ORIGINAL ARTICLES—Laboratory science

DNA ploidy pattern in choroidal melanoma: correlation with survival. A flow cytometry study on archival material

Paolo Toti, Giuseppe Greco, Paola Mangiavacchi, Alessandra Bruni, Marie Louise Desirèe Palmeri, Pietro Luzi

Abstract

***Background/aims*—Paraffin embedded samples have provided an important source of material for retrospective cytofluorimetric studies, useful in establishing the predictive value of DNA content measurements. The aim of this study was to investigate the incidence and type of aneuploidy in choroidal malignant melanomas (CMM) and the significance in the clinical outcome (median follow up 55 months).**

***Methods*—DNA content was quantified by flow cytometry in 61 CMM from archival material. Non-tumour ocular tissue was used as the reference diploid standard. Cases in which the coefficient of variation (CV) of the diploid peak was >8% were excluded. The CMM were classified as spindle A, spindle B, mixed spindle and epithelioid, epithelioid, and necrotic.**

***Results*—The frequency of the aneuploid DNA pattern was 38%. Necrotic tumours showed a worse clinical outcome independent of the ploidy pattern. Spindle A tumours were found to be diploid. Spindle B and mixed tumours showed a prevalent diploid and near diploid aneuploid pattern (DI <1.3), yet aneuploidy was not correlated with a worse prognosis. The epithelioid tumours were prevalently diploid. However, 83% of the aneuploid tumours were hypodiploid (DI <0.95), and showed the worst prognosis.**

***Conclusion*—These results indicate that increasing DNA abnormalities in CMM, especially in the epithelioid histotype, were associated with an increasing mortality.**

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Institute of Pathological Anatomy and Histology, University of Siena, Siena, Italy
P Toti
P Mangiavacchi
A Bruni
M L D Palmeri
P Luzi

Department of Ophthalmology and Neurosurgery, University of Siena, Siena, Italy
G Greco

Correspondence to:
Professor Pietro Luzi, Institute of Pathological Anatomy and Histology, University of Siena, Via delle Scotte, 53100 Siena, Italy.

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Choroidal malignant melanoma (CMM) is the most common primary intraocular malignancy in adults. The estimated 15 year survival rate after detection of the tumour is 53%.[1] Callender classification of cell types,[2] as simplified by the Armed Forces Institute of Pathology (AFIP),[3 4] remains one of the most reliable prognosticators in CMM. Unfortunately, it lacks reproducibility, primarily because there is no objective cut off point that could be used to categorise tumours within the mixed cell class.[5 6] Moreover, it does not differentiate between the various tumours within the different classes.[5]

In search of more objective prognostic factors, a number of adjunctive factors have been found, including largest tumour dimension,[7] nuclear and nucleolar areas,[6 8 9] necrosis,[4] neovascularisation,[10 11] mitotic activity, and proliferation index[12 13] (for review see Mooy and De Jong[14]).

Ploidy status of uveal melanomas has been investigated by flow cytometry (FCM) and image analysis (IA) both in fresh and paraffin embedded material. Controversial results concerning the incidence of aneuploidy and the correlation between ploidy status and prognosis have been reported.[5 13 15–18] However, there is increasing evidence that in uveal melanomas, as well as in a variety of neoplasms, an elevated DNA index (>1.4) is strongly correlated with higher tumour related mortality.[16 19]

We have retrospectively studied a group of patients with choroidal melanomas who were treated with enucleation more than 5 years ago and for whom complete follow up data were available. The purpose of this study was to investigate the incidence, type of aneuploidy, and effect of DNA ploidy in clinical outcome.

Materials and methods

In order to correlate ploidy status with prognosis, a retrospective analysis of 117 cases of formalin fixed (split bulb in 4% buffered formalin for 48 hours) paraffin embedded uveal malignant melanoma, selected from the archives of the Institute of Pathology, University of Siena, was performed. The following criteria were used: (a) uveal melanomas had to be located in the choroid, (b) enucleation had to be performed without previous therapy, (c) adequate paraffin embedded material had to be available in each case. Cases were rejected if insufficient tissue was available, or if the tissue had been processed before 1982. Follow up data (minimum 5 years) were obtained by contacting the registry office, the general practitioner, and/or the patients' relatives. These

Table 1 Summary of DNA ploidy quantification of 61 choroidal melanomas according to Callender cell types and survival

