Metastatic Disease in Polyploid Uveal Melanoma Patients Is Associated With BAP1 Mutations

Serdar Yavuziçitoglu, Hanneke W. Mensink, Kyra N. Smit, Jolanda Vaarwater, Robert M. Verdiç, Berna Beverloo, Hennie T. Brüggenwirth, Ronald van Marion, Hendrikus J. Dubbink, Dion Paridaens, Nicole C. Naus, Annelies de Klein, and Emine Kılıç

1Department of Ophthalmology, Erasmus University Medical Center, Rotterdam, The Netherlands
2Department of Clinical Genetics, Erasmus University Medical Center, Rotterdam, The Netherlands
3The Rotterdam Eye Hospital, Rotterdam, The Netherlands
4Department of Pathology, Erasmus University Medical Center, Rotterdam, The Netherlands

Correspondence: Emine Kılıç, Erasmus University Medical Center, Department of Ophthalmology, Room Number Fc-1610, P.O. Box 2040, 3000 CA Rotterdam, The Netherlands; e.kilic@erasmusmc.nl.

Purpose. Most of the uvea melanoma (UM) display a near-diploid (normal, ~2N) karyotype with only a few chromosomal changes. In contrast to these simple aberrations, 18% of the UM samples show a polyploid character (>2N) and this was associated with an unfavorable prognosis. This study attempts to gain insight in the prognostic value of polyploidy in UM.

Methods. In 202 patients the ploidy status of the UM was determined using cytogenetic analysis, fluorescence-in-situ-hybridization (FISH), multiplex ligation dependent probe amplification (MLPA), and single nucleotide polymorphism (SNP) array analysis. Immunohistochemistry was used to determine the BAP1 expression and mutation analyses of BAP1 (coding regions) and the mutation hotspots for the SF3B1, EIF1AX, GNAQ, and GNA11 genes was carried out using Sanger sequencing or whole-exome sequencing.

Results. Twenty-three patients had a polyploid UM karyotype (11.4%). Patients with a polyploid tumor had larger tumors (15.61 vs. 13.13 mm, P = 0.004), and more often loss of heterozygosity of chromosome 3 (P = 0.003). No difference in occurrence of mutations between polyploid and diploid tumors was observed for BAP1, SF3B1, EIF1AX, GNAQ, and GNA11. Polyploidy did not affect survival (P = 0.143). BAP1 deficiency was the only significant independent prognostic predictor for patients with polyploid tumors, with a 16-fold increased hazard ratio (HR 15.90, P = 0.009).

Conclusions. The prevalence of mutations in the UM related genes is not different in polyploid UM compared with diploid UM. Moreover, similar to patients with diploid UM, BAP1 mutation is the most significant prognostic predictor of metastasis in patients with polyploid UM.

Keywords: uveal melanoma, BAP1, polyploidy, chromosomal abnormalities, oncology

Ocular melanoma (UM) is the most common primary intraocular malignancy in adults with an annual incidence of approximately 7 to 10 per million.1 In approximately one-half of the patients UM metastasizes via the blood with a preference for the liver.1 The prognostic factors linked to metastatic disease include clinical variables (increased age, large tumor size), histopathologic findings (epithelioid cell type, closed vascular patterns), genetic, and chromosomal abnormalities (loss of chromosome 3, gain of chromosome 8q).2–5

For UM, the karyotype is usually near-diploid (normal, ~2N) with only few nonrandom chromosomal changes, such as loss of chromosome 3 (monosomy 3) and gain of chromosome 8q.6 Besides these near-diploid (~2N) tumors, UM with polyploidy (>2N) have also been described. Based on DNA content, a prevalence of 13% to 18% of polyploid UM has been observed.7–9 In addition to the prevalence, the prognostic value of the ploidy was also described, in which polyploidy was associated with an unfavorable prognosis.7–9 However, despite the impact on survival, polyploidy in UM is not mentioned in recent literature or investigated with the current knowledge of UM.

Nowadays in UM research, the focus is more on genetic variations. Monosomy 3 in combination with the loss of function of the tumor suppressor BAP1 (BRCA-associated protein 1) is strongly associated with metastases.10–13 In contrast, mutations in the SF3B1 (splicing factor 3 subunit B1) gene and the EIF1AX (eukaryotic translation factor 1A) gene are reported mainly in disomy 3 (no loss of chromosome 3) tumors.14–16 Therefore, mutations in SF3B1 or EIF1AX have been suggested as favorable prognostic factors in UM, with low risk of metastasis.10,14–17 Mutations in the oncogenes GNAQ (Guanine nucleotide-binding protein, q polyepitope) and GNA11 (Guanine nucleotide-binding protein, subunit alpha-11) are present in the majority of UM and are not associated with patient prognosis.18–20

This study attempts to describe the differences between polyploid and diploid UM regarding clinical variables, histopathologic findings, chromosomal abnormalities, and genetic variations. Mutations in the SF3B1, EIF1AX, GNAQ, and GNA11 genes were analyzed. Immunohistochemistry was used to determine BAP1 expression. In 202 patients, the ploidy status of UM was determined using cytogenetic analysis, fluorescence-in-situ-hybridization (FISH), and single nucleotide polymorphism (SNP) array analysis.
TABLE 1. Patient and Tumor Characteristics of the Study Cohort, and Associations Between Ploidy of the Tumor With Other Clinical, Histopathologic, and Genetic Variables

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<td>Diploid, Mean or n</td>
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</tr>
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<tr>
<td>Mutated</td>
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<td>47</td>
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* P values ≤ 0.005 was considered significant after correction for multiple testing. TNM classification was not compared since tumor diameter and thickness are analyzed separately. Bold numbers indicate significant P values at P ≤ 0.005.
† Mann-Whitney U test was used for associations with continuous data.
‡ χ² test or § Fisher’s exact test was used for associations with categorical data.

Mutational Analyses

Mutation analyses using Sanger sequencing or obtained from whole-exome sequencing (WES) was available for 126 samples. Variants found in the WES data were validated by Sanger sequencing. BAP1, SF3B1, EIF1AX, GNAQ, and GNA11 mutation analyses and BAP1 immunohistochemistry (IHC) were carried out as reported previously.13,18,25,26 For five polyploid samples, no fresh or frozen tissue was available, therefore DNA was isolated from formalin-fixed, paraffin-embedded (FFPE) tissue.
Polyploidy in Uveal Melanoma

**RESULTS**

**Patient and Tumor Characteristics**

Polyploid UM was detected in 23 of 202 patients (11.4%). Nine patients were male and 14 were female with a mean age at diagnosis of 62.5 years. Mean tumor diameter was 15.6 mm with a mean tumor thickness of 8.9 mm. Histopathologically, 19 tumors contained epithelioid cells and 11 formed extracellular matrix patterns. All tumors (n = 23) showed a relative loss of chromosome 3 (>4N), resulting in LOH for chromosome 3 in 20 tumors (Fig. 2), whereas three tumors (S28, S102, and S156) still contained two different alleles despite the relative loss (Figs. 1, 2). For chromosome 8q all polyploid UM had more than two copies. 19 tumors had a relative gain (>4N), all copies were present in three tumors (4N; S147, S156, and S161) and one tumor (S75) had three copies of chromosome 8q (Fig. 1). An overview of the clinical, histopathologic, and chromosomal variables are shown in Table 1.

**Statistical Analyses**

Disease-free survival (DFS) was calculated as date of first initial treatment to date of clinically proven metastasis from UM. The Log-rank test was used for categorical variables, and Cox proportional hazard analysis for continuous variables. Statistical significant variables conducted from univariate analysis were analyzed using Cox proportional hazard multivariate analysis. P values of 0.05 or lower were considered significant for survival analyses. χ² or Fisher’s exact test was used for associations with categorical data; Mann-Whitney U test was used for associations with continuous data. P values of 0.005 or lower were considered significant for correlation analyses. All statistical analyses were performed with SPSS 21.0 Software (IBM, Armonk, NY, USA).

**Genetic Analyses UM Genes**

Within the patients with polyploid tumors 12 patients harbored a BAP1 mutation, which were hemi- or homozygous in all cases. In 13 of 22 patients, the tumors did not express BAP1 (examples provided in Fig. 3). In one case (S111) the lack of tumor material restricted us to investigate BAP1 both for mutations and expression. One patient (S76) could not be investigated for BAP1 mutations, but did reveal loss of BAP1 expression. In one patient (S125), the tumor did not harbor a mutation in the coding exons, but had a loss of expression of BAP1 in the tumor. One patient (S121) harbored a missense mutation in the tumor, p.E30G, whereas the IHC did show expression of BAP1. This mutation was predicted as ‘Deleterious’ by SIFT software and ‘Probably damaging’ by PolyPhen-2 software (Harvard Medical School, Boston, MA, USA). All three patients (S76, S121, and S123) were treated as BAP1-deficient tumors in further analysis. SF3B1 was mutated in four samples targeting the hotspot p.R625 in three cases and p.V576Del in one case. EIF1AX harbored a missense mutation, p.G15D, in one case, which was predicted as ‘Deleterious’ by SIFT software and ‘Probably damaging’ by PolyPhen-2 software. Twelve tumors harbored a GNAQ p.Q209 hotspot mutation, 10 harbored a GNA11 p.Q209 hotspot mutation, and one tumor was wild-type for exon 4 and 5 of both genes. An overview of the mutations with the corresponding polyploid tumor is shown in Figure 1.

Mutations in BAP1, SF3B1, EIF1AX, GNAQ, and GNA11 in the patients with diploid UM were described previously.13,18,25

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**Figure 1.** Overview of mutations and copy number variation in polyploid UM. The TNM classification is represented on the first line. **First row** of blocks represent the GNAQ and GNA11 mutation status; dark gray, GNAQ mutation; light gray, GNA11 mutation; n.d. = not determined. All mutations were exclusive. **Second row** of blocks represent the BAP1, SF3B1, and EIF1AX mutation status; black, BAP1 mutation; striped, SF3B1 mutation; gray, EIF1AX mutation; and white, wild-type for the three genes. All mutations were exclusive. **Third row** of blocks represent the EIF1AX expression; white, EIF1AX expressed; black, EIF1AX not expressed. **Fourth row** represent the alleles of chromosome 8q; CNV = copy number variation (baseline is four copies); black, allele A; light gray, allele B; white, loss of chromosome. **Fifth row** represent the alleles of chromosome 3; CNV = copy number variation (baseline is four copies); black, allele A; light gray, allele B; white, loss of chromosome; dark gray, gain chromosome(s). * In these samples the BAP1 mutation status could not be determined.
Statistical Analyses

Based on tumor ploidy, patients did not differ in age at diagnoses, sex, tumor localization, tumor thickness, cell type, and presence of extracellular matrix patterns. Patients with a polyploid tumor had significantly larger tumors than patients with a diploid tumor (15.6 vs. 13.1 mm, \( P = 0.004 \); Table 1).

For chromosomal abnormalities we classified the copy number changes for polyploid UM in two ways. For chromosome loss, we determined the relative loss from baseline and also loss with LOH. For chromosome gain, we determined absolute gain from disomy state and relative gain from baseline. Patients with polyploid UM showed more loss of chromosome 3 (\( P < 0.001 \)), which was still significant after correcting for LOH (\( P = 0.003 \)). For chromosome 8q, the polyploid UM contained more often absolute gain (>2N; \( P < 0.001 \)). Relative gain of chromosome 8q was observed more often in polyploid UM (\( P = 0.047 \)), and after correcting the \( P \) value for multiple testing to \( P \) less than or equal to 0.005 this was not considered significant. Mutational frequencies did not differ between polyploid and diploid UM for BAP1, SF3B1, EIF1AX, GNAQ, and GNA11.

Survival Analyses

To test whether polyploidy in UM was associated with worse disease-free survival we performed survival analyses for the total group (\( n = 202 \)). Ploidy was not associated with prognosis, because patients with polyploid tumors, as a group, did not differ from patients with diploid tumors based on the survival (Fig. 4A). Univariate analyses results are shown in Table 2. Also in the multivariate Cox-regression analyses, polyploidy was not associated with disease-free survival. Larger basal tumor diameter (HR 1.110; \( P = 0.015 \)) and BAP1 deficiency (HR 5.152; \( P < 0.001 \)) were the only independent significant predictors for disease-free survival in the total cohort (Table 2).

Survival analysis was also performed for patients with polyploid UM to investigate prognostic predictors within this subset (\( n = 23 \)). Loss of heterozygosity of chromosome 3 (\( P = 0.050 \)), BAP1 deficiency (\( P = 0.001 \)), and SF3B1 wild-type mutation status (\( P = 0.035 \)) were significantly associated with decreased disease-free survival. Other variables were not significantly associated with disease-free survival (Table 2).
Chromosome 3, BAP1, and SF3B1 status, together with tumor diameter (because this was associated with polyploid UM) were included into the multivariate Cox analyses. This showed BAP1 deficiency as the only significant independent prognostic predictor for patients with polyploid tumors, with a 16-fold increased HR (HR 15.90, \( P = 0.009 \); Table 2).

**DISCUSSION**

In our cohort polyploidy occurred in 11.4% of the patients with UM. Previously, we as well as other groups have reported ranges between 13% and 18%,\(^8,9\) and this difference in prevalence can be explained by the different methods which were used to determine the ploidy status and the DNA index (DI) thresholds which were adapted to classify a tumor as polyploid. Meecham et al.\(^7\) report polyploidy in 13% of the UM with flow cytometry measurements and a threshold of DI greater than 1.4 for polyploidy. Toti et al.\(^9\) report polyploidy in 18% of the UM cases, while maintaining a threshold of DI greater than 1.3 for polyploidy, which would explain the higher prevalence of polyploidy. Mooy et al.\(^8\) reports tetraploidy (4N) in 17% of the cohort; however, this subset also contains preirradiated tumors, which they also correlate to a higher prevalence of aneuploidy.

When compared with patients with diploid UM, we found larger tumor diameter in the polyploid patient group. Polyploid UM also contained more LOH of chromosome 3. We could not confirm previous findings, which stated that polyploidy as a group is associated with worse patient survival.\(^7,9\) However, we did find that BAP1 deficiency was the most significant factor associated with survival in patients with a polyploid UM, similar to diploid UM.

BAP1 expression has been shown as an independent prognostic marker in UM.\(^10,12,13\) The gene is located on chromosome 3, and is mutated mainly in tumors with loss of chromosome 3,\(^15\) resulting in the loss of BAP1 expression.\(^13\) One sample harbored a missense mutation (p.E30G), whereas the staining did reveal BAP1 expression. This mutation was predicted ‘Deleterious’ and ‘Probably damaging’ by the prediction software’s. Moreover, this mutation is located at the first \( \beta \)-sheet of the protein and also next to three amino acids (p.E31–p.Y33), which form a binding site for ubiquitin,\(^27\) making it likely that the replacement of the negatively charged

**FIGURE 3.** Examples of BAP1 immunohistochemistry of two polyploid uveal melanoma cases. *Left picture:* BAP1 expression in the tumor cells of S28 (×400). *Right picture:* lack of BAP1 expression in the tumor cells of the case S162 (×400). Note the positive staining in the retinal pigment epithelium cells and macrophages.

**FIGURE 4.** Kaplan-Meier survival analyses for: (A) polyploid UM compared to diploid UM (\( P = 0.143 \)) and (B) survival analyses between polyploid and diploid UM stratified for BAP1 status (\( P < 0.001 \)).
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<th>Multivariate</th>
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<td>P Value</td>
<td>HR</td>
<td>95% CI</td>
<td>P Value</td>
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<tr>
<td>Largest tumor diameter</td>
<td>0.937–1.261</td>
<td>0.270</td>
<td>-</td>
<td>-</td>
<td>0.156</td>
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</tr>
<tr>
<td>Tumor height</td>
<td>0.909–1.229</td>
<td>0.471</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Cell type</td>
<td></td>
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<tr>
<td>Spindle</td>
<td>22.0–149.2</td>
<td>0.624</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Mixed/epithelioid</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Closed vascular loops</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Present</td>
<td>17.1–96.2</td>
<td>0.129</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>Absent</td>
<td>52.5–128.6</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
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<tr>
<td>Ploidy</td>
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<td></td>
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<tr>
<td>Diploid</td>
<td>99.6–128.0</td>
<td>0.143</td>
<td>-</td>
<td>-</td>
<td>0.568</td>
<td></td>
</tr>
<tr>
<td>Polyploid</td>
<td>49.2–117.9</td>
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<td></td>
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<tr>
<td>Chromosome 3</td>
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<tr>
<td>Loss, with LOH</td>
<td>66.5–167.4</td>
<td>0.156</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>Normal</td>
<td>35.0–107.0</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
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<tr>
<td>Chromosome 8q</td>
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<tr>
<td>Normal</td>
<td>123.7–211.5</td>
<td>0.001</td>
<td>15.90</td>
<td>1.97–128.3</td>
<td>0.009</td>
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<tr>
<td>Gain, relative</td>
<td>16.4–42.5</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>BAP1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>123.7–211.5</td>
<td>0.001</td>
<td>15.90</td>
<td>1.97–128.3</td>
<td>0.009</td>
<td></td>
</tr>
<tr>
<td>Deficient</td>
<td>16.4–42.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>SF3B1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutated</td>
<td></td>
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</tbody>
</table>
glutamic acid with the neutral glycine causes a structural malformation of the protein resulting in a deficiency of BAP1. The BAP1 expression can be explained, because the mutation is a missense mutation and does not lead to protein degradation. Also, the affected amino acid is located in the N-terminal UCH domain, whereas the antibody used for the staining target the C-terminal end of the BAP1 protein. In this study, tumors with BAP1 mutations and/or loss of BAP1 expression were categorized as deficient BAP1. In this way we observed that deficient BAP1 was the only independent prognostic marker in patients with polyploid UM.

For the other UM relevant genes we found GNAQ or GNA11 mutations in all but one polyploid UM as one would expect based on the occurrence described in diploid UM.2,19,20 SF3B1 mutations were observed in four of our polyploid tumors. Patients with SF3B1 mutation in the UM have been associated with the low-risk prognostic features; disomy 3, spindle-cell type, and low age at diagnoses.15,16 None of the patients in our polyploid group with SF3B1 mutations had developed metastatic disease and were alive at the end of the study (follow-up range, 1–192 months). Nevertheless, both to our own experience as well as by other groups patients can be identified with disomy 3 tumors harboring an SF3B1 mutation that developed metastasis.15,16,25,28 In our polyploid cohort the numbers are too low and the follow-up for some tumors is too short in order to draw conclusions regarding the influence of SF3B1 mutations in the tumor on disease-free survival. EIF1AX mutations are mainly reported in disomy 3 tumors and are correlated with a good prognosis for these patients, and present in the tumor of one patient in our series of polyploid UM, who is metastasis-free and alive at a follow-up of 136 months.10,16,17,25 The missense mutation found in the tumor of this patient affects amino acid 15 (p.G15D), a missense mutation described in other UM samples as well (the COSMIC database; id = COSM3973544; in the public domain, http://cancer.sanger.ac.uk/cosmic/). The first 18 amino acids at the N-terminus of the eIF1A protein are essential in the interaction with the 40S subunit,29 thus making it very likely that this mutation results in an altered function of the protein. In this current study, we have shown that the prevalence of mutