DNA flow cytometry in uveal melanoma: the effect of pre-enucleation irradiation

Cornelia Mooy, Kees Vissers, Gregorius Luyten, Andries Mulder, Theo Stijnen, Paul de Jong, Fred Bosman

Abstract

Background—For uveal melanoma it has been demonstrated that aneuploidy correlates with worse clinical outcome. However, a striking variation in incidence of aneuploidy is reported for uveal melanomas.

Methods—Flow cytometry was used to study retrospectively DNA-ploidy of 132 uveal melanomas on paraffin embedded material. Thirty five patients received 2×4 Gy doses of irradiation 24 and 48 hours before enucleation. Correlation between DNA-ploidy and histopathological grading, largest tumour diameter, tumour height, tumour location, scleral invasion, and TNM classification was assessed. Survival analysis methods were used to investigate the predictive value of these variables on clinical outcome.

Results—Of the tumours 37% were aneuploid and 63% were diploid. Intratumour ploidy heterogeneity was minimal (92% concordance). A strong correlation (p=0.009) was found between DNA-ploidy and cell type. No correlation was found between DNA-ploidy and other conventional prognostic variables. Irradiated melanomas were significantly more aneuploid than non-irradiated tumours (p≤0.01).

Conclusion—In survival analysis DNA-ploidy and the largest tumour diameter were significant in predicting metastatic outcome (p≤0.03 and 0.01 respectively); histological cell type and tumour location were of borderline significance.


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Ciliary body and choroidal melanomas are the most common types of primary intraocular malignancy in the adult. The estimated 15 year survival rate after detection of the tumour is 53%.1 The metastatic potential of uveal melanomas varies, depending on tumour cell type, largest tumour diameter (LTD), the standard deviation of the nucleolar area, and the mean of the largest nucleoli.2 New treatment methods, including preoperative irradiation, have not substantially reduced the mortality of people with these tumours.3 In a variety of solid tumours, including cutaneous malignant melanoma4 5 DNA flow cytometry has proved to be a useful and objective prognostic variable in addition to conventional histopathological classification.6 7 For uveal melanoma, analysis of recent paraffin embedded material has shown that aneuploidy correlates with poor prognosis,8 but on older (>15 years) archival paraffin embedded material this was not confirmed.9 The percentage of aneuploidy in different studies on uveal melanomas varies between 16%10 and 78%.11 In the largest study interpretable DNA histograms were obtained in 64 cases, from which 36% were aneuploid.8 It has been shown that pre-enucleation irradiation reduces the proliferative activity in uveal melanomas.12 13 Effects of preoperative irradiation on tumour ploidy have not been extensively investigated, however. In an attempt to resolve these inconsistencies, we have studied archival (aged 13 years max) paraffin embedded tissue of 136 uveal melanomas to test the predictive value of aneuploidy on clinical outcome. In the study material we included patients who had received 2×4 Gy doses of irradiation before enucleation in order to reduce the risk of haematogenous metastases during the enucleation procedure.14

The aims of this study were (1) to investigate the incidence of aneuploidy in a large series of cases, (2) to assess intratumour heterogeneity, (3) to investigate the effect of pre-enucleation irradiation on DNA-ploidy, and (4) to investigate the effect of DNA-ploidy on clinical outcome. Estimation of S-phase fractions was not performed, because of unreliability of this method on paraffin embedded material.15

Materials and methods

From 1976 to 1989, 98 paraffin blocks and five frozen specimens from patients with choroidal

<table>
<thead>
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<th>Table 1 Patient selection</th>
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<tbody>
<tr>
<td>Total No</td>
</tr>
<tr>
<td>2×4 Gy irradiation</td>
</tr>
<tr>
<td>No irradiation</td>
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<tr>
<td>146</td>
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*Excluded because of a high coefficient of variation (CV).
and ciliary body melanomas were collected from the department of pathology, Erasmus University Rotterdam. Thirty-eight patients received 2x4 Gy doses of irradiation before enucleation. Fortythree paraffin blocks from non-irradiated tumours from the same period were selected from the department of pathology, University of Nijmegen, totalling 146 cases (Table 1). Until 1993 adequate follow up of 97 patients could be obtained by contacting the ophthalmologist or the general physician. Follow up data were requested and verified. Thirty-four patients died from tumour related causes, 50 patients were still alive, and 13 patients died of other causes (Table 2).

Three 50 µm sections were cut from the paraffin blocks. Additional 7 µm sections were obtained from each side of the experimental material and stained with haematoxylin and eosin for histopathological evaluation. Eighty blocks contained more than 75% tumour tissue and 66 blocks contained normal ocular tissue as well. Seven normal eyes without tumour were also examined. Of the 50 µm sections, nuclear suspensions were processed by the method of Hedley et al. The paraffin was dissolved with two washes of xylene. All sections were rehydrated with two 10 minute washes in 100% alcohol, one wash with 96% alcohol, and two washes with 70% and 50% alcohol. The samples were rinsed with distilled water, and centrifuged for 10 minutes at 800 g. The centrifuged tissue was suspended in a test tube containing 0.5% pepsin in 0.9% sodium chloride (pH 1.9 plus 0.02% azide) and incubated for 1 hour at 37°C with repeated vortexing, centrifuged at 800 g and subsequently the cells were resuspended in Hank's balanced salt solution containing ethidium bromide (50 µg/ml). The samples were filtered through a 40 µm nylon mesh filter. The stained samples were measured on a FACS Scan (Becton Dickinson, Sunnyvale, CA, USA). For each histogram 10^6 nuclei were analysed. In these paraffin embedded tissues, artefactual low staining debris and cell clumps tend to be present. Confounding effects of these signals were eliminated by setting a gate. Of 15 cases, a second paraffin block for a different area of the neoplasm was obtained to investigate intratumour heterogeneity.

The five freshly obtained tumours had all been irradiated preoperatively. These samples were prepared and stained according to the method of Vindelov et al. 17

**DATA ANALYSIS**

The DNA-ploidy of the tumour sample was estimated by DNA index, which was calculated as the ratio between the median channel numbers of the first and the subsequent peaks in the sample. Tumour samples were accepted as diploid where there was a single G0/G1 peak. Samples with a coefficient of variation (CV) of the single diploid peak of more than 9% were excluded. The CV was calculated as the full width of the G0/G1 peak at half maximum divided by the mean channel number. In cases with multiple peaks, the population with the lowest DNA content was assumed to represent the diploid population. In the samples from normal eyes the percentage G2M ranged from 3% to 6%. Therefore we defined a histogram as tetraploid if the second peak had a DNA index between 1-9 and 2-1 and the fraction contained more than 8% of the nuclei measured. Samples with a DNA index of the second peak of more than 2-1 or less than 1-9 or with a first peak with a shoulder were defined as DNA aneuploid.

**HISTOPATHOLOGICAL GRADING**

The tumours were classified as spindle cell, epithelioid cell, or mixed cell type according to modified Callender's classification. Five other variables were measured: largest tumour diameter (LTD, <7 mm, 7–10 mm, 10–15 mm, >15 mm); tumour height (<2 mm, 2–3 mm, 3–5 mm, >5 mm); tumour localisation (ciliary body, equator, posterior, diffuse), and scleral invasion (none, less than 50%, 75%, episcleral growth). In addition the tumours were classified according to the TNM system (WHO).

**STATISTICAL ANALYSIS**

Cross tabulation together with the χ2 test were used to compare DNA-ploidy with the six above described variables. For ordinal variables, the Cochran-Mantel-Haenszel trend version of the χ2 , as implemented in spss, was performed. Distribution of (tumour related) survival is described by Kaplan-Meier curves.
which were compared with the log rank test. Where appropriate a trend version of this test was performed.

Results
Seventy two patients were male and 74 were female. The mean age was 61.6 years. A total of 146 tumour samples were analysed. Fourteen cases were excluded, because of a high CV – three of whom had received preenucleation irradiation (Table 1); in 12 of these follow up was known (Table 2). On the remaining 132 cases possible correlations between the various parameters were analysed. Influence of histological cell type, LTD, tumour prominence, tumour localisation, scleral invasion, and TNM classification on survival was studied in 97 patients with adequate follow up (Table 2), whereas the influence of DNA-ploidy on survival was studied in 85 patients with adequate follow up (Table 2). The total mean follow up was 62 months. DNA-ploidy, and LTD were significant in predicting metastatic potential (p≤0.03, and 0.01 respectively) (Figs 1 and 2); histological cell type and tumour location were of borderline significance (p≤0.05).

Thirty seven per cent of the samples were aneuploid, including 17% of the tumours which were tetraploid; 63% were diploid (Table 3). A strong association (p≤0.0009) was found between DNA-ploidy and cell type (Table 3). This association remained significant after correction for LTD (p≤0.009) and TNM classification (p≤0.008) by stratification. No correlation was found between DNA-ploidy and LTD, tumour height, scleral invasion, tumour location, and TNM classification.

A significant association was found between aneuploidy and pre-enucleation irradiation (p≤0.01) (Table 3).

Normal eye tissue was diploid, as assessed on paraffin blocks of seven eyes without abnormalities. Of 12 patients two tumour samples were analysed: in 11 of these (92%) only one DNA peak was found. The five fresh tumours were all aneuploid, including one tumour of the epithelioid cell type.