	Total		Diploids		Aneuploids		Living		Dead	
Cell type	No	%	No	%	No	%	No	%	No	%
Spindle A	1	2	1	100	0	0	1	100	0	0
Spindle B	35	57	23	66	12	34	26	74	9	26
Epithelioid	14	23	8	57	6	43	7	50	7	50
Mixed	8	13	5	62	3	38	5	62	3	38
Necrotic	3	5	1	33	2	67	0	0	3	100
Total	61	100	38	62	23	38	39	64	22	36

Table 2 DNA indexes (DI) in aneuploid tumours by cell types

	DI					
	1.05–1.3		>1.3		<0.95	
Cell type	No of patients	%	No of patients	%	No of patients	%
Spindle	5	42	6	50	1	8
Epithelioid	0	0	1	17	5	83
Mixed	1	34	2	66	0	0
Necrotic	0	0	2	100	0	0

Table 3 Tumour related deaths and ploidy status

Ploidy status	No of patients (living)	%	No of patients (dead)	%
Diploid	29	77	9	23
Aneuploid	10	45	13	55
DI 1.05–1.3	6	100	0	0
DI >1.3	4	45	7	55
DI <0.95	0	0	6	100

data were reviewed in order to determine if the death was tumour related or a result of other causes. Seventy seven patients met these criteria and thus were included in the study.

Haematoxylin and eosin stained sections were prepared for each block and analysed for their histological type. For the purpose of this study, the Callender classification[2] was modified as follows: spindle A, spindle B, epithelioid, mixed cell (predominantly spindle with few epithelioid cells),[5] and necrotic. The area in the block containing the tumour was then marked (tumour enriched area). The tumour enriched and non-tumour areas were isolated by perpendicular and horizontal cutting of the block. They were placed in separate vials and sent to the FCM laboratory. All FCM analyses were performed without previous knowledge of tumour characteristics. Tissues from five non-neoplastic eyes were also processed and evaluated.

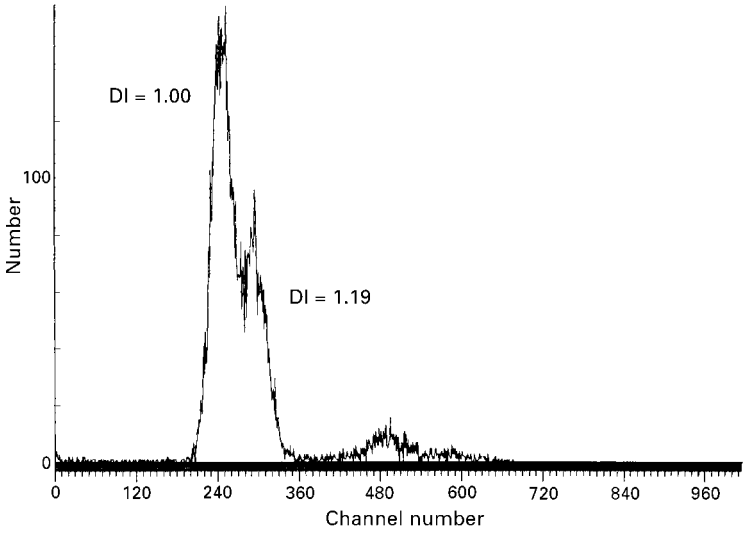

Figure 1 Histogram of spindle B choroidal malignant melanoma (CMM) showing a near diploid aneuploid peak. DI indicates the DNA index.

Nuclei were isolated according to the method of Hedley and coworkers,[20] though some modifications were made in the process. Neoplastic fragments were placed in glass tubes and dewaxed overnight in 10 ml of xylene at room temperature. The fragments were then rehydrated in a sequential series of ethanol solutions: 100%, 95%, 70%, and 50%, taking 50 minutes for each step. The fragments were left in distilled water overnight. The fully rehydrated tissue was then minced with surgical scissors, incubated in 2 ml 0.5% pepsin (Sigma P 6887; Sigma, St Louis, MO, USA) in saline solution at pH 1.5 for 60 minutes. The preparations were placed in a 37°C water bath for 60 minutes with frequent vortexing. The enzymatic reaction was controlled under phase contrast microscopy and stopped with the addition of 2 ml of ice cold PBS buffer. After washing, the pellet was resuspended in 2 ml of PBS and filtered through 50 μm mesh to remove aggregates. The nuclei were simultaneously counted in a haemocytometer, adjusted to 1×10^6/ml and stained with 500 μl of Lysis/DNA solution (propidium iodide, Sigma, 50 μg/ml; RNase, Sigma R-4875, 0.2 mg/ml; Nonidet P40, Sigma, 0.5%; EDTA, Sigma, 0.5 mM in PBS calcium and magnesium free, at pH 7.2).

The presence of tumour cells in the material used was immediately verified after disaggregation by examination of a May–Grunwald Giemsa stained cytospin. A cytologist performed the cytospin reading. In each case, 10 000 nuclei were measured. FCM was performed on a FACStar plus flow cytometer (Becton Dickinson, San Jose, CA, USA) using a 488 nm argon laser (100 mW). The flow cytometer was connected to a Hewlett Packard 300 microcomputer (Hewlett Packard, Fort Collins, CO, USA) using FACStar plus software for instrument control and data acquisition. To obtain a reference diploid standard, the ocular non-tumour tissue treated as described above was added. The analysis of the histograms generated by FCM was carried out by means of the Modfit DNA modelling system (Verity Software House, Topsham, ME, USA). The mathematical model was based on the Marquardt non-linear least squares algorithms.[21] In particular, G0/G1 and G2M phases were fitted by Gaussians and SPF with a trapezoid. Debris filtering was performed using debris subtraction routines based on a multicut for the beginning and end of the debris components. The software was implemented on a Compaq 386/20e (Compaq Computer Corporation, Houston, TX, USA).

In accordance with current literature,[19 22] tumours were classified as diploid ($0.95<DI<1.05$) or aneuploid ($DI<0.95$; $DI>1.05$). The degree of DNA content abnormalities was given according to the DNA index (DI). For practical purposes, tumours with a

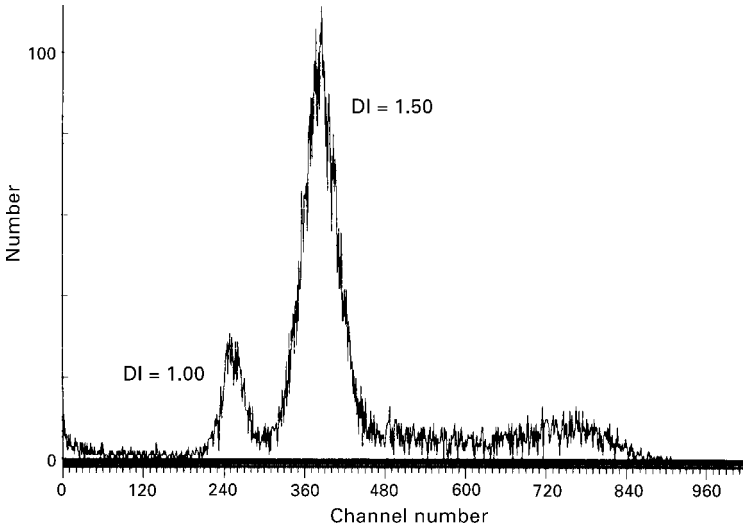

Figure 2 Histogram of spindle B choroidal malignant melanoma (CMM) showing a hyperdiploid peak. DI indicates the DNA index.

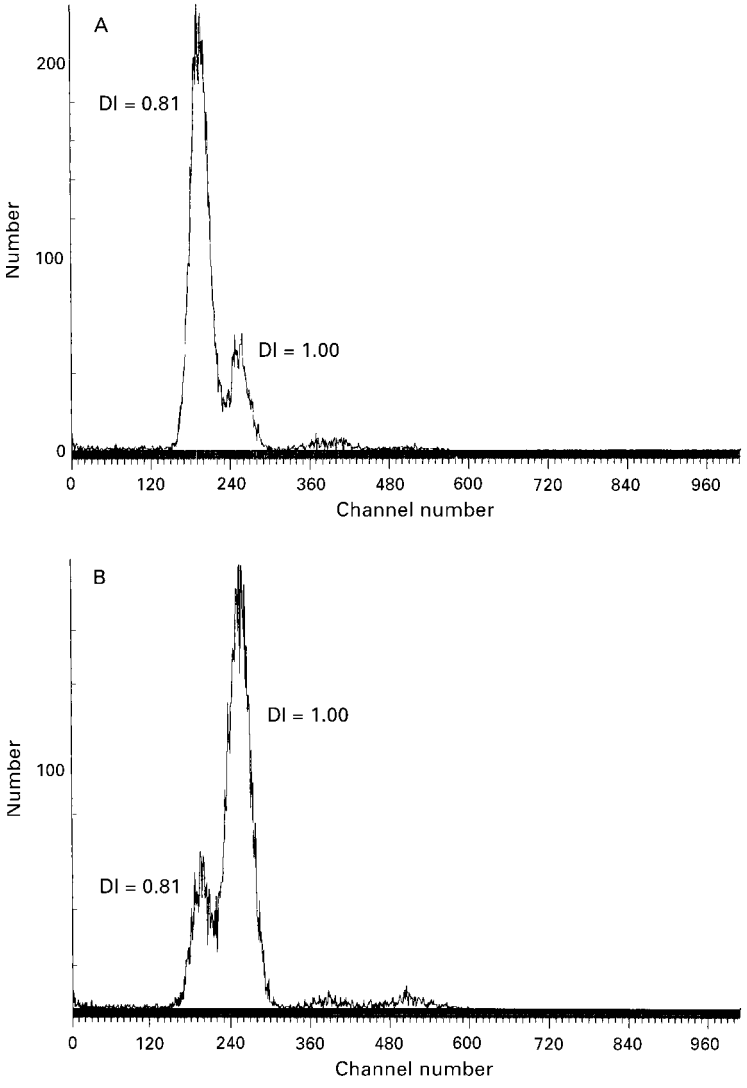

Figure 3 Histogram of epithelioid choroidal malignant melanoma (CMM) showing a hypodiploid peak (A). Diploid peak increased while hypodiploid peak proportionally decreased after the reference diploid standard (non-tumour ocular tissue) was added (B). DI indicates the DNA index.

DI >1.05 were considered hyperdiploid; tumours with a DI between 1.05 and 1.3 were classified as near diploid aneuploid; and tumours with a DI <0.95 were considered hypodiploid. However, no direct measurements of changes in the number and composition of individual chromosomes were made. Samples with a coefficient of variation of the diploid peak of more than 8% were excluded. Acceptable DNA histograms were obtained in 61 cases.

The results were statistically analysed by the two tail Fisher's exact test. Values of $p < 0.05$ were considered significant.

Results

Sixty one tumours were included in the study; 27 patients were male and 34 female. Their ages ranged between 32 and 73 years (mean of 59 years). The coefficient of variation of sample histograms ranged between 4 and 7.60 (mean 5.1).

In an overall analysis (Table 1), 35 of the 61 analysed tumours were spindle B (57%), 14 were epithelioid (23%), eight were mixed (13%), three were necrotic (5%), and one was spindle A (2%). Of these tumours, 38 were shown to be diploid (62%) and 23 were aneuploid (38%). Normal cell tissue, studied in paraffin blocks of five non-neoplastic eyes, was diploid. Among the aneuploid tumours, there were 12 spindle B, three mixed, six epithelioid, and two necrotic tumours. A near diploid DNA pattern was found in one mixed and five spindle B tumours. An aneuploid hypodiploid DNA pattern was found in one spindle B and five epithelioid tumours (Table 2). The DIs ranged from 0.77 to 0.89 (mean 0.84). All the remaining tumours showed a hyperdiploid DNA profile. No correlation between ploidy pattern and cell type was found (p=0.74). Furthermore, 22 of the 61 patients survived (36%). The patients who died were 25% with spindle B, 38% with mixed, 50% with epithelioid, and all three patients with necrotic tumours (p=0.176; NS). Necrotic and epithelioid tumours considered together showed a significantly worse prognosis than spindle and mixed cell tumours (p=0.036).

Ploidy status appeared correlated with survival (p=0.038; Table 3). Moreover, patients with diploid and near diploid tumours had a higher survival rate than those with aneuploid tumours ($p<0.005$). However, only in epithelioid and necrotic tumours was an aneuploid pattern significantly correlated with a worse prognosis ($p<0.005$). Patterns of DNA aneuploid histograms obtained in this study are shown in Figures 1–3.

Discussion

Since recent technologies now allow for the study of DNA ploidy pattern in paraffin embedded tissue,[20] retrospective studies of DNA profile of intraocular melanomas have been performed.

In this study, we investigated ploidy pattern in 61 choroidal melanomas. The frequency of aneuploid tumours was 38%, which is consistent with more recent findings.[5 13 16] Mooy *et al*[13]

found an increased aneuploidy in the preirradiated tumours, and a strong correlation between ploidy status and cell types, as well as between ploidy status and metastatic outcome.

We found a DNA diploid pattern in the spindle A tumour and in most of the spindle B, mixed cell, and epithelioid tumours. Thus, we cannot confirm the correlation between aneuploidy and epithelioid cell type.[13] Spindle B, mixed cell, and necrotic aneuploid tumours were prevalently hyperdiploid. Moreover, in most of the spindle B tumours, the DI was in the near diploid range as also found by Meecham and Char.[16] Surprisingly, 30% of the aneuploid tumours and 83% of the epithelioid aneuploid tumours were hypodiploid.

Aneuploidy correlated with poor prognosis in the series of Meecham and Char[16] and of Mooy *et al*,[13] but not in those of McMillan *et al*[15] and Coleman *et al*.[5] We found correlation between aneuploidy and poor prognosis only in the epitheliod hypodiploid tumours. A hypodiploid DNA pattern has been said to be associated with a poorer prognosis than other aneuploid types in breast cancer.[23 24] Monosomy of chromosome 3 has been reported in uveal melanoma with a poor prognosis.[25] The high incidence of diploid and near diploid (low degree aneuploidy) pattern for many aneuploid spindle B tumours may explain why the spindle tumours usually show a more favourable prognosis. In some types of tumours the prognosis for near diploid tumours does not differ from that of a DNA diploid tumour.[19]

The percentage of hypodiploid tumours observed in our series of CMM can be considered unusual. Only in one previous study on ear melanoma was such a high percentage reported.[26] The lack of a known DNA diploid reference population in nuclei from paraffin embedded tissues renders the identification of the nuclei populations with a DI from 0.8 to 0.95 a truly difficult problem.[19]

The general acceptance of the first peak as a normal diploid G0/G1 may have led to the misinterpretation of the second peak as a hyperdiploid or near diploid tumour G0/G1 peak, disregarding the hypodiploid population.[19] This may explain why hypodiploid tumours are rarely reported. In the present study, we always added normal tissue obtained from the same block to augment the diploid population after analysing the tumour enriched tissue. Given the variations in degree of fixation within a single paraffin embedded tissue, it is not possible to completely separate the non-tumour diploid cells from the aneuploid tumour cells with this procedure. However, a normal tissue component representing the normal counterpart of neoplastic cells is presently considered the best DNA content standard.[13 27 28] Moreover, as shown in the figures, the relative proportions of each diploid and aneuploid population in our cases dramatically changed after the normal tissue was added.

Technical problems in DNA content assessment of paraffin embedded specimens become more likely as the sample age increases.[19] The increase in the background noise from broken nuclei and cellular debris may cause wider CV. Thus, the interpretation of overlapping peaks may be more difficult. As such, we processed material no more than 15 years old and rejected cases in which the CV of the diploid peak was >8%. Furthermore, a routine debris filtering and subtraction were implemented in the software. Such methods probably allowed us to better interpret the cell cycle.

In conclusion, the percentage of aneuploid tumours in our series was consistent with the more recent literature. However, we found an unusually high percentage of tumours with a hypodiploid DNA pattern, especially in epithelioid tumours. This pattern was strongly correlated with the worst prognosis. To our knowledge, this study is the first to show such a peculiar result in CMM.

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1 Gamel JW, McLean IW, McCurdy JB. Biological distinction between cure and time to death in 2892 patients with intraocular melanoma. *Cancer* 1993;**71**:2299–305.
2 Callender GR. Malignant melanotic tumors of the eye: a study of histologic types in 111 cases. *Trans Am Acad Ophthalmol Otolaryngol* 1931;**36**:131–42.
3 McLean IW, Foster WD, Zimmerman LE, *et al*. Modifications of Callender's classification of uveal melanoma of the Armed Forces Institute of Pathology. *Am J Ophthalmol* 1983;**96**:502–9.
4 McLean IW, Burnier MN, Zimmermann LE, *et al*. *Tumors of the eye and ocular adnexa.* Bethesda: AFIP Atlas of tumor pathology, third series, 1994.
5 Coleman K, Baak JPA, Van Diest PJ, *et al.* DNA ploidy status in 84 ocular melanomas: a study of DNA quantitation in ocular melanomas by flow cytometry and automatic and interactive static image analysis. *Hum Pathol* 1995;**26**:99–105.
6 Seddon JM, Polivogianis L, Chung-Cheng Hsiech SD, *et al.* Death from uveal melanomas: number of epithelioid cells and inverse SD of nucleolar area as prognostic factors. *Arch Ophthalmol* 1987;**105**:801–6.
7 Augsburger JJ, Gamel JW. Clinical prognostic factors in patients with posterior uveal malignant melanoma. *Cancer* 1990;**66**:1596–600.
8 McLean IW, Gamel JW. Prediction of metastasis of uveal melanomas: comparison of morphometric determination of nuclear size and spectrophotometric determination of DNA. *Invest Ophthalmol Vis Sci* 1988;**29**:507–11.
9 Gamel JW, McCurdy JB, McLean IW. A comparison of prognostic covariates for uveal melanoma. *Invest Ophthalmol Vis Sci* 1992;**33**:1919–22.
10 Greco G, Toti P, Motolese E, *et al.* Morphometric evaluation of vascularization of uveal melanoma. In: Frezzotti R, Balestrazzi E, Falco, Esente S, eds. *International symposium of intraocular and epibulbar tumors.* Bologna: Monduzzi, 1994:91–3.
11 Folberg R, Rummelt V, Parys-Van Ginderdeuren R, *et al.* The prognostic value of tumor blood vessel morphology in primary uveal melanoma. *Ophthalmology* 1993;**100**:1389–98.
12 Karlsson M, Boeryd B, Carstensen J, *et al.* Correlation of Ki-67 and PCNA to DNA ploidy, S-phase fraction and survival in uveal melanoma. *Eur J Cancer* 1996;**32A**:357–62.
13 Mooy CM, Vissers K, Luyten G, *et al.* DNA flow cytometry in uveal melanoma: the effect of pre-enucleation irradiation. *Br J Ophthalmol* 1995;**79**:174–7.
14 Mooy CM, De Jong PTVM. Prognostic parameters in uveal melanoma: a review. *Surv Ophthalmol* 1996;**41**:215–28.
15 McMillan J, Char DH, McLean IW, *et al.* DNA content analysis of uveal melanoma. *Arch Ophthalmol* 1989;**107**:1278.
16 Meecham WJ, Char DH. DNA content abnormalities and prognosis in uveal melanoma. *Arch Ophthalmol* 1986;**104**:1626–9.
17 Rennie JG, Rees RC, Parsons MA, *et al*. Estimation of DNA content in uveal melanomas by flow cytometry. *Eye* 1989;**3**:611–17.
18 Shapiro BE, Felberg NT, Donoso LA, *et al*. Flow cytometry of uveal melanomas. *Cancer Biochem Biophys* 1986;**8**:235–8.
19 Shankey TV, Rabinovitch PS, Bagwell B, *et al.* Guidelines for implementation of clinical DNA cytometry. *Cytometry* 1993;**14**:472–7.
20 Hedley DW, Friedlander ML, Taylor JW. Application of DNA flow cytometry to paraffin-embedded archival material for the study of aneuploidy and its clinical significance. *Cytometry* 1985;**6**:327–33.

21 Marquardt DI. An algorythm for least squares estimation of nonlinear parameters. *J Soc Ind Appl Math* 1963;**2**:431–41.
22 Luzi P, Bruni A, Mangiavacchi P, *et al.* Ploidy pattern and cell cycle in breast cancer as detected by image analysis and flow cytometry. *Commun Clin Cytometry* 1994;**18**:79–87.
23 Coulson PB, Thornthwaite JT, Woolley TW, *et al.* Prognostic indicators including DNA histogram type, receptor content, and staging related to human breast cancer survival. *Cancer Res* 1987;**44**:4187–96.
24 Ferno M, Baldetorp B, Olsson H, *et al.* Flow cytometric DNA index and S-Phase fraction in breast cancer in relation to other prognostic variables and to clinical outcome. *Acta Oncol* 1992;**31**:157–65.
25 Prescher G, Bornfeld N, Hirche H, *et al.* Prognostic implications of monosomy 3 in uveal melanoma. *Lancet* 1996;**347**:1222–5.
26 Cohen C, Walker BF, Solomon AR, *et al.* DNA ploidy and cell cycle analysis in ear malignant melanoma by flow and image cytometry. *Anal Quant Cytol Histol* 1992;**14**:81–8.
27 Kute TE, Quadri Y, Muss H, *et al.* Flow cytometry in node-positive breast cancer: cancer and leukemia group B protocol 8869. *Cytometry* 1995;**22**:297–306.
28 Shuttle B, Reynders MMJ, Bosman FT, *et al.* Flow cytometric determination of DNA ploidy level in nuclei isolated from paraffin embedded tissue. *Cytometry* 1995;**6**:26–30.