The Prognostic Value of Extraocular Extension in Relation to Monosomy 3 and Gain of Chromosome 8q in Uveal Melanoma

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Submitted: November 25, 2013
Accepted: January 24, 2014

Keywords: uveal melanoma, extraocular extension, monosomy 3, gain of chromosome 8q, survival

Purpose. To identify the prognostic value of extraocular extension in enucleated uveal melanoma (UM) patients and to correlate extraocular extension to chromosomal aberrations, metastasis-free survival (MFS), and clinico-histopathological risk factors.

Methods. Retrospective study of patients with UM treated with enucleation between 1987 and 2011. Melanoma-related metastasis and death were recorded. Statistical analysis (log-rank test or Cox regression analysis) was performed to correlate MFS with tumor characteristics, extraocular extension, episcleral diameter of the extraocular extension, cell type, extracellular matrix patterns, inflammation, loss of chromosome 3, and gain of chromosome 8q.

Results. In 43 (12%) of 357 patients, extraocular extension was observed. In this subset of patients, we noted a reduced survival of 70 months (105.5 months, P = 0.010) compared with patients without extraocular extension (175.8 months). Patients with gain of chromosomal region 8q in UM with extraocular extension had an increased risk of metastatic disease (P < 0.001). In multivariate Cox proportional hazard analysis, largest basal tumor diameter (P = 0.001), extracellular matrix patterns (P = 0.009), episcleral diameter of the extraocular extension (P = 0.016), loss of chromosome 3 (P < 0.001), and gain of 8q (P < 0.001) were independent predictors for MFS.

Conclusions. Larger episcleral diameter of the extraocular extension and additional gain of chromosome 8q in extraocular extension UM correlates to a worse prognosis. MFS is significantly reduced in UM with a large basal tumor diameter, extracellular matrix patterns, loss of chromosome 3, and gain of chromosome 8q.

The age-adjusted incidence of uveal melanoma (UM) is 5.1 per million since 1973.1 During the past decades, several risk factors have been identified and related to survival. Clinical factors that correlate with poor survival are a large tumor thickness and tumor basal diameter, ciliary body localization, mushroom configuration, and older age.2,3 The tumor size is of great importance, as each millimeter increase in tumor thickness seems to increase the risk of metastases by approximately 5%.4 Histopathological risk factors associated with decreased survival are epithelioid cell type, high mitotic activity, presence of extracellular matrix patterns, and extraocular extension.5–7 UM with epithelioid cells tend to have a more aggressive behavior and are therefore related to a poor clinical outcome. From the known prognostic parameters, the genetic alterations are by far the most strongly associated with metastatic disease. Loss of chromosome 3 or monosomy 3 is observed in approximately 50% of the UM and is not only associated with clinical but also with histopathological prognostic factors and metastatic death.8–11 A higher percentage of monosomy 3 leads to a poorer disease-free survival.12 The same is true for gain of chromosome 8q, and when these abnormalities occur simultaneously, the prognosis is even worse.13 Van den Bosch et al.12 showed that gradual increase in copy number of chromosome 8q shortened survival. Extraocular extension occurs in 2% to 15% of the UM.3,5,14–16 Tumors with extraocular extension are classified in a different subclass of the TNM classification and are associated with a worse prognosis.3,14 Moreover, the larger the extension diameter, the shorter the survival will be. The 5-year survival of UM patients with an extraocular extension of 5.1 mm or more is between 18% and 22%.14,16 Extraocular extension has been correlated with monosomy 3; however, no associations have been found between extraocular extension and chromosome 8q alterations.3,13 Therefore, the aim of this study was to identify monosomy 3 and gain of 8q as additional risk factors, besides clinical and histopathological...
factors, in UM with extraocular extension and correlate these with metastasis-free survival (MFS).

**METHODS**

**Patients and Clinical Characteristics**

A retrospective study was carried out by the Rotterdam Ocular Melanoma Study group, in patients with a choroidal or ciliary body UM who underwent primary or secondary enucleation from 1987 until 2011. We excluded patients with iris melanoma and cases in which no sufficient tumor material was available to describe the histopathological characteristics of the tumor. The following data were recorded: sex, location of the tumor, date of enucleation, age at time of enucleation, development of metastases, and date and cause of death. If measurements of the tumor thickness and largest basal diameter from B-scan ultrasonography (US) were not available, we used the tumor's measurements before histological preparation. Tumor measurements obtained before fractionated stereotactic radiotherapy (FSRT) were used if patients had received primary FSRT. Data on extraocular extension was registered during US, surgery, or histopathologically. Patients with extraocular extension were selected based on their pathology report.

Informed consent was obtained before treatment and the study was performed according to guidelines of the Declaration of Helsinki. Until 1999, all patients were enucleated; hereafter, enucleation was performed only if the tumor was too large for FSRT (basal tumor diameter > 16 mm and tumor thickness > 12 mm) or if the patient requested enucleation. MFS was defined as the time in months from enucleation until the development of metastasis. We obtained survival data up to April 2013 by reviewing patients' charts and contacting their primary physician. Patients were screened for the presence of metastasis by testing liver enzymes in peripheral blood every 6 months for the first 5 years and thereafter annually. If these were elevated, an abdominal US or computed tomography scan was carried out.

**Histopathology**

Fresh tumor material was obtained within 1 hour of enucleation and processed for further histopathological and cytogenetic analysis. Conventional histopathological examination with hematoxylin and eosin (H&E) staining of formalin-fixed and paraffin-embedded eyes was performed on all tumors and confirmed the origin of the tumor. The intraocular part of the tumors was evaluated for the presence of inflammation and necrosis. Inflammation was defined as any obvious clusters of lymphoid inflammatory cells in the tumor assed by H&E staining. Microfoci of necrosis were accepted as positive. H&E staining was used to differentiate between an epithelioid, mixed, or spindle-cell type according to the modified Callender classification. Extracellular matrix patterns were visualized in tumor specimens stained with periodic acid-Schiff reagent. The mitotic rate was determined only in tumors with extraocular extension by counting the mitosis in 8 mm² equal to 50 high-power fields. Extraocular extension was confirmed by revision of all histopathological sections by an ophthalmic pathologist (RV), and was defined as tumor growth through the sclera and beyond the outer scleral surface. Subsequently, the largest diameter of the extension of the tumor on the scleral surface was measured. The surgical margin was examined for infiltration of UM cells extending from the extraocular extension. We determined the route of extraocular spread and involvement of optic nerve, ciliary body, or choroid.

**Cytogenetic Analysis**

We determined the copy number status of chromosomes 3 and 8 of the intraocular part of the primary tumor with fluorescence in situ hybridization (FISH) analysis by using centromeric and locus-specific probes on directly fixated tumor cells for chromosomes 3 and 8. A deletion was scored if more than 15% of the nuclei showed one signal for chromosome 3 (probe P33.5) and/or 3q24 (probe YAC 827D3). Amplification was scored if more than 10% of the nuclei had three or more signals for 8q22 (probe RP11-88J22). For tumor samples collected from December 2000, we used a probe located on 3q25 (RP11-64F6). FISH analysis was performed in most tumors. In some tumors, the chromosome status was solely based on comparative genomic hybridization (CGH) (n = 8), karyotyping (n = 18), or single nucleotide polymorphism (SNP) array (n = 21). In 78 tumors, both FISH and SNP array were used to determine monosomy 3 or gain of 8q. CGH and FISH analysis were performed according to the protocol described by Naus et al. For whole genome analysis, we used an SNP array (Illumina HumanCytoSNP-12 v2.1 BeadChip and Illumina 610Q BeadChip; Illumina, San Diego, CA). Two hundred nanograms of fresh tumor DNA was used as input. The data were analyzed with version 6 of the Nexus software (Biodiscovery, Inc., El Segundo, CA). BioDiscovery’s SNP-Rank Segmentation Algorithm, an extension of the Rank Segmentation algorithm (a statistically based algorithm similar to the Circular Binary Segmentation algorithm) was used to make copy number as well as loss of heterozygosity (LOH) calls. SNP-Rank Segmentation takes into account both the log-R as well as the B allele frequency value at each probe location to create a segment. The significance threshold for segmentation was set at 5.0E-7, also requiring a minimum of three probes per segment and a maximum probe spacing of 1000 kilobase pairs (Kbp) between adjacent probes before breaking a segment. The log ratio thresholds for single copy gain and single copy loss were set at 0.15 and -0.15, respectively. The log ratio thresholds for two or more copy gain and homozygous loss were set at 0.41 and -1.1, respectively. The homozygous frequency threshold was set to 0.95. The homozygous value threshold was set to 0.8. The heterozygous imbalance threshold was set to 0.4. The minimum LOH length was set at 100 Kbp. Polyploid tumors with a relative loss of chromosome 3 were also considered as monosomy 3 UM. This is also applicable for relative gain of chromosome 8q.

**Statistical Analysis**

Tumors with an epithelioid and mixed cell type were classified as tumors containing epithelioid cells for further statistical analysis. The primary end point for MFS was the development of metastatic disease. Cases in which the cause of death was unknown or not related to their UM, were treated as censored. The importance of prognostic factors on MFS was assessed using the logrank test (for categorical variables) or Cox regression analysis (for continuous variables). The significance of associations between clinico-histopathological, chromosomal variables and extraocular extension were calculated with the Pearson’s χ² test or Fisher’s exact test (for categorical variables) and the Mann-Whitney test (for continuous variables). Multivariate analysis using the forward stepwise method was conducted for the variables that were significant in univariate analysis. A two-tailed P value less than or equal to 0.05 was considered significant. Statistical analyses were performed with SPSS version 20.0 software (SPSS, Inc., Chicago, IL).
**RESULTS**

**Patients**

In total, 357 patients were included in this study. The mean age was 61 years at time of enucleation (range, 21–90). The mean largest basal tumor diameter was 12.5 mm (range, 2.0–22.0) and the mean tumor thickness was 7.3 mm (range, 1.0–24.0). Overall, 20 patients received brachytherapy and one patient received proton beam radiation before enucleation. These patients did not have extraocular extension. Genetic testing of the UM patient who received proton beam radiation was conducted 20 months after the enucleation. These patients did not have extraocular extension. Genetic testing of the UM patient who received proton beam radiation was conducted 20 months after the enucleation.

The tumor characteristics for the patients with extraocular extension versus patients without extraocular extension are shown in Table 1. Extraocular extension was identified in 43 (12%) of 357 patients (Fig. 1A). The mean age of the patients with extraocular extension was 64 years (range, 29–86). The mean largest basal tumor diameter and mean tumor thickness for this group of patients with extraocular extension were 14.2 mm (range, 6.0–22.0) and 7.7 mm (range, 1.5–22.0), respectively. Tumor localization (P = 0.045) and largest basal tumor diameter (P = 0.002) correlated with extraocular extension (Table 1).

**Histopathology**

Several histopathological features were determined for the extraocular extension (Fig. 1B). For instance, the (largest) episcleral diameter of the extraocular extension ranged from 0.1 to 40.0 mm, with a mean of 2.9 mm. Necrosis was found in 23 of 43 histopathological slides. The mean mitotic rate was 9.95/8 mm² (range, 0.00–29.00). Absence of inflammation (P < 0.001) was associated with extraocular spread (Table 1). Eleven of the choroidal tumors invaded the ciliary body and all the ciliary body tumors invaded the choroid. The tumors did not show a significant difference in size of the mean largest basal tumor diameter and mean tumor thickness within the extraocular extension group (P = 0.001). The tumors with extraocular extension were located posteriorly; 11 UMs invaded through the long posterior ciliary nerves. Besides these routes of invasion, 41 tumors also invaded perivascularly and 29 tumors invaded perineurally. In total, three UMs with extraocular spread invaded the lamina cribrosa through three different routes:
transciliary, and short and long ciliary nerves. However, the optic nerve resection margin was free of malignant cells.