Discussion
There is increasing evidence for a variety of neoplasms that DNA aneuploidy may correlate with poor prognosis. In primary cutaneous melanomas DNA aneuploidy has been shown to correlate with tumour thickness, incidence of recurrence, and survival. Studies in uveal melanoma demonstrated that an elevated DNA index (>1.4) is strongly correlated with higher tumour related mortality. We found aneuploidy in 37% of the cases, which is consistent with the findings of Meehan and Char. The striking variation in incidence of aneuploidy in earlier studies can partly be explained by the relatively small number of cases studied and the use of fresh tissue. Another explanation may be the differences in the applied techniques, like assessing DNA content on older archival paraffin embedded specimens. In older material increased background noise from fragmented nuclei and cellular debris is important, and as sample age increases, such problems for DNA content seem to increase. Up to a period of 10 years the age of the block does not appear to influence the CV because comparison of DNA-ploidy of fresh tissue samples with formalin fixed paraffin embedded specimens in solid tumours showed a concordance of 87%. None the less, in the latter study near diploid aneuploid peaks observed in histograms from fresh tissues were sometimes not apparent in histograms from paraffin embedded tissues, because of the higher CV for the latter samples. Another problem in paraffin embedded specimens is that, if both diploid and near diploid aneuploid peaks are present, it is difficult to determine which peak is diploid. To obviate this particular problem in 66 cases we measured a mixture of nuclei from a paraffin block of normal tissue and tumour tissue from the same specimen as recommended by Schutte et al.

A further explanation for the variable percentage of aneuploid cases reported is tumour heterogeneity. In a small tumour sample an aneuploid subpopulation of cells might remain undetected. However, in the cases in which we could study two blocks, 92% were concordant. We found the predictive value of aneuploidy for

Table 3 Cross tabulation DNA-ploidy and clinicopathological features

<table>
<thead>
<tr>
<th></th>
<th>Aneuploid</th>
<th>Diploid</th>
<th>Total No</th>
</tr>
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<tbody>
<tr>
<td><strong>Histological type</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epithelioid</td>
<td>15 (60%)</td>
<td>10 (40%)</td>
<td>25 (100%)</td>
</tr>
<tr>
<td>Mixed</td>
<td>24 (42%)</td>
<td>33 (58%)</td>
<td>57 (100%)</td>
</tr>
<tr>
<td>Spindle</td>
<td>19 (18%)</td>
<td>41 (82%)</td>
<td>60 (100%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>48 (37%)</td>
<td>84 (63%)</td>
<td>132 (100%)</td>
</tr>
<tr>
<td>Pre-enucleated irradiation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No irradiation</td>
<td>29 (30%)</td>
<td>68 (70%)</td>
<td>97 (100%)</td>
</tr>
<tr>
<td>2×4 Gy irradiated</td>
<td>19 (54%)</td>
<td>16 (46%)</td>
<td>35 (100%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>48 (37%)</td>
<td>84 (63%)</td>
<td>132 (100%)</td>
</tr>
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</table>
survival to be significant (p=0.03), contrasting with a study on older formalin fixed paraffin embedded specimens.\textsuperscript{9} Our findings indicate that specimens can be used for flow cytometry at least up to 10 years after preparation.

Although simplification of the original Callender classification has improved histological correlation with malignancy, interobserver error can be large.\textsuperscript{23} Subsequently, a more quantitative system was developed to estimate subjectively the percentage of epithelioid cells in each tumour,\textsuperscript{24} however still relying on subjective judgment. Therefore, methods have been developed to measure more objective features. One of these methods is determining DNA-ploidy. We have demonstrated that archival paraffin embedded material can be used for flow cytometry analysis to predict clinical outcome. We found that aneuploidy in uveal melanomas strongly correlates with epithelioid cell type; none of the less, aneuploid, appropriate to have better predictive value for prognosis than subjective histopathological classification. In our study we found that cell type and tumour location were of borderline significance in predicting clinical outcome. This is in contrast with earlier reports on prognostic factors in uveal melanoma,\textsuperscript{25} but in agreement with recent findings of Folberg et al.\textsuperscript{26}

Interestingly, we found irradiated melanomas to be significantly more often aneuploid than non-irradiated melanomas. Radiation induces mitotic delay and both numerical and structural chromosome aberrations.\textsuperscript{27} It is conceivable that during cell cycle progression alterations of chromatid condensation and DNA fluorochrome labelling lead to DNA damage which, in flow cytometry, may give rise to the appearance of pseudo-aneuploid cell populations.\textsuperscript{28} Numerical chromosomal aberrations may give rise to near diploid or near tetraploid histograms. However, the decline in radiation induced aberrations in the initial 24 hours is rapid, presumably as a result of DNA repair.\textsuperscript{29} In vitro studies on a K-1735 melanoma cell line exposed to 7 Gy x irradiation revealed that increase in karyotype diversity generated by radiation, when non-lethal, may accelerate tumour progression.\textsuperscript{30} Unfortunately no paraffin tissue was left to study the relation between DNA aneuploidy and chromosomal aberrations in irradiated melanomas.

We demonstrated a strong correlation (p=0.009) between aneuploidy and epithelioid cell type. Other studies indicated a similar correlation, but were too small to draw statistical conclusions.\textsuperscript{10,31} Aneuploidy may reflect a genetically more unstable population with an enhanced ability to metastasise.

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