Higher Percentage of FISH-Determined Monosomy 3 and 8q Amplification in Uveal Melanoma Cells relate to Poor Patient Prognosis

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PURPOSE. To investigate the relation between patient survival and incrementally increasing percentages of fluorescence in situ hybridization–determined complete loss of chromosome 3 (monosomy 3) and gain of chromosome 8q in primary uveal melanoma cells.

METHODS. Clinicopathological factors were related to disease-free survival. Fluorescence in situ hybridization was performed using probes on chromosomes 1, 3, 6, and 8. The percentages of UM cells with monosomy 3 or chromosome 8q gain were classified in groups with incrementally increasing percentages and related to disease-free survival. Correlations between clinical factors and cytogenetic aberrations were also analyzed.

RESULTS. Two-hundred twenty choroidal and ciliary body melanomas were analyzed. The following proved to be significant predictors of survival in univariate analysis: older patient age (P = 0.005); large tumor diameter (P < 0.001); mixed cell type (P = 0.001); presence of closed microvascular loops (P < 0.001); loss of chromosome 1p (P = 0.006); monosomy 3 (P < 0.001); gain of 6p (P < 0.001); and gain of chromosome 8q (P < 0.001). Multivariate Cox analysis displayed monosomy 3 (Hazard ratio [HR] 2.83, P = 0.002) and gain of chromosome 8q (HR 3.13, P = 0.002) as the most important independent prognostic factors of poor survival, followed by older patient age (HR 1.02, P = 0.017). Increasing percentages of monosomy 3 and gain of chromosome 8q in tumor cells showed a correlation with worse prognosis (Log-rank test 45.7, P < 0.001). Increasing number of additional copies of 8q correlated with shorter disease-free interval (Log-rank test 45.7, P < 0.001).

CONCLUSIONS. A high percentage monosomy 3 and chromosome 8q gain in primary UM cells showed a strong relation with poor disease-free survival compared with low percentage aberrations. (Invest Ophthalmol Vis Sci. 2012;53:2668–2674) DOI:10.1167/iovs.11-8697

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Uveal melanoma is the most common primary intraocular malignancy in adults with an annual incidence of 5–7 cases per million. Nearly half of all patients with UM eventually die of metastases which are most often late-appearing.¹–² In search for prognostic factors, several clinical, pathological, and genetic parameters have been identified. Genetic factors have proven to be the most significant factors of all and can reliably indicate high risk of metastasis and poor survival in patients with UM.³–⁴ Nonrandom chromosomal alterations are present in more than 80% of cases with most frequently a complete loss of chromosome 3 (monosomy 3) in 50% of cases. Monosomy 3 is the most important chromosomal factor relating to a 4-year overall survival of only 30%.⁵–⁶ Other known but less frequently occurring alterations that also relate with prognosis are gain of chromosome 6p or 8q, loss of chromosome 1p or 6q, and co-occurrence of chromosome 1p and 3 loss.⁷–⁹

Fluorescence in situ hybridization is a reliable technique for assessing chromosomal aberrations in UM; therefore, many large referral centers routinely use fluorescence in situ hybridization (FISH) for the analysis of the chromosome 3 status of a tumor. FISH enables in situ analysis of chromosomal aberrations in tumor cells and by using a cutoff threshold for identification of loss, tumors are either classified as chromosome 3 disomic or monosomic. By classifying tumors in one of these groups using a cutoff threshold, information on exact percentages of aneuploidy and their possible relation to prognosis is disregarded. Using FISH analysis, this study assessed the percentages of tumor cells with loss of chromosome 3 or gain of chromosome 8q for each tumor separately and correlated these findings with patient prognosis. Tumors were classified according to the FISH counts in groups of incrementally increasing percentages of chromosome 3 or 8q aneuploidy: 15%–33% (10%–33% for gain of chromosome 8q), 33%–66%, and 66%–100%, and investigated whether a high percentage of aneuploidy in the tumor is related to a decreased survival. If so, this could provide a more precise prognosis for patients with low, intermediate, and high percentages of chromosome 3 or 8q aneuploidy in their tumor and could be used for selecting patients eligible for adjuvant therapy.