In 20 patients, infiltrating UM cells extending from the extraocular extension were observed at the surgical margin. Of these patients, seven received postoperative irradiation and one patient with a 40-mm extraocular extension underwent an orbital exenteration. Thus far, orbital recurrence was noticed in one patient with a free surgical margin. Seven of the 20 patients with irradial enucleation were still alive at the last follow-up date. There was no significant difference in mean survival between patients with (72.7 months, 95% confidence interval [CI] 46.9–98.5, log-rank test, \( P = 0.660 \)) and without (120.6 months, 95% CI 67.7–173.6) a free surgical margin.

**Cytogenetic Analysis**

Loss of chromosome 3 was present in 61.5% (176/286) of all UMs and in 64.1% (25/39) of the tumors with extraocular extension (Table 1). This was not statistically different from cases without extraocular extension. Gain of chromosome 8q was present in 67.4% (188/279) of all UMs and in 71.1% (27/38) of the UMs with extraocular extension. Forty-five patients had gain of 8q with disomy of chromosome 3, and 141 patients had gain of 8q combined with monosomy 3. An example of a case with loss of chromosome 3 and gain of chromosome 8q on SNP array is depicted in Figure 2. Twenty-five extraocular extension patients showed gain of chromosome 8q combined with monosomy 3. Due to lack of material, chromosome 3 and 8 status could not be examined in all extraocular extension patients.

**Survival Analysis**

The mean MFS of the overall group was 76.6 months (range, 0.0–508.5). Irrespective of extraocular extension, 145 patients (40.6%) developed metastasis with a mean survival of 41.8 months (range, 0.0–207.7) and 158 patients (44.3%) were alive at the end of the follow-up with a mean survival of 104.3 months (range, 0.8–308.5). Forty-three patients (12.0%) died due to other disease causes, such as a ruptured aneurysm or a myocardial infarction. The mean survival of this group was 93.4 months (range, 0.2–270.0). Eleven patients were lost to follow-up, of which five patients moved abroad and the other six patients moved to another city and did not provide their general practitioner with information or withdrew from ophthalmologic follow-up.

The follow-up of patients with extraocular extension is shown in Table 2. The survival was significantly reduced in patients with extraocular extension versus without extraocular extension (105.5 vs. 175.8 months, respectively, log-rank test, \( P = 0.010 \)).

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Univariate analyses of prognostic factors showed a significantly shorter MFS in tumors with a larger episcleral diameter of the extraocular extension (hazard ratio [HR] 1.120, \( P < 0.001 \)), epithelioid cells (154.6 vs. 188.2 months, \( P = 0.002 \)), extracellular matrix patterns (87.1 vs. 167.6 months, \( P < 0.001 \)), monosomy 3 (96.8 vs. 151.6 months, \( P < 0.001 \)), and gain of 8q (97.1 vs. 169.8 months, \( P < 0.001 \)) (Table 1). In addition, we conducted univariate survival analysis for extraocular extension UM patients only and the episcleral diameter of the extraocular extension remained significant (HR 1.079, \( P = 0.040 \)).

The MFS was significantly longer in the overall group without chromosomal aberrations compared with patients with these aberrations (Figs. 3A, 3C). UM with disomy 3 and normal 8q versus gain of 8q (171.6 vs. 123.2 months, \( P = 0.004 \)) showed a prolonged survival compared with UM with monosomy 3 and normal 8q versus gain of 8q (143.5 vs. 78.1 months, \( P < 0.001 \)).

In the subgroup of extraocular extension, patients with and without monosomy 3 had a survival of 73.5 months and 92.0 months, respectively (\( P = 0.056 \)) (Fig. 3B). Patients with extraocular extension and gain of 8q had a reduced survival compared with patients with normal chromosome 8q (\( P < 0.001 \)) (Fig. 3D). We validated the interaction between extraocular extension and gain of 8q and its effect on the MFS in a separate multivariate model.

In multivariate analysis, the largest basal tumor diameter, extracellular matrix patterns, episcleral diameter of the extraocular extension, chromosome 3 loss (HR 2.654, \( P < 0.001 \)), and chromosome 8q gain (HR 2.874, \( P < 0.001 \)) were independent prognostic factors on MFS (Table 3). Prognostic factors, such as presence of epithelioid cells, extraocular spread in general, tumor thickness, and age, were rejected after multivariate analysis.

**DISCUSSION**

UM patients with extraocular extension are a clinically challenging group of patients, as there are only a few studies that have a large cohort of patients for analysis and often a
limited duration of follow-up. In our study, we reviewed 357 ciliary body and choroidal UMs, of which 43 (12%) had extraocular extension with a mean follow-up of 6.4 years (range, 0.0–25.7 years). As observed in previous studies, we also found that UM patients with loss of chromosome 3 and/or gain of chromosome 8q in their melanoma have a significantly reduced MFS ($P < 0.001$). In addition, we observed that gain of chromosome 8q was associated with a worse prognosis in patients with extraocular extension. Besides that, patients with extraocular extension developed metastases or died due to metastases almost 6 years earlier, on average, compared with patients without extraocular extension (log-rank test, $P = 0.01$).

Monosomy 3 and gain of chromosome 8q (or concurrent presence of abnormalities on chromosomes 3 and 8) and extraocular extension have already separately been identified as risk factors in several other studies. Nevertheless, gain of chromosome 8q in combination with extraocular extension has not been related to survival. A near significant trend ($P = 0.056$) was observed between monosomy 3 and extraocular extension regarding survival. With a larger patient group, a relation to patient survival could be noted. Histopathological factors have been described and related to survival in patients with extraocular extension. Coupland and associates found that epithelioid cell type and high mitotic rate were related to extraocular spread and poor prognosis. In our series, UMs with an epithelioid cell type were also related to a reduced survival, though this did not correlate with extraocular extension ($P = 0.868$). Because the percentages of epithelioid cells in the group of extraocular extension and without extension were similar, and although a difference in survival was measured, epithelioid cell type appeared not to be the most important prognostic factor in our population. In our multivariate analysis, the presence of epithelioid cells, extraocular spread in general, tumor thickness, and age were rejected. These prognostic factors were significant predictors of survival in the univariate analysis. In the multivariate analysis, age nearly reached statistical significance as an independent prognostic marker ($P = 0.051$). In previous studies, older age and presence of epithelioid cells have proven to have a significant effect on survival.

In concordance with previous studies, we also found that clinical factors, such as a larger basal tumor diameter and the presence of extracellular matrix patterns, were associated with a decreased survival, whereas the size of the extraocular extension did not correlate significantly with metastatic death in all studies. With an increasing size of extraocular extension diameter, the 5-year survival seems to decline: 81% in patients without extension, 49% in patients with a 0.1- to 5.0-mm extension diameter, and 18% in patients with 5.1-mm

| Table 2. Follow-up of Patients Stratified for the Presence of EXE |
|------------------------|------------------------|------------------------|
| Patients | Without EXE, $n = 314, n (%)$ | Patients With EXE, $n = 43, n (%)$ |
| Alive | 144 (45.9) | 14 (32.6) |
| Melanoma-related death and metastases | 121 (38.5) | 24 (55.8) |
| Death due to other cause | 38 (12.1) | 5 (11.6) |
| Lost to follow-up | 11 (3.5) | 0 (0.0) |
or more extension diameter. In our analysis, we found that an increase of 1 mm in episcleral diameter led to a nearly 1.1 times increase in risk of developing metastatic disease (HR 1.078). We had only three patients in the subcategory of greater than or equal to 5.1 mm, and for this reason we could not perform statistical analyses for this group. In these three patients, one patient had metastasis at time of diagnosis (diameter extension of 40 mm) and another patient died due to a non-melanoma-related cause without metastasis (diameter extension of 9 mm) with a follow-up of 110.4 months at an age.

**Table 3.** Cox Multivariate Regression Analysis Correlating With Metastatic Disease

<table>
<thead>
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<th>P Value</th>
<th>HR</th>
<th>95% CI</th>
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<tr>
<td>Largest basal tumor diameter</td>
<td>0.001</td>
<td>1.094</td>
<td>1.037</td>
</tr>
<tr>
<td>Extracellular matrix patterns</td>
<td>0.009</td>
<td>1.674</td>
<td>1.137</td>
</tr>
<tr>
<td>Largest episcleral diameter of the extraocular extension</td>
<td>0.016</td>
<td>1.078</td>
<td>1.014</td>
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<tr>
<td>Loss of chromosome 3</td>
<td>&lt;0.001</td>
<td>2.634</td>
<td>1.570</td>
</tr>
<tr>
<td>Gain of chromosome 8q</td>
<td>&lt;0.001</td>
<td>2.874</td>
<td>1.651</td>
</tr>
</tbody>
</table>

**Figure 3.** Survival probability plots for chromosomes 3 and 8q in the overall group (A, C) and in the extraocular extension group (B, D).
of 86 years. The third patient with a 6-mm extension was still alive at 41.9 months and had other favorable prognostic factors, such as the absence of genetic aberrations, absence of extracellular matrix patterns, absence of mitotic figures, and a free surgical margin. Interestingly, all three UMs contained epithelioid cells.

Orbital recurrence has been reported in 3% to 25% of the patients undergoing enucleation for UM with extraocular extension. In our study, only one patient, with an initial tumor-free surgical margin, had an orbital recurrence after 7 months and was exenterated. Nevertheless, orbital recurrence is described even 20 and 42 years after enucleation. In 20 of 43 UM patients with extraocular spread, melanoma cells extending from the extraocular extension were found at the surgical margin. Of the irradiated patients, the mean survival was 71.2 months (range, 10.4–257.1), and was almost similar to patients without additional treatment, 79.2 months (range, 0.0–254.2). Nevertheless, incomplete surgical removal of the tumor, especially if the extraocular part of the tumor is nonencapsulated, remains one of the most important risk factors for orbital recurrences. In our group of patients with incomplete resection, we found no cases with orbital recurrence. Nowadays most patients with extraocular extension will be treated with additional therapy or surgery.

In this study, we associated extraocular extension with chromosomal abnormalities of chromosomes 3 and 8 in UM. Compared with other studies, our patient group has a long follow-up with a mean MFS of 6.4 years, and only a few patients were lost to follow-up. Extraocular extension was histologically proven and reviewed by an oculomotor pathologist in a relatively large group of UMs. Because this is a retrospective study, some data were missing. For example, we could not detect histopathological or chromosomal aberrations in all patients due to necrosis or lack of material. Because we studied only enucleated eyes and not patients who have had eye-conserving treatments, our group contained relatively large UMs. This selection bias could influence survival, because in general larger tumors have a worse prognosis. Still, in our multivariate analysis, other parameters remained significantly associated with a decreased survival. Chromosome 3 and 8q status was determined in almost all patients with FISH, and in some cases with additional SNP array analysis. Intraduct heterogeneity has been described in a small number of UMs in our research group previously, although no structural difference in monosomy 3 distribution occurred between the base and the apex of the tumor. On the other hand, genetic heterogeneity of chromosomes 3 and 8 has been reported between the intracocular and extraocular part of the UM, and for monosomy 3 between the apex and base of the tumor. This variation of monosomy 3 in intrac- and extraocular parts of UM was demonstrated by Lake et al. with multiplex ligation-dependent probe amplification (MLPA) in only 10 patients. Despite a certain heterogeneity, tumors can be classified correctly for monosomy 3 or gain of chromosomal region 8q, as is the case in our study, as we used either FISH and/or confirmed these results with SNP array in a large group of our patients. Moreover, from previous studies we know that the percentage of chromosomal aberrations does not influence the development of metastases, but can influence the time to development of metastatic disease. In our series, we found that MFS is significantly reduced in UMs with a large basal tumor diameter, extracellular matrix patterns, loss of chromosome 3, and gain of chromosome 8q. Loss of chromosome 3 itself is not related to extraocular extension, but a gain of chromosomal region 8q in tumors with extraocular extension increases the risk of metastatic disease.

Acknowledgments

Supported by the Stichting Nederlands Oogheelkundig Onderzoek. The authors alone are responsible for the content and writing of the paper.

Disclosure: J.G.M. van Beck, None; A.E. Koopmans, None; J. Vaarwater, None; J.J. de Rooi, None; D. Paridaens, None; N.C. Naus, None; A. de Klein, None; R.M. Verdiijk, None; E. Kiliç, None

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