmented area. It is notable that gross

differences in pigmentation are not frequently present to alert the sampler to morphological and cytogenetic heterogeneity when cells are to be cultured for standard cytogenetics. Techniques that involve extraction of DNA from tissue sections should theoretically represent the tumour cell population more accurately (Aalto *et al.*, 2001, Tsechentscher *et al.*, 2001, Scholes *et al.*, 2001, Scholes *et al.*, 2003, Parrella *et al.*, 1999). However some subclones or even contaminating normal DNA may be preferentially amplified during polymerase chain reaction. The CISH technique differs from other technique used to assess monosomy 3 because chromosomal losses are assessed in both interphase and metaphase nuclei within a population of cells in a tissue section. This allows the direct correlation of genotype with phenotype.

In this study I selected tumours with discrete populations of spindle and epithelioid cells to aid counting of the different areas. The CISH technique can only be applied to a defined population of tumour cells and the assessment of chromosomal loss cannot be made on individual tumour cells. These 22 cases were selected from 64 choroidal melanomas of mixed cell type. This supports the impression that such morphological subclones are not uncommon. However since this does not represent our entire archive there may be an element of selection bias. In the other 42 tumours there were insufficiently large areas of each cell type to allow accurate counting.

Although the number of cases studied was small we have demonstrated heterogeneity for chromosome 3 copy number in 7 of 22 (32%) cases. This has important implications for other methods of cytogenetic analysis such as short-term culture for metaphase spreads, since often only a sample of tumour is submitted for analysis. For

example, a small biopsy of the tumour may be taken before submitting the remaining tumour for histopathological examination (Sisley *et al.*, 1997, Horsman and White, 1993).

Since the importance of cytogenetic assessment of uveal melanoma will increase as new therapies become available tumours may be biopsied by FNA in order to obtain cytogenetic information prior to treatment with modalities other than surgery (Damato, 2004). The possibility of morphologically non-representative material in FNA has already been reported by Folberg *et al.* in a study comparing average nucleolar area in FNA with enucleation specimens of uveal melanoma (Folberg *et al.*, 1985). Similarly Augsburger *et al.* demonstrated a needle track that just missed an epithelioid region in a tumour removed after FNA (Augsburger *et al.*, 1985). Based on the results of this study the confidence attributed to any prognostic assessment undertaken on a small sample of tumour would be greater if a morphological assessment showed the tissue sampled to contain epithelioid cells.

In conclusion, mixed choroidal melanomas with discrete spindle and epithelioid cell populations may display heterogeneity for chromosome 3 copy number that correlates with populations of different cell type. CISH is a useful technique to identify this clonal heterogeneity in excision specimens. However the genetic information obtained from small samples using other techniques such as classical cytogenetics may not be representative. This in turn will affect the degree of certainty in patient counselling and potentially patient selection for novel treatments.

6 FINAL DISCUSSION

6.1 Summary

This thesis describes the adaptation of the technique of chromosome *in situ* hybridisation to assess chromosome 3 copy number in choroidal melanoma, and the utilisation of this to study the presence of monosomy 3 in an archival series. The findings were interpreted in conjunction with histological features, survival and time to death. This *in-situ* method of assessing chromosomal changes in tumours has both advantages and disadvantages compared with other techniques, and these studies utilising CISH have provided additional information on chromosome 3 changes in choroidal melanoma.

The main advantage of CISH is that it is applicable to archival material. This is important for the study of tumours such as choroidal melanoma. Choroidal melanoma is a relatively rare tumour so retrospective studies are likely to include larger numbers than prospective studies. In addition, the majority of tumours are currently dealt with using vision sparing techniques such that tissue is not often available for cytogenetic analysis. Therefore study of archival tissue may give a better cytogenetic picture of choroidal melanoma as a whole.

Choroidal melanoma is renowned for the occurrence of very late metastases which may take many years to reveal themselves and these late metastasising tumours may not be recognised in prospective studies with only a 3 to 5 year follow up. Another advantage of CISH is that it allows the direct correlation of chromosomal changes with histology. This is helpful for choroidal melanoma where there are clearly defined cell types (spindle and epithelioid) which have been shown in numerous previous studies to correlate with prognosis.