METHODS

Between July 1994 and November 2010, tumor material was collected from 248 patients who underwent enucleation for UM. Thirteen iris melanomas and fifteen hyperaneuploid cases were excluded from this study because of the differences in molecular behavior.¹⁰,¹² Routine clinical systemic evaluation including blood liver function tests was conducted before enucleation was performed. Fresh tumor tissue was harvested within 1 hour after enucleation of the remaining 220 ciliary-body and choroidal melanomas, and was processed for histopathologic and genetic research. Histopathologic examination...
was conducted according to standardized protocols and confirmed the origin of the tumor as well as tumor size, cell type, and presence of microvascular patterns (closed vascular loops). Informed consent was obtained prior to enucleation and the study was performed according to guidelines of the Declaration of Helsinki. Clinical data and follow-up data regarding metastases and tumor-related death were obtained from medical records and by contacting the general physician. In total, three patients were lost to follow-up: the first patient was 57 years old when he was lost to follow-up after 28 months because he moved abroad to an unknown destination. The second patient was 89 years old and was lost to follow-up after 69 months; the third patient was 93 years old and lost to follow-up after 18 months. These three patients had no sign of metastasis at the last follow-up moment.

**Fluorescence In Situ Hybridization**

Fluorescence in situ hybridization allows interphase cytogenetic analysis of fresh or archival tumor tissue by using differentially labeled fluorescent probes mapping to specific chromosomal regions. With this technique, copy number alterations can be determined in a large number of cells. Fresh tumor tissue from enucleated eyes containing UM was routinely used for direct interphase FISH (chromosome 1, 3, 6, and 8) as described previously. The following probes were used: RP11-48E9 (1p36); RP11-384L8 (3p22) or RP11-384L8 (3p22); D8Z2 (centromere 8); and RP11-88J22 (6q21); RP11-24P4 (8p21); and a 3q25 probe, to allow for assessment of monosomy of chromosome 3 and chromosome 8q status in 201 cases (91%). Monosomy 3 was present in 12 of 19 tumors where data on chromosome 8q status was missing. Co-occurrence of monosomy 3 and gain of chromosome 8q was present in 102 of 201 cases (51%); monosomy 3 without gain of 8q was present in 20 cases (10%); and gain of 8q without monosomy 3 was present in 32 cases (16%). Of the 220 patients recruited for this study, 121 were male. The mean age of all patients was 62 years (median 62 years, range 21–87 years). The mean duration of follow-up, from diagnosis to end of study, was 4.7 years (range 0.3–15.9 years), with metastases occurring at a mean follow-up of 3.1 years (range 0.3–11.0 years). Eighty-one patients died from metastatic disease and five were diagnosed with metastases at the time of evaluation.

Univariate analysis of the single prognostic risk factors showed a significantly decreased survival for patients with UM with the presence of epithelioid cells, closed vascular loops, loss of chromosome 1p, monosomy 3, and gain of chromosome 8q (Table 1). Large tumor diameter and older patient age were significantly related to poor survival as well. A gain of chromosome 6p was related to a more favorable prognosis.

Kaplan-Meier survival analysis displayed poor survival probabilities for patients having tumors with monosomy 3 (Log-rank test 36.5, P < 0.001) (Fig. 1a). Survival probabilities were even worse if the patients’ tumors had a high percentage of monosomy 3 in analyzed cells, and Kaplan-Meier survival analysis showed a significantly worse survival for patients with high percentage of monosomy 3 in their tumor compared with patients having tumors with low or intermediate percentage monosomy 3 (Log-rank test 49.9, P < 0.001) (Fig. 1b). The disomy 3 group was analyzed next to medium and high percentage aneuploidy groups as well and displayed a more favorable prognosis than the higher percentage aneuploidy groups (Fig. 1c). Presence of chromosome 8q gain in tumors also correlated with worsening patient survival (Log-rank test 31.6, P < 0.001) (Fig. 1d). The high and intermediate percentage gain of chromosome 8q groups displayed comparable survival probabilities, which were worse than with low percentage gain (Log-rank test 40.4, P < 0.001) (Fig. 1c). The disomy 8q group was analyzed next to higher percentage aneuploidy groups as well, displaying a more favorable prognosis (Fig. 1f).

