Concurrent Loss of Chromosome Arm 1p and Chromosome 3 Predicts a Decreased Disease-Free Survival in Uveal Melanoma Patients

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PURPOSE. Uveal melanoma is a highly malignant disease with a mortality rate of 50% at 10 to 15 years. Previous studies have shown that chromosomal changes are associated with decreased survival of the patient. However, in these studies the small number of tumors analyzed did not allow robust statistical analysis. In the present study, the independent numerical changes in chromosomes 1, 3, 6, and 8 on disease-free survival (DFS) was assessed in a large series of patients with uveal melanoma.

METHODS. One hundred twenty tumors from patients with uveal melanoma were analyzed for numerical changes in chromosomes 1, 3, 6, and 8, with cytogenetic analysis, fluorescent in situ hybridization, and/or comparative genomic hybridization. Data were correlated with disease outcome in univariate and multivariate analyses, by Kaplan-Meier and Cox regression analyses.

RESULTS. At a mean follow-up time of 45 months, 42 patients had died or had metastatic disease. In the univariate analysis, loss of chromosome 3, gain of 8q, largest tumor diameter, or the presence of epithelioid cells was associated with a decreased DFS. In the multivariate analysis, the effect of monosomy 3 on survival was largely modified by changes in 1p36. Regarding all chromosomal changes, only the concurrent loss of the short arm of chromosome 1 and all of chromosome 3 was an independent prognostic parameter for disease-free survival (P < 0.001).

CONCLUSIONS. In uveal melanoma, concurrent loss of the short arm of chromosome 1 and all of chromosome 3 is an independent predictor of decreased DFS. (Invest Ophthalmol Vis Sci. 2005;46:2253–2257) DOI:10.1167/iovs.04-1460

Uveal melanoma is the most common form of primary ocular cancer in adults, with a mortality rate of 50% after 10 to 15 years. Metastases occur predominantly in the liver. Early identification of patients at high risk of metastasis may allow detection of metastases at a stage in which adjuvant therapy can be justified. Several prognostic factors based on clinical and histologic features are known—for instance, gender, age at time of diagnosis, largest tumor diameter (LTD), involvement of the ciliary body, and the presence of epithelioid cells. Nevertheless, none of these prognostic factors is specific enough for identification of patients at risk of metastatic disease.

Cytogenetic abnormalities correlate with the clinical outcome of patients with leukemia and lymphoma. Uveal melanomas are highly amenable to cytogenetic analysis and show mostly simple karyotypes in contrast to most other solid tumors. Nonrandom chromosomal abnormalities, such as variation in the short arm of chromosome 1 and in chromosomes 3, 6, and 8 were detected in these tumors. Loss of chromosome 3 and gain of 8q have been associated with a high mortality rate, whereas abnormalities in chromosome 6 were found to correlate with a good prognosis. However, these data were obtained from studies with relatively small samples. Furthermore, the independent value of these chromosomal changes and the effect of the loss of the short arm of chromosome 1 on survival remain to be determined.

The purpose of the present study was to investigate the association between chromosomal changes and clinical and histologic variables. Furthermore, we sought to examine the independent effect and interactions of numerical changes in chromosomes 1, 3, 6, and 8 on disease-free survival (DFS) of patients with uveal melanoma.

METHODS

Patients and Tumor Samples
From March 1992 to April 2003, we collected tumor tissue of 152 consecutive patients who underwent enucleation for ciliary body or choroidal melanoma. Informed consent was obtained before enucleation, and the study was performed according to the tenets of the Declaration of Helsinki. Fresh tumor tissue was obtained within 1 hour after enucleation, according to a standardized protocol. An incision was made through the tumor, leaving the optic nerve intact. The quantity of tissue obtained (5–8 mm³) depended on the tumor’s size. A sample was taken from the side opposite the optic nerve and divided in two; one part was processed for cytogenetic analysis and/or fluorescent in situ hybridization (FISH), and the other part was stored in liquid nitrogen. Until January 1995, only cytogenetic analysis was performed, which was successful in 15 of 46 cases. From that time on, tumors (n = 106) were analyzed with FISH and, if metaphases could be obtained, with cytogenetic analysis. In the latter ones, comparative genomic hybridization (CGH) analysis (n = 30) was performed on tumor tissue that could not be completely analyzed by these two
techniques. Conventional histopathologic examination was performed on all tumors and confirmed the origin of each one. Cytogenetic studies were also performed on stimulated peripheral blood samples of each patient to exclude the presence of congenital chromosomal abnormalities. Follow-up data from the time of diagnosis until the end of the study in April 2004 were obtained by reviewing each patient’s charts and contacting their general physicians. At that time, three patients were lost to follow-up. From two of these patients, however, a late date of follow-up was obtained, and they were therefore also included in the study. From the 120 patients included in the survival analysis, there were 67 men and 53 women. The age at the time of diagnosis ranged from 21 to 87 years (mean, 61). The mean duration of follow-up, from diagnosis to presence of metastases or end of study, was 45 months (range, 6–142).

### Histologic Findings

The mean and median tumor diameter and thickness were 12.7 ± 3.3 (SD) and 13.0 mm (range, 4.5–19), and 7.8 ± 3.7 and 8.0 mm (range, 1.5–22), respectively. Twenty tumors showed involvement of the ciliary body, and 100 were located in the choroid. Cell type was classified as mixed/epithelioid in 69 tumors and as spindle cell type in 51 tumors.

### Cytogenetic, Fluorescent In Situ Hybridization, and Comparative Genomic Hybridization Analyses

#### Cytogenetic Analysis

Chromosome preparations were made according to standard procedures and stained with acridine orange or Atabrine to obtain R or Q banding. Cytogenetic abnormalities were described in accordance with the International System for Human Cytogenetic Nomenclature (ISCN, 1995).

#### Fluorescent In Situ Hybridization Analysis

Dual color FISH was performed on uncultured tumor tissue, by using centromeres, locus-specific cosmids, P1, or YAC as probes for chromosomes 1, 3, 6, and 8, respectively, as described previously. Seven probes were used: p1-79 (mapped to chromosome band 1p36), P2 3.5 (centromere 3), YAC82T3 (3q24), cos85 (6p21), and cos52 (6q23) (all from Yusuke Nakamura, Tokyo, Japan) and DR22 (centromere 8) and ETO (8q22). The probes were validated on normal peripheral blood cell metaphase spreads, and 10 metaphases were analyzed for each probe. Cutoff limits were less than 3%. The concentration for centromeric probes was 5 ng per slide; for cosmid, P1, and YAC probes, 50 to 75 ng per slide was used. After hybridization and washing, the slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and mounted in anti-fade medium (Dabco-Vectashield 1:1; Vector Laboratories, Burlingame, CA). Signals were counted in 500 interphase nuclei, according to the criteria of Hopman et al.9 Scoring for deletion (>15% of the nuclei with one signal) or amplification (>10% of the nuclei with three or more signals) was adopted from the available literature.

#### CGH Analysis

DNA was isolated from 40 formalin-fixed, paraffin-embedded 10-μm tumor sections. The pigmented tumor was scraped off the glass slides with a fine scalpel. Excised material was deparaffinized in xylene and ethanol and air-dried. Isolation of DNA was performed by a DNA tissue kit (Qiagen, Hilden, Germany). Concentration was determined with a fluorometer (Bio-Rad, Veenendaal, The Netherlands), and molecular weight was estimated on ethidium-bromide–stained agarose gels. Tumor DNA and reference DNA (0.5 μg) were labeled with a kit (Bio-prime; Invitrogen, Breda, The Netherlands, with Spectrum Green; Vysis, Hoofddorp, The Netherlands, or Alexa 594; Molecular Probes, Leiden, The Netherlands, respectively). An equal probe mixture of tumor and reference DNA was denatured and hybridized in the presence of human cot-1 DNA to normal male metaphase chromosomes for 3 days at 37°C. Samples were counterstained with DAPI in anti-fade solution. Images were acquired with a microscope (Axioplan; Carl Zeiss Meditec, Jena, Germany) with software from Metasystems (Iris; Metasystems, Altlussheim, Germany). In each case, 10 metaphases were analyzed. Loss of DNA sequences was defined as chromosomal regions where the mean green-to-red ratio was below 0.8, and gain was defined as chromosomal regions where the ratio was above 1.2. Threshold levels were determined on the basis of analysis of known chromosomal aberrations.

### Data Classification

Using cytogenetic and FISH analysis, we subdivided the variation in chromosomes 1, short arm; 3, short arm and long arm; and 8, long arm, into three categories: loss of one copy, normal copy numbers (two), and gain of one or more copies. Monosomy 3 was defined, using FISH, as when there was only one signal for both the centromere 3 and 3q24 probes. Gains 6p and 8q were scored when more than two signals were found for both the 6p21 and 8q22 probe, and loss of 1p and 6q when the probes for 1p36 and 6q23 showed only one signal. When different subclones were identified, only the FISH findings of the largest clone were classified. Cytogenetic and CGH results were classified for those regions studied with FISH analysis. All major chromosomal changes detected by cytogenetic analysis could also be detected by FISH analysis.

### Statistical Analysis

The primary end point for DFS was the time until development of metastatic disease compared with the time of enucleation, wherein death due to other causes was censored. The influence of single prognostic factors on DFS was assessed using the log rank test (for categorical variables) or Cox proportional hazard analysis (for continuous variables), and Kaplan-Meier curves were used to illustrate the differences in survival. Comparisons of the distributions of clinical and chromosomal variables were performed with the Fisher exact test (for categorical variables) and the Mann-Whitney test (for continuous variables). To identify the independent value of the prognostic factors on DFS, we used a multivariate Cox proportional hazard analysis and the likelihood ratio test. Possible prognostic factors were age at time of diagnosis, cell type (spindle cell versus mixed/epithelioid cell), largest tumor diameter, mutual loss of 1p36 and 3, and gain of 8q. All tests were two-sided. An effect was considered significant at $p \leq 0.05$. The statistical analyses were performed on computer (SPSS-11; SPSS, Chicago, IL).

### Results

Of the 152 uveal melanomas, 32 cases could not be analyzed for the short arm of chromosome 1, or chromosome 3, 6, and 8 abnormalities. A total of 120 uveal melanoma cases were analyzed for chromosomal changes by using cytogenetic, FISH, and/or CGH analyses. Cytogenetic analysis was successful in 69 of 120 tumors. For 55 tumors, cytogenetic and FISH data were available, whereas for 47 tumors, only FISH was performed. In addition, in 30 tumors, CGH analysis was performed. Not all probes could be tested on all tumors because there was not enough tissue. The mean number of probes successfully used for FISH was 5.5. Combining the results of cytogenetic, FISH, and CGH analyses showed genomic abnormalities in 88% of the 120 tumors. Results for all chromosome regions (1p, 3, 6p, 6q, and 8q) were obtained for 108 tumors (varying from 108 to 118 successful analyses per region, Table 1). Thirty-eight patients had died of metastatic disease and four had metastases at the time of evaluation.

Univariate analysis of the single prognostic factors showed significantly lower DFS in patients with loss of chromosome 3 and gain of 8q and a mixed/epithelioid cell type in the tumor compared with patients without these chromosomal changes or with a spindle cell type (Table 1). The largest tumor diameter was also significant in the univariate analysis. Other potential prognostic factors such as gender, age at time of diagnosis, tumor thickness, and tumor location (i.e., involvement of...
To examine the possibility that other chromosomal variations may affect the prognosis of the patients bearing monosomy 3, we constructed Kaplan-Meier curves of changes in chromosome 3, stratified for the other chromosomal changes, and performed log rank tests (results not shown). We found that the effect of monosomy 3 on DFS was substantially modified by changes in the copy number of 1p36. In tumors with a normal copy number of 1p36, a small difference in DFS was observed between those patients with and without loss of chromosome 5 (P = 0.064), whereas this difference was significant in patients with tumors who also had loss of 1p36 (P < 0.001). The interaction term between tumors with loss of 1p36 and 3 and the remaining patients (i.e., patients with tumors with a normal number of 1p36 and chromosome 3 or with loss of either 1p36 or 3) was highly suggestive (hazard ratio [HR] = 3.61), but did not reach significance (P = 0.155). In addition, we compared the DFS of patients with a concurrent loss of 1p36 and 3 with the remaining patients, by using the log rank test. The difference in survival was significant (P < 0.001; Fig. 1). Remarkably, a gain of 1p36 occurred in five patients, but this number was too small to perform statistical analysis.

Considering the strong interaction between losses of 1p and chromosome 3, we validated whether this concurrent loss is an independent parameter for DFS. Significant correlations between age at time of diagnosis, tumor diameter, cell type, and gains in 6p and 8q were observed, according to the Mann-Whitney and Fisher exact tests (Table 2). Monosomy 3 was associated with age at time of diagnosis (P = 0.050), cell type (P = 0.013), and mean tumor diameter (P = 0.002). A gain of 6p correlated with cell type (P = 0.008) and gain of 8q with mean tumor diameter (P < 0.001). These chromosomal changes and confounding variables were analyzed in a multivariate model. After correcting for these variables, we found that patients with tumors with concurrent loss of 1p36 and 3 have an almost 7.8 times higher chance of having metastases than do those without these losses or with loss of either 1p36 or 3 (P = 0.039; Table 3). A gain in 8q (HR = 2.43, P = 0.054) and mixed/epithelioid cell type (HR = 2.24, P = 0.077) almost reached significance, and the other variables (gain of 6p, largest tumor diameter, and age at time of diagnosis) were not significant. Furthermore, the interaction term between monosomy 3 and gain of 8q was not significant (HR = 0.53; P = 0.469). When analyzed in a multivariate model with the confounding variables, such as age at time of diagnosis, gain of 6p, cell type, and mean tumor diameter, we found an HR of 0.67 with P < 0.001 (data not shown).

### DISCUSSION

Previous studies have demonstrated the nonrandom occurrence of cytogenetic abnormalities of chromosomes 1, 3, 6, and 8 in uveal melanoma. Monosomy 3 and a gain of 8q have been shown to be associated with poor survival after treatment for uveal melanoma.6,11,12 Using univariate analysis, we confirmed these findings. In addition, we demonstrated in the present study, which is the largest series described so far and therefore allows multivariate statistical analysis, that tumors with a concurrent loss of chromosome 3 and of 1, region p36, are at high risk of metastasizing (HR = 7.81; Table 3). The molecular genetic changes that underlie these chromosomal changes have not yet been determined.

Loss of 1p occurs frequently in many solid tumors such as cutaneous melanoma and neuroblastoma. In the latter tumor type, loss of 1p is known to be a predictor of an unfavorable outcome.13,14 In uveal melanoma loss of 1p has been described, but any prognostic significance had not been determined up to now. Contrary to Sisley et al.,15 in our study, loss of 1p36 was not associated with large ciliary body melanomas, but was rather detected in metastasizing tumors, in agreement with Aalto et al.16 Eighty-nine percent of the metastasized tumors with loss of 1p36 loss had concurrent monosomy 3. Concurrent loss affecting survival suggests an interaction of proteins encoded by genes located on these chromosomes that may promote tumorigenesis and metastatic disease and, consequently, reduce survival. However, we cannot exclude the possibility that these sites encode for proteins that may independently promote tumorigenesis and metastasis.

Gain of 8q was a significant predictor of survival in the univariate analysis. In the multivariate analysis, it did not reach statistical significance as an independent prognostic marker. Previous studies have suggested that the acquisition of the long arm of isochromosome 8 is a secondary event and that the gain of additional copies is related to tumor size.11,17,18 Moreover, monosomy 3 seemed to predispose to isochromosome formation.4 This may explain the correlation of the gain of 8q with...
survival, as observed in other studies.\textsuperscript{6,11} We demonstrated a strong correlation between the largest tumor diameter and the presence of abnormalities in 8q, suggesting that acquisition of additional copies of 8q may result in an increased size of the tumor.

Similarly, the abnormalities in chromosome 6 were not independently associated with survival, in contrast to previous claims.\textsuperscript{6} We found a strong correlation between gain of 6p and the spindle cell type. Sisley et al.\textsuperscript{12} and White et al.\textsuperscript{6} associated chromosomal changes, such as loss of chromosome 3; gain of chromosome 8, long arm; and abnormalities in chromosome 6 with prognosis.\textsuperscript{6,15} However, as far as we know, their findings were not corrected for tumor diameter or cell type, as in the present study. This could have influenced their findings, leading to contradictory observations. Another known prognostic marker for a poor outcome of patients with uveal melanoma is the presence of epithelioid cells. We found a strong correlation between chromosomal aberrations (chromosomes 3, 5, 6, and 8) and cell type (Table 2). Even though epithelioid or mixed cell type was significantly associated with decreased DFS in the univariate analysis, it was not in the multivariate analysis.

Although loss of an entire chromosome is a common change in uveal melanoma, partial deletions of chromosome 3 have been reported, leading to the hypothesis that two regions, one on the short arm and one on the long arm, may be involved in metastasis.\textsuperscript{19} Seven patients in our study had a partial deletion of chromosome 3 (either one copy of the centromeric region or the 3q region), from which two died of metastatic disease. In five tumors, two signals for the centromere and only one signal for the 3q probe were observed, whereas two tumors had one copy of the centromere and two of the 3q probe. Because these changes were observed with FISH analysis and karyograms of these tumors were not available, we were not able to identify any breakpoints. These and more subtle structural aberrations can be resolved with techniques with higher resolution, such as genomic arrays or loss of heterozygosity. However, changes such as base substitutions, very small deletions, or insertions will still be missed.

Our study of chromosomal abnormalities in uveal melanoma is, to our knowledge, the largest series reported in the literature. The study may be biased, because we examined only tumors from patients treated by enucleation, as no tumor tissue is available from patients treated with radiotherapy protocols. There is a need to stratify patients prospectively into low- and high-risk groups for metastases. Our findings suggest that chromosomal abnormalities may be useful in identifying patients at high risk of metastases. Previous studies by Sisley et al.\textsuperscript{20} have shown a correspondence between major clonal alterations in fine-needle aspiration biopsies (FNABs) and the main tumor, by using cytogenetic techniques. Furthermore, they showed that with short-term cultures of FNABs conventional cytogenetic analysis was possible in 60% of the cases. In addition, Naus et al.\textsuperscript{18} indicated that application of FISH on FNABs is a reliable method for assaying genetic prognostic parameters. Only in 0.8% a small variation that could have led to a misclassification was found.

There are at least two potential challenges involved in the application of our data to patients on a prospective basis. First, our study involved samples from relatively large tumors that were treated by enucleation. It remains to be seen whether our data can be applied to smaller tumors that are to be treated by radiation therapy. Second, despite correspondence between chromosomal abnormalities detected from FNAB samples and tissue retrieved at enucleation, there are no studies to date that confirm the uniform distribution of cytogenetic abnormalities in uveal melanoma, and it is at least theoretically possible that an FNAB may capture tissue that does not contain the cytogenetic markers of interest. Nevertheless, data from our study, the largest cohort of patients studied to date for cytogenetic abnormalities in primary uveal melanoma, suggest the feasibility of studying patients with uveal melanoma in prospective trials. using samples retrieved by FNAB.

<table>
<thead>
<tr>
<th>Variable</th>
<th>HR</th>
<th>P†</th>
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<tbody>
<tr>
<td>Loss of 1p36 with loss of 3</td>
<td>7.81</td>
<td>0.039</td>
</tr>
<tr>
<td>Gain of 8q</td>
<td>2.43</td>
<td>0.054</td>
</tr>
<tr>
<td>Mixed/epithelioid cell type</td>
<td>2.24</td>
<td>0.077</td>
</tr>
<tr>
<td>Gain of 6p</td>
<td>1.33</td>
<td>0.558</td>
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<td>Largest tumor diameter</td>
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<td>0.588</td>
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<td>Age at time of diagnosis</td>
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<td>0.900</td>
</tr>
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HR, hazard ratio.
\textsuperscript{a} Multivariate analysis using Cox proportional hazard analysis.
\textsuperscript{†} Likelihood ratio test.
Acknowledgments

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References