CISH does, however, presents some limitations. The adaptation of CISH for choroidal melanoma, which in archival tissue is often gluteraldehyde fixed, has required relatively rigorous pretreatments. Despite pre-bleaching I was unable to obtain adequate signal in some heavily pigmented tumours. The counting technique is time consuming and requires a certain amount of experience. In addition to these practical problems, and contrary to some other techniques, fine mapping of changes or characterisation of specific tumour suppressor genes that may be involved cannot be achieved with CISH.

CISH is only applicable to a defined tumour population and not to individual tumour cells. This is because of the effects of nuclear truncation which explains why some cells will contain only 1 spot even if they are disomic. Other cells will appear polysomic during mitoses. Nonetheless the advantages of CISH allow the assessment of monosomy 3 in choroidal melanoma from a different perspective to that previously cited in the literature.

Having developed the technique of CISH for archival choroidal melanoma, I attempted the technique of FISH to assess if this would offer any advantages over CISH. Theoretically, FISH has the advantage of allowing the use of multiple fluorochromes and therefore the detection of more than one chromosome in one tissue section. However despite rigorous pretreatments I was not able to obtain consistent results with FISH. This can be explained by the many recognised limitations of FISH when applied to archival paraffin tissue samples. It is well recognised that poor probe penetration is a problem in gluteraldehyde fixed tissue although I was able to obtain good signal in 8 cases. Another common problem with

FISH is the presence of background autofluorescence. In many tumours this is commonly due to the effects of endogenous enzyme activity. In addition pigmentation in choroidal melanoma may cause problems with background autofluorescence. The above limitations reflect many of the problems already encountered in the literature and this may be why it has only previously been reported on disaggregated tumour cells (Patel *et al.*, 2001, Naus *et al.*, 2002, Naus *et al.*, 2001). This study confirmed that CISH was a more consistent and effective technique to apply to an archival series of choroidal melanoma.

In the first study I identified 2 patient groups with choroidal melanoma. The first group represented those where death from metastatic choroidal melanoma had been recorded. The second represented those who were alive or had died from other causes following primary treatment of choroidal melanoma. This study confirmed the work of previous studies that monosomy 3 is associated with poor survival. As previously discussed it is not possible to directly compare the results obtained with CISH with other techniques. In addition, the relative biases introduced by case selection in a retrospective study means that results are difficult to compare with other studies which all contain an element of bias. For example many prospective studies include only melanomas for which primary treatment has failed or large tumours that required enucleation. Many small tumours are now dealt with by vision sparing techniques and as such tissue is not available. Significantly as the cases in this retrospective study were selected on outcome it was possible to identify a small but significant number of patients with disomy 3 who died from metastatic melanoma, suggesting that other molecular events are important in tumour invasion and metastatic cascade. In addition, this study showed that monosomy 3 does not

correlate with time to death as there was no significant difference in time to death between patients with MMs containing one or two copies of chromosome 3 and survival beyond 10 years (with the longest time reaching 14 years). This study also found that several histological features correlated with monosomy 3; specifically, monosomy 3 could be partly predicted by the presence of epithelioid cells and closed vascular loops. These results have important implications for screening because given that patients without monosomy 3 are not ensured survival this means that there is an increasing need for a suitable screening method that can identify and follow up those tumours that carry a high risk of metastasising. Furthermore, if patients with monosomy 3 may still survive up to 14 years, elderly patients at time of diagnosis may make additional screening and therapies undesirable.

The majority of tumours are treated by some form of vision sparing technique, and if cytogenetics is to be performed as part of the prognostic/therapeutic work up some form of biopsy may be required. In the second study, using CISH, I showed that monosomy 3 was heterogeneous in a subpopulation of choroidal melanomas. The majority of cytogenetic techniques, contrary to CISH, do not assess cell type in conjunction with the cytogenetic abnormalities and clearly this is important. A cell block preparation made from a fine needle aspirate wash-out or the recently described microbiopsy technique (Sen *et al.*, 2006) would allow the assessment of cell morphology in conjunction with assessment of monosomy 3 (using any recognised technique). Based on the results of this study in a biopsy composed purely of spindle cells the presence of 2 copies of chromosome 3 would therefore carry less prognostic significance than one containing both spindle and epithelioid cells. CISH is therefore a useful technique to prove heterogeneity of monosomy 3

with cell type. The technique can however only be applied to a defined population of tumour cells since the assessment of chromosomal loss can not be made on individual tumour cells.

Although CISH may not be the technique of choice for screening there are not any other described techniques, of which I am aware, that can assess the individual cells, cytogenetic and morphological make up.

It has also been suggested that cytogenetic screening will allow patients a better idea of outcome and it will also help to rationalise scarce NHS resources. However the best way to do this is still not clear. Whilst it is entirely possible that an FNA could capture both monosomic and disomic subclones, the operator could not be certain that a monosomic epithelioid subclone had not been missed. Furthermore, the majority of patients still do not have their tumours biopsied and the question whether these patients should have it done remains. Disomy 3 does not mean definite survival and the presence of clonality within a tumour has implications for screening methods such as FNA. Whether it would be appropriate to limit more invasive biopsy to those that have undergone surgery is yet another unknown but again these are a minority. Such an approach would have implications for the assessment of the less invasive technique of FNA, if applied as a screening method.

In conclusion, CISH is an alternative method to assess chromosomal gains and losses in choroidal melanoma. Like other techniques it has both advantages and disadvantages. Acknowledging these and capitalising on the advantages it has proven a useful tool for the study of monosomy 3 in archival choroidal melanomas.

6.2 Further studies

This thesis has raised several questions that could form the basis of future research projects.

Firstly, I identified a group of melanomas that caused death by metastases, yet did not show monosomy 3. Further genetic studies such as CGH using tumour and normal DNA samples, simultaneously hybridised to normal metaphase chromosomes, would be worthwhile on this group to see if there were any common features and to exclude or identify other cytogenetic causes. There is also increasing evidence for the involvement of alternative mechanisms at the molecular level in the development of metastases in choroidal melanoma. Some of the molecular targets involved are cell adhesion molecules, growth factors and matrix metalloproteinases. A more detailed study of these prognostic factors may guide therapeutic strategies, including drug design.

It is assumed that tumour progression in patients with monosomy 3 is due to loss of TSGs but it is still not known what these genes are. Further areas of study might include the identification of the specific genes and pathways involved, directly or as a result of inactivation of *trans*-acting factors following loss of chromosomal material. Thus molecular studies may provide information about genes that are overexpressed in choroidal melanoma and activated in mitogenesis and which could be potential targets for the treatment of micrometastases.

Secondly, I identified clonal heterogeneity in a subgroup of tumours with distinct subpopulations. Whilst in my experience tumours with these defined areas of

epithelioid and spindle cells are relatively common, there are still more tumours with truly mixed populations of cell types. After identifying the presence of monosomic subclones in choroidal melanomas it may be of interest to examine how small these clones can be and what size a clonal population has to be before it carries a poor prognosis. This is difficult to answer and is certainly not possible with the CISH technique since a clonal population could range from 1 up to many thousands of cells. Whilst cell morphology can be viewed by the CISH technique, a result as applied to an individual cell is meaningless. The answer to these questions regarding clonal population size may answer many of the questions regarding the role of biopsy in screening and how to assess if a tumour sample is representative. Assessment of this will require alternative techniques. For example, small group of cells could be removed using a laser capture microdissection system and after extraction of DNA a PCR based approach could be used to amplify relevant areas on chromosome 3.

Ultimately this is a relatively rare tumour for which there is currently no effective treatment for metastatic disease. However with further advancement in treatments it may become increasingly important to identify patients who are at risk of metastatic disease. Whilst there is potential for cytogenetic screening to identify these 'at risk' patients further research on the best technique to undertake this is required. As with all screening techniques it is improbable that any technique will identify all patients at risk of metastases however early recognition and treatment could alter the course of the disease for many patients and the possibility of such treatment would certainly be a prerequisite for offering screening.

Finally, the mechanistic implications of monosomy 3 in the development of choroidal melanoma remains to be fully understood using traditional research methodology. However, loss of all or part of chromosome 3 seems to suggest that one or more tumour suppressor genes involved in the development of choroidal melanoma are located in this chromosome. Furthermore, minimal regions of abnormalities have been described (Parrella *et al.*, 1999, Tsechentscher *et al.*, 2001) and although the exact role of these areas in the development of uveal melanoma is not clear, they might harbour important "candidate genes". Identifying those important discriminating genes and understanding the changes occurring that lead to metastases is currently the subject of intense investigation.

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