Abnormalities of Chromosomes 3 and 8 in Posterior Uveal Melanoma Correlate With Prognosis

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Posterior uveal melanomas have nonrandom alterations affecting chromosomes 3, 6, and 8. Loss of chromosome 3 in uveal melanoma has been shown to act as a predictor of disease-free and overall survival. To confirm the significance of chromosome 3 loss and to extend the observations to include those of the associated alterations of chromosome 8, we have conducted a cytogenetic analysis on a series of 42 tumours from patients with primary uveal melanoma who were followed up for a median of 31 months (range = 8–96 months). Abnormalities of chromosomes 3 and 8 were the commonest changes and were confirmed in 10 tumours using fluorescence in situ hybridization. Monosomy of chromosome 3 was found in 21 (50%) of the tumours, and 23 (54%) tumours had additional copies of 8q. Alterations of chromosomes 3 and 8 were found occurring together in 19 (45%) of the tumours and were significantly associated with a ciliary body component ($P < 0.0001$). Prognostic indicators and changes of chromosomes 3 and 8 were analysed for correlation with patient survival. Of the chosen parameters, only ciliary body involvement ($P = 0.003$), monosomy of chromosome 3 ($P = 0.0007$), and additional copies of 8q ($P = 0.003$) correlated with reduced survival. Evaluation of the dosage effect of additional copies of chromosome arm 8q showed a significant association with reduced survival ($P = 0.0001$), which was also predictive of a decreased disease-free interval ($P = 0.01$). Thus, the cytogenetic analysis of uveal melanoma may provide a valuable predictor of prognosis. *Genes Chromosom. Cancer 19:22–28, 1997.* © 1997 Wiley-Liss, Inc.

INTRODUCTION

Solid tumours constitute the majority of malignancies worldwide; however, in comparison to leukaemia, little is known about the relevant cytogenetic aberrations that may characterise them (Mitelman, 1994). In leukaemia, chromosome changes are relevant to both the classification of the malignancy and the determination of the progression of the disease (Sandberg and Chen, 1995). The application of cytogenetics to the assessment of prognosis in solid tumours has, with a few notable exceptions, been severely limited (Mertens et al., 1994; Choong et al., 1996). This limitation is due in part to the technical difficulties associated with obtaining good quality metaphase spreads for cytogenetic analysis and is further complicated by the extreme complexity of chromosome aberrations frequently observed in these solid malignancies (Sandberget al., 1988; Teyssier, 1989; Johansson et al., 1996). In a recent survey of cytogenetic studies conducted on malignancies, only 27% were from solid tumours, and of these fewer than 1% were from melanomas (Mitelman, 1994).

Posterior uveal melanomas are primary ocular tumours that arise from the ciliary body and choroid of the eye. They are more amenable to cytogenetic analysis than other solid neoplasias (approximately 100 reported cases), mainly because of their ability to establish short-term cultures and produce good quality metaphases (Prescher et al., 1990, 1995; Sisley et al., 1990, 1992; Horsman and White, 1993; Wiltshire et al., 1993; Singh et al., 1994). This ability has enabled the identification of nonrandom alterations of chromosomes 3, 6, and 8 in these tumours (Prescher et al., 1990, 1992, 1994, 1995; Sisley et al., 1990, 1992; Horsman and White, 1993; Wiltshire et al., 1993; Singh et al., 1994), with abnormalities of chromosomes 3 and 8 present in approximately 50% of cases, whereas chromosome 6 anomalies are detected in roughly 45% of cases. In particular, changes affecting chromosomes 3 and 8 (loss of one copy of chromosome 3 and gain of the long arm, or q, of chromosome 8) occur together in the same tumour (Prescher et al., 1990, 1992, 1994, 1995; Sisley et al., 1990, 1992; Horsman and White, 1993; Wiltshire et al., 1993; Singh et al., 1994).

Supported by Yorkshire Cancer Research Campaign; Contract Grant number S 253; Medical Research Council; Contract Grant number G9106078CA.

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Received 2 August 1996; Accepted 5 November 1996

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Although posterior uveal melanomas are rare compared with cutaneous melanomas, with an annual incidence of one case per 5–7 million population, they account for almost 80% of all noncutaneous melanomas and pose a severe threat to life (Scotto et al., 1976; Egan et al., 1988; Rennie, 1991). The 15-year survival rate for patients treated by enucleation is approximately 50% (Egan et al., 1988). Classical prognostic indicators include tumour size, cell type, ciliary body involvement, measurement of nucleolar size, and assessment of tumour vascular patterns (Paul et al., 1962; Shammas and Blodi, 1977; Gamel et al., 1985; Augsburger et al., 1989; Rummelt et al., 1995), but none of these parameters are reliable. The potential value of chromosome changes to the clinical assessment of uveal melanoma has recently been confirmed by comparing monosomy 3 and survival rate in a series of 54 patients with uveal melanoma, and a significant association with survival was determined (Prescher et al., 1996). However, in primary uveal melanomas, abnormalities of chromosomes 3 and 8 tend to occur in association, which would suggest that alterations of chromosome 8 may also be an important prognostic indicator. In this study, we have performed cytogenetic analysis on 42 uveal melanomas, 16 of which have been reported previously (Sisley et al., 1990, 1992). The patients were monitored postoperatively, with 17 patients monitored until their death, and a minimum follow up of 8 months up to a maximum of 8 years for the survivors. The incidence of particular cytogenetic anomalies was determined, and the results were analysed for any relationship between these alterations and patient survival.

MATERIALS AND METHODS

Clinical Details

Patients were referred for treatment to the Department of Ophthalmology and Orthoptics, University of Sheffield, during the period 1987–1995. Because fresh tumour tissue is required for cytogenetic analysis, only those patients undergoing surgical procedures, local resection (six patients), or enucleation, with no prior treatment, were included in the investigation. Therefore, the series had an intrinsic bias towards those patients with large tumours (which represents approximately 25% of all patients referred) that could not be reasonably treated by more conservative regimes, such as radiotherapy. Patient consent was obtained for the collection of material, and histopathological diagnosis and treatment were not compromised by biopsy collection. Of the 42 patients recruited into this study, 29 were male. The age of the patients ranged from 43 to 90 years (median = 66 years). Ciliary body involvement was observed in 24 patients (57%). Tumour size was estimated preoperatively by B scan ultrasonography (Cooper Vision), and the longest tumour diameter in contact with the sclera (LTD) was estimated. The median LTD was 16 mm (range = 8–24 mm). Conventional histological examination was performed on all tumours, which were classified by AFIP classification (Spencer, 1986). Thirty-one tumours were mixed cell melanomas (74%), and the remainder were spindle cell melanomas. Patients were followed up by routine assessment at a clinic or by contacting family doctors. Only patients who had succumbed to metastatic disease were considered to have died from uveal melanoma.

Short-Term Culture and Cytogenetics

Where possible (36 cases), heparinised blood samples were collected and cultured for 72 h in TC199 medium containing phytohaemaglutinin (20 µg/ml). Metaphase cells were obtained from the lymphocyte cultures by following standard protocols (Gosden et al., 1992) and were used to assess the constitutional karyotypes. Tumour specimens were collected from the theatre immediately after surgery and were transported to the laboratory in phosphate buffered saline (PBS). Upon receipt, the specimen was processed by fine mincing with scalpel blades, washing in PBS, and resuspending in media. Tumour specimens were cultured in RPMI supplemented with 20% foetal calf serum, glucose (2 mg/ml), fungizone (3 mg/ml), penicillin (100 U/ml), streptomycin (100 µg/ml), and epidermal growth factor (10 ng/ml). Tumour cells were seeded into 25-cm^3 tissue culture flasks and grown in short-term culture (37°C with 5% CO_2) for a minimum of 1 week. Divisions were harvested by adding Colcemid (0.05 µg/ml) for 4 h, treatment with 0.075 M KCl for 15 min, and fixation with 3:1 methanol:acetic acid. Chromosome preparations were made on clean, cold, wet slides, which were G banded, using trypsin and Leishman stain. Between 5 and 30 cells were analysed for each tumour. Karyotypes were described following the recommendations for cancer cytogenetics (ISCN, 1995).

Fluorescence In Situ Hybridization

In 10 cases, dual-colour fluorescence in situ hybridization (FISH) was performed for chromosomes 3 and 8 to confirm the abnormalities detected by conventional cytogenetics. Chromosome prepara-

tions were made 1 week prior to use. Slides were treated by adding RNase (100 µg/ml) for 1 h in a humid chamber at 37°C, washed twice with 2× SSC, and digested with pepsin (50 µg/ml) in 0.01 M HCl at 37°C for 10 min. After washing for 5 min with PBS and PBS with 50 mM $MgCl_2$, slides were fixed in 1% formaldehyde in PBS + 50 mM $MgCl_2$ for 10 min, washed in PBS for 5 min, and dehydrated in an ethanol series (70%, 95%, and 100%) for 3 min each. Slides were left to air dry and were then denatured in 70% formamide, 2× SSC, pH 7, at 70°C for 2 min, followed by dehydration through an ethanol series (−20°C) for 3 min each. Chromosome 3 (biotin labelled) and 8 Fluorescein Isothiocyanate (labelled with FITC) paints were supplied by Cambio. To an Eppendorf tube containing 3 µg of COT 1 DNA (Life Technologies) and 6 µl of ethanol, which had been vacuumed to dryness, 5 µl of each paint were added, and the mixture was denatured at 75°C for 5 min and incubated at 37°C for 30 min. Ten microlitres of the paint were added to the slides, which were incubated in a humid chamber at 37°C for 24 h.

After hybridisation, the slides were washed at 42°C for 5 min (three times) in 50% formamide, 2× SSC (pH 7), followed by three washes in 2× SSC (pH 7) at 42°C for 5 min. Slides were immersed in 4× SSC and 0.05% Tween 20 (SSCT) for 3 min and blocked with SSCT +5% Marvel dried milk powder (SSCTM) for 5 min at room temperature. After washing in SSCT for 3 min, the chromosome 3 paint was detected by incubating for 20 min at 37°C with avidin–Texas red (2 µg/ml) in SSCTM. On completion, slides underwent three washes in SSCT for 3 min, followed by two washes in PBS for 5 min each. The slides were immersed for 3 min each in 70%, 95%, and 100% ethanol and dried at room temperature. Chromosome spreads were counterstained with DAPI (100 ng/ml) in antifade and observed by using a Cooled CCD camera attached to a microscope and controlled by a Macintosh computer.

Statistical Analysis

Fisher's exact test was used to determine any association between the clinical and cytogenetic parameters, and the Mann-Whitney test was used to determine any difference in chromosome copy number and disease-free interval. Kaplan–Meier survival curves were constructed to determine the relationship among the cytogenetic abnormalities, clinical and histological features, and patient survival. The statistical significance of the survival curves was evaluated using the log-rank test. Values of $P < 0.05$ were considered significant.

TABLE 1. Clinical Details of the Patients and Presence of Alterations Affecting Chromosomes 3 and 8

Case no.	Tumour site[b]	Cell type[c]	Tumour diameter[d] mm (LTD)	Survival[e] (months)	3 and 8 changes[f]
1[a]	C	S	14	A: 96	none
2[a]	CB	M	11	D: 42	−3, +8q (×2)
3	C	M	17	D: 32	none
4[a]	C	M	15	U: 65	none
5[a]	CB/C	M	14	U: 44	none
6	C	M	12	A: 82	−3,
7[a]	CB/C	M	14	D: 9	−3, +8q (×3)
8[a]	CB	M	12	D: 28	−3, +8q (×3)
9[a]	CB/C	M	18	D: 8	−3, +8q (×3)
10	C	M	11	A: 72	none
11	C	M	16	A: 71	none
12	CB/C	M	16	A: 71	−3, +8q (×2)
13[a]	C	M	14	A: 70	none
14[a]	CB/C	M	19	U: 46	−3, +8q (×3)
15[a]	CB/C	M	17	D: 54	−3
16[a]	CB/C	M	16	D: 38	−3, +8q
17[a]	C	M	17	A: 61	+8q
18[a]	C	M	11	U: 34	none
19[a]	C	M	14	A: 61	none
20[a]	CB/C	M	19	D: 19	−3, +8q (×2)
21[a]	C	S	17	A: 58	none
22	CB/C	M	23	D: 33	−3, +8q
23	CB	M	18	A: 39	−3, +8q
24	C	S	13	A: 37	none
25	CB	S	12	A: 36	none
26	CB	M	16	D: 29	none
27	CB/C	S	20	D: 9	−3, +8q (×2)
28	CB/C	M	21	A: 25	−3, +8q
29	CB	M	11	D: 20	−3, +8q (×4)
30	C	S	17	A: 20	+8q
31	C	M	24	A: 19	none
32	CB	M	8	D: 14	−3, +8q (×6)
33	CB	M	13	A: 17	−3, +8q (×6)
34	CB/C	S	23	A: 17	+8q
35	C	M	21	D: 8	−3, +8q (×7)
36	CB/C	M	18	A: 13	none
37	C	S	17	A: 12	+8q
38	C	S	11	A: 11	none
39	CB	M	20	A: 9	−3, +8q (×3)
40	C	S	12	A: 9	none
41	CB	M	18	A: 9	−3, +8q (×2)
42	CB/C	S	14	A: 8	−3, +8q

[a]Previously published (Sisley et al., 1990, 1992).
[b]CB, ciliary body; C, choroid.
[c]S, spindle; M, mixed.
[d]LTD, longest tumour diameter in contact with the sclera.
[e]A, alive; D, dead; U, unrelated death.
[f]The number of additional copies of 8q is given in parentheses.

RESULTS

From the analysis of lymphocyte cultures, all 36 patients examined were found to have a normal constitutional karyotype. Table 1 presents the clini-

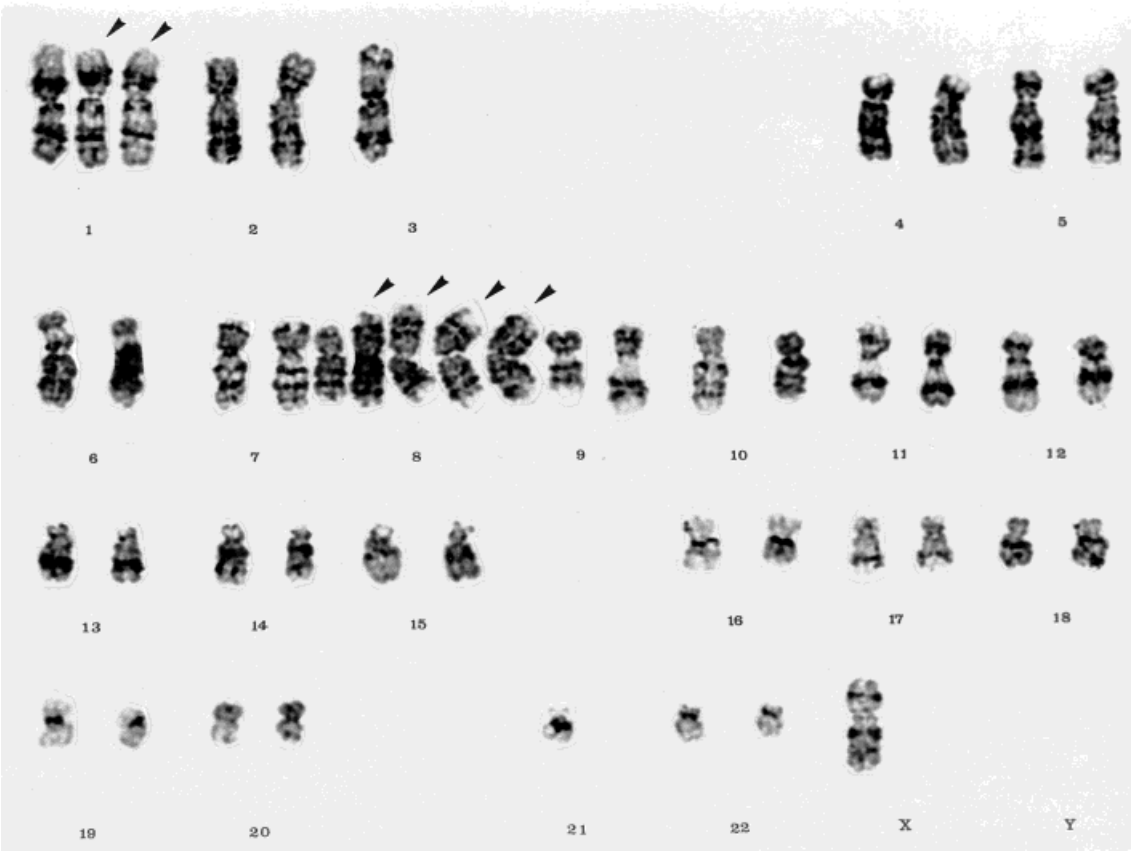

Figure 1. Representative karyotype from a primary uveal melanoma, 47,X,-X,del(1)(p36),+del(1)(p36), -3,i(8)(q10),+i(8)(q10)x2,+der(8)t(8;8)(p23;q23),-21. Structural abnormalities are indicated by arrowheads.

cal details of the patients and identifies those patients with alterations of chromosomes 3 and 8. Cytogenetic analysis on 16 of the tumours in the series has been reported previously (Sisley et al., 1990, 1992; Table 1). The most consistent alterations found were those affecting chromosomes 1, 3, 6, and 8. Of these abnormalities, changes of chromosomes 3 and 8 were observed most frequently. Twenty-three tumours (54%) had one or more additional copies of the long arm of chromosome 8, either by translocation or in the form of an isochromosome i(8q), and of these, 14 had two or more copies. Monosomy for chromosome 3 was found in 21 (50%) of the tumours examined. Other abnormalities included deletion of chromosome arm 1p in 12 patients (29%), deletion of 6q in 13 patients (31%), and trisomy 6p in 8 patients (19%). A uveal melanoma karyotype is presented in Figure 1, and Figure 2 shows an analysis of a uveal melanoma using FISH for chromosomes 3 and 8. FISH analysis in all cases confirmed the conventional cytogenetic analysis.

This study confirmed the strong association between involvement of the ciliary body and alterations of chromosomes 3 and 8. Of the 23 tumours with additional copies of 8q, 19 involved the ciliary body ($P = 0.0004$, Fisher's exact test). Furthermore, 19 of the 21 tumours exhibiting monosomy 3 also affected the ciliary body ($P < 0.00001$, Fisher's exact test). Nineteen tumours (45%) had karyotypes that contained abnormalities of both chromosomes 3 and 8; this result was again highly significant ($P < 0.0001$, Fisher's exact test). No association was found between chromosome changes and the age or sex of the patient or the tumour size. A weak association was found between monosomy 3 and mixed cell type ($P = 0.02$).

Kaplan–Meier survival curves were constructed with respect to patient's age (older/younger than 60 years), sex, tumour size (LTD), location, histological cell type, monosomy 3, and additional copies of 8q. Statistical significance was compared by using the log-rank test. Of the chosen parameters, only ciliary body involvement ($P = 0.003$; Fig. 3a),

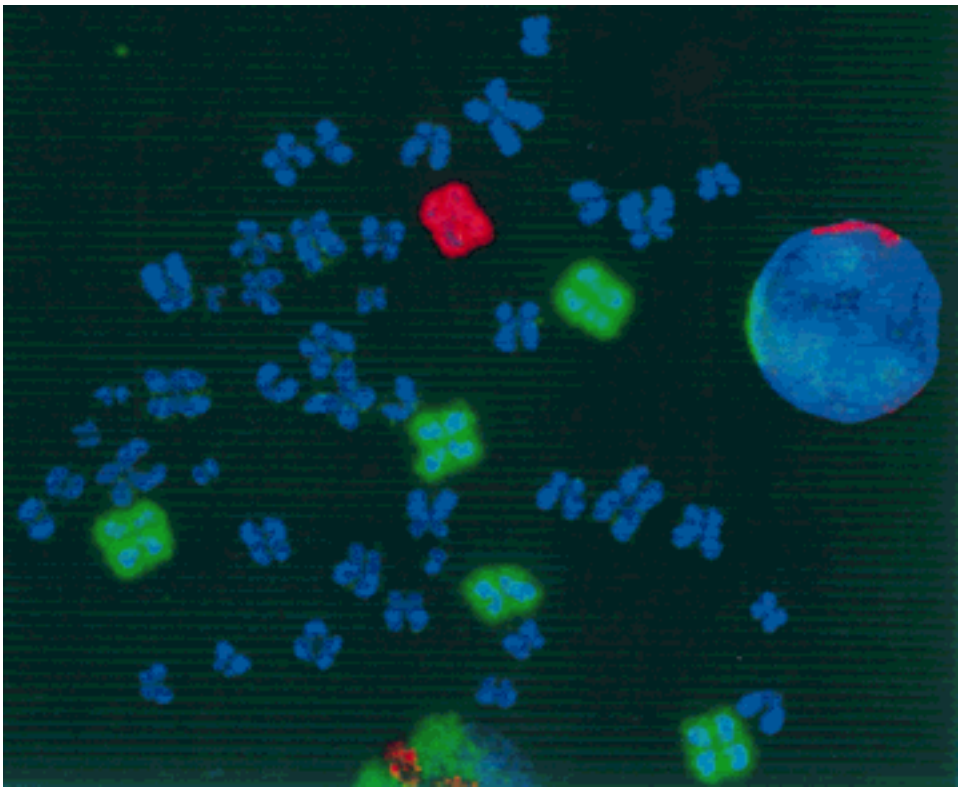

Figure 2. Chromosome painting for chromosomes 3 (red) and 8 (green) in primary uveal melanoma, demonstrating monosomy 3 and additional copies of chromosome 8.

monosomy of chromosome 3 ($P = 0.0007$; Fig. 3b), and additional copies of 8q ($P = 0.003$; Fig. 3c) were correlated with reduced survival. In addition, Kaplan–Meier curves were constructed to evaluate the influence of dosage of 8q on survival (Fig. 3d). The results showed that increasing copies of 8q correlate with reduced survival ($P = 0.0001$). The effect of increasing dosage of chromosome arm 8q on disease-free interval was also examined. Patients with tumours containing only one or two additional copies of 8q had significantly longer disease-free intervals (median = 32 months, range = 9–54 months) than did patients with tumours possessing three or more additional copies of 8q (median = 14 months, range = 8–27.8 months; $P = 0.01$, Mann Whitney test).

DISCUSSION

The frequency of specific clonal chromosome abnormalities, as found in posterior uveal melanoma, is most unusual (Prescher et al., 1990, 1992, 1994, 1995; Sisley et al., 1990, 1992; Horsman and White, 1993; Wiltshire et al., 1993; Gordon et al., 1994; Singh et al., 1994; Speicher et al., 1994). The relevance of these abnormalities is compounded by the fact that two abnormalities (monosomy 3 and additional copies of 8q) often coexist in the same tumour and have a predilection for a particular anatomical location, namely, the ciliary body (Sisley et al., 1990, 1992; Prescher et al., 1992, 1996; Horsman and White, 1993; Singh et al., 1994).

Previous investigations have suggested that monosomy 3 and aberrations of chromosome 8 are associated with clinical and histological parameters that are known to confer a poor prognosis (Prescher et al., 1992; Horsman and White, 1993), and until recently, no direct comparison of cytogenetic abnormalities with survival has been made (Prescher et al., 1996). In this study, we have shown that poor prognosis correlates with ciliary body involvement, monosomy 3, and additional 8q. In contrast, other previously recognised prognostic parameters (LTD and histological cell type) did predict survival, which is perhaps not surprising when the inherent basis of this study is considered. The majority of the tumours were large and had a mixed cell morphology, and as a result, the power of these parameters to predict survival must be diminished.

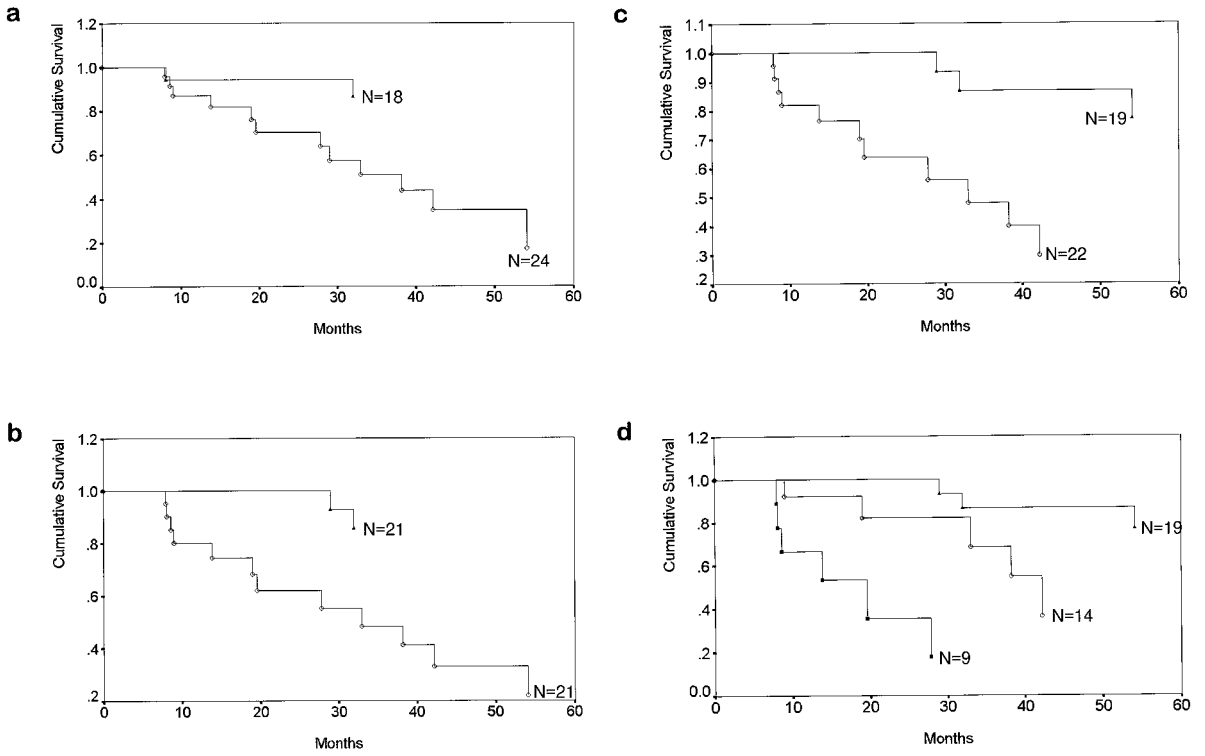

Figure 3. Kaplan–Meier survival curves for death from metastatic melanoma. **a:** Choroid melanomas (black triangle) versus cilairy body involvement (open circles) (log rank, $P = 0.003$). **b:** Melanomas with a normal copy number of chromosome 3 (black triangle) versus tumours with monosomy of chromosome 3 (open circles) (log rank, $P = 0.0007$). **c:** Tumours with a normal copy number of chromosome 8 (black triangle) versus tumours with additional copies of 8q (open circles) (log rank, $P = 0.003$). **d:** The effect of increasing dosage of 8q on survival: normal copies (black triangle), one or two extra copies (open circles), and three or more extra copies (black square) (log rank, $P = 0.0001$).

The intrinsic bias in this study is unavoidable and reflects a shift in clinical practise from simple enucleation to other treatment modalities, such as radiotherapy, which preserve the globe and from which histological material is not available.

Ciliary body involvement has been shown in previous studies to confer a poor prognosis (Mclean et al., 1977; Seddon et al., 1985), although the reason for this has been unclear. Our study has confirmed this association. It is apparent from this and previous studies (Sisley et al., 1990, 1992; Prescher et al., 1992; Horsman and White, 1993; Singh et al., 1994) that abnormalities of chromosomes 3 and 8 are intimately involved with tumours of the ciliary body, and thus the strong association between the cytogenetic abnormalities and survival may explain the relationship between tumour location and prognosis. This hypothesis has been strengthened latterly by the observations of Prescher et al. (1996) who found a significant correlation of monosomy 3 with overall survival, and this relationship could also be predictive of disease-free interval. Because tumour location and chromosome alterations are intimately linked, other factors unrelated to the cytogenetic alterations may be responsible for the poor prognosis seen in these melanomas, and chromosomal abnormalities may achieve significance by virtue only of secondary association. However, the evidence from this and previous studies would appear to refute this theory (Prescher et al., 1992, 1996; Horsman and White, 1993). In particular, the finding that dosage of 8q predicts not only survival but also disease-free interval suggests that this chromosome change may be crucial to the development of metastases by these tumours. This notion is supported by previous studies that have suggested that the acquisition of an isochromosome 8q is a secondary event (Gordon et al., 1994; Prescher et al., 1994) and appears to be an ongoing process, whereby many additional copies of 8q can be gained (Fig. 1). Our finding that increasing copies of 8q affect disease-free survival suggests that a gene, or genes, may reside on the long arm of chromosome 8, the overexpression of which is linked to the development of the metastatic phenotype.

ACKNOWLEDGMENTS

We thank Janet White and Julia Garnham for their excellent technical support, Professor Ian

Dunsmore for statistical advice, and Robin Farr for photographic assistance.

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