Cox proportional hazard analysis was performed with all factors that were significant after univariate analysis to exclude confounding variables and identify the independent prognostic value of chromosome 3 and 8q in this cohort. Older age, monosomy 3, and gain of 8q proved to be independent negative prognostic factors. If these factors were stratified for the different age groups and increasing percentages of monosomy 3 or gain of chromosome 8q, the highest age group and highest percentage tumor aneuploidy groups...
showed the strongest correlation with poor survival (Table 2). General prognostic factors such as tumor diameter, epitheloid cell type, presence of closed vascular loops, loss of chromosome 1p, and gain of 6p lost significance after multivariate analysis.

Tumors with high percentage monosomy 3 were larger in diameter than tumors with low percentage monosomy 3 ($P = 0.028$) (Table 3). The correlation between high percentage gain of chromosome 8q and large tumor diameter was slightly stronger than for monosomy 3 ($P = 0.024$). There was no direct correlation found between older patient age and larger tumor size. Tumors with monosomy 3 and a high percentage of chromosome 8q gain frequently had additional copies of chromosome 8q present (Fig. 2), which related to worse patient survival (Fig. 3, Log-rank test 45.7, $P < 0.001$). Moreover, an increased number of additional copies of chromosome 8q related to a shorter disease-free interval.

**DISCUSSION**

Study findings confirm the importance of monosomy 3 as an important prognostic cytogenetic factor for metastatic disease in UM. Additionally, gain of chromosome 8q and older patient age were classified as important independent prognostic factors in this study. Our FISH results show a gradually worsening patient prognosis for UMs with incrementally increasing percentages of monosomy 3. A higher percentage gain of chromosome 8q (more than 33% of tumor cells) correlates more with worsening survival than low percentage gain.

**TABLE 1.** Univariate Analysis of Prognostic Markers on Disease-Free Survival in 220 Uveal Melanomas

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean, Median (range)</th>
<th>No. of Patients (%)</th>
<th>Missing Data (%)</th>
<th>$P$ Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis</td>
<td>62 yrs, 62 (21–87)</td>
<td></td>
<td>−</td>
<td>0.003</td>
</tr>
<tr>
<td>Largest tumor diameter</td>
<td>12.8 mm, 13.0 (2–30)</td>
<td></td>
<td>−</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Tumor height</td>
<td>7.5 mm, 7.0 (1–22)</td>
<td></td>
<td>−</td>
<td>0.178</td>
</tr>
<tr>
<td>Male gender</td>
<td>121 (55)</td>
<td>−</td>
<td>0.218</td>
<td></td>
</tr>
<tr>
<td>Mixed/epitheloid cell type</td>
<td>154 (70)</td>
<td>−</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Involvement ciliary body</td>
<td>30 (14)</td>
<td>4 (2)</td>
<td>0.065</td>
<td></td>
</tr>
<tr>
<td>Closed vascular loops</td>
<td>90 (41)</td>
<td>15 (7)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Loss of chromosome 1p</td>
<td>66 (30)</td>
<td>−</td>
<td>0.006</td>
<td></td>
</tr>
<tr>
<td>Monosomy 3</td>
<td>134 (61)</td>
<td>−</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Gain of chromosome 6p</td>
<td>93 (42)</td>
<td>26 (12)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Loss of chromosome 6q</td>
<td>65 (30)</td>
<td>33 (15)</td>
<td>0.332</td>
<td></td>
</tr>
<tr>
<td>Gain of chromosome 8q</td>
<td>134 (61)</td>
<td>19 (9)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>

* Cox-regression analysis.
† Log-rank test.
8q gains (in 10%–33% of tumor cells), while the intermediate and high percentage 8q aneuploidy groups display comparable patients’ survival (Fig. 1). High percentage monosomy 3 in our study relates to a 4-year overall survival probability of 15%–20% (Figs. 1B, 1C). This is comparable to or slightly worse than the reported 4-year overall survival of 30% for monosomy 3 tumors in general.5,6 Also in the low percentage monosomy 3 group, patients had an increased risk of metastasis compared with the disomy 3 group (HR = 1.9) (Table 2); and 3 out of 9 patients from the low percentage group died from metastasis after a mean follow-up of 2.1 years (range 0.4–3.8 years). With a longer follow-up, even more patients may develop metastases. Interestingly, these patients with metastases all had low percentage monosomy 3 next to a high percentage gain of chromosome 8q. Figure 2 illustrates that a low percentage of monosomy 3 may coincide not only with higher percentages of gain of chromosome 8q, but also additional copies of chromosome 8q in tumor cells. This supports previous results of Sisley et al.16 where additional copies of chromosome 8q predicted a worse disease-free survival.

Considering polysomy 8q, low percentage gain showed borderline favorable prognosis after multivariate analysis (HR = 0.923), which is remarkable as all other aneuploidy groups are correlated with poor prognosis. This result could be due to the small number of patients in this group and may, therefore, need more cases to allow for a reliable estimation of the risk of metastasis for this group. At present, 2 out of 12 patients from this group of low percentage gain of chromosome 8q died from metastasis. Of the remaining patients within this group, eight have a tumor with simultaneous loss of chromosome 3 and four patients have a follow-up of less than 2 years. Considering this, these patients might develop metastasis in the near future too.

Co-occurrence of chromosome 3 and 8 was reported before16,17 and may be referred to as genetic imbalance, as stated by Patel et al.18 Current study findings indicate the importance of determining chromosome 8q status when there is no monosomy 3 or only low percentage monosomy 3 in a tumor, as this may coincide with a high percentage gain of chromosome 8q (or increased copies of 8q) and lead to worsening patient survival. This hypothesis is also supported by Patel et al.18 where a genetic imbalance (monosomy of chromosome 3, gain of chromosome 8, or both) was associated with worsening survival. However, the cutoff limits in the study by Patel et al. were 30% due to the sensitivity of the probes used. The authors report two patients with genetic imbalance in 20% of tumor cells who survived for over 100 months, but also hypothesized that these patients might develop metastases in the long run, and that minimal genetic imbalances (of 5%–10%) could lead to development of metastatic disease. Bronkhorst et al. also report that tumors with 5% of monosomy 3 correlate to a high risk of metastatic disease using a centromere probe. However, in their study, they state that a threshold of 30% for monosomy 3 predicts high risk of metastasis more accurately. Even though the low and intermediate percentage aneuploidy groups in our study were small, still a significant number of patients with monosomy in less than 30% of tumor cells died due to metastasis. Therefore, using a threshold of 30% and higher for monosomy 3 will not lead to identification of these high-risk patients who would consequently be excluded from any adjuvant treatment.

In contrast to monosomy 3, chromosome 8 alterations are known to be a late event in UM development, relating to large tumor size.20 This relation with tumor size was confirmed with the present study as high percentage chromosome 8q gain in tumor cells related to a larger tumor diameter than low

### Table 3. Correlation between Tumor Size and Abnormalities of Chromosome 3 and 8q

<table>
<thead>
<tr>
<th>Clinical Data</th>
<th>Chromosome-3 Loss</th>
<th>P Value</th>
<th>Chromosome-8q Gain</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15%–33% Cells</td>
<td>66%–100% Cells</td>
<td></td>
<td>10%–33% Cells</td>
</tr>
<tr>
<td>Mean tumor diameter (mm)</td>
<td>12.2</td>
<td>13.4</td>
<td>0.028</td>
<td>11.8</td>
</tr>
<tr>
<td>Mean tumor thickness (mm)</td>
<td>7.5</td>
<td>8.0</td>
<td>0.341</td>
<td>6.5</td>
</tr>
</tbody>
</table>
percentage 8q gain (Table 3). The high percentage gain of chromosome 8q group also frequently showed increased copy numbers of 8q (Fig. 2), which in turn correlated with reduced patient survival and shorter disease-free interval (Fig. 3). This could indicate that when UMs grow larger, cytogenetic alterations accumulate in an increasing number of cells, leading to additional copies of chromosome 8q and worsening survival. On the other hand, presence of cytogenetic alterations in tumor cells may give the tumor a growth advantage.

It remains a major issue whether actual percentages of aberrations found in analyzed tumor sections reflect the situation in all parts of the tumor. Several groups already reported intra-tumor heterogeneity to be present in UM, and biopsy taking may therefore result in sampling error. However, discordance of chromosome 3 results was only found in a minority of cases analyzed by fine needle aspiration biopsy specimens and direct single-cell suspensions and paraffin sections of different parts of the tumor. This leads to misclassification in less than 1% of patients. Dopiera et al. analyzed 32 UMs by multiplex ligation-dependent probe amplification for different parts of the tumor and reported heterogeneity of chromosome 3 in 47% of cases, not leading to misclassification when compared with the whole tumor. The MLPA technique provides a relative quantification of monosomy 3 (and multiple other chromosomal regions) and cells with disomy 3 in the different tumor regions may dilute the obtained results. Fluorescence in situ hybridization, on the other hand, enables absolute quantification of monosomy 3 in single tumor cells. The problem of sampling error and misclassification is thought to be less important if larger enucleation specimens are used, as these
are more representative of the tumor than biopsies.\textsuperscript{21,23} In this study, larger enucleation specimens were used from the patient tumors, minimizing the risk of misclassification.

Single nucleotide polymorphism array is a recent molecular genetic technique based on a series of DNA segments orderly arranged on a chip to which fluorescently labeled DNA can be hybridized. With this technique, rapid assessment of copy number alterations as well as zygosity changes on a genome-wide level with a high resolution is possible. MLPA allows for copy number analysis of up to 50 chromosomal regions in one experiment and is also less labor-intense than the FISH technique. Nevertheless, an important advantage of the FISH technique is that absolute copy numbers can be assessed and low mosaic cases (alterations in low percentage of cells) can be detected, which is more difficult with SNP-array and MLPA. This study demonstrated that even patients with low percentage aneuploidy of chromosomes 3 and 8q, who are at risk for developing metastasis, can be identified by FISH with absolute quantification of additional copies of chromosome 8q as well.

This study is, based on current literature and data, the first to use incrementally increasing FISH counts of chromosome 3 losses and 8q gains, and evaluate its impact on disease-free survival. In total, 220 patients were studied and analyzed by FISH, providing first steps toward a more individualized prognosis for UM patients. Future studies are needed in order to obtain more cases with low and intermediate percentages of chromosome 3 loss and chromosome 8q gain and enable an even more reliable comparison of these groups. There is a bias toward the larger tumors, since only enucleated eyes were included in this study. In the future, in-vivo biopsy of UMs treated by eye-sparing techniques may provide new information on the distribution of chromosome 3 and 8q alterations in small and medium-sized tumors. The importance of chromosome 3 alterations in UM was recently further demonstrated by Harbour et al.,\textsuperscript{24} who reported on frequent somatic and one germline BAP1-mutation, located on chromosome 3p21.1, in class 2 metastasizing melanomas. In a previous study from this group, monosomy 3 was detected in four out of five class 2 tumors.\textsuperscript{25} It would be interesting to assess whether patients with monosomy 3 tumors from this present study have a mutant BAP1-gene on the remaining allele as well. If so, then it is worthwhile to determine whether BAP1 mutations have a better predictive value than monosomy 3.

In conclusion, patients having tumors with a high percentage of monosomy 3 have a slightly worse 4-year overall survival probability than patients with monosomy 3 tumors in general. The patients with an increased number of additional copies of chromosome 8q in their tumor are at risk of early metastasis. Since patients with a high percentage monosomy 3, intermediate or high percentage gain of chromosome 8q, and additional copies of 8q in their tumor cells have a high risk of early metastasis, this group could be eligible for adjuvant treatment preventing the development of metastasis. Therefore, results of adjuvant therapies may be observed much earlier within this group than with classic long-term studies.

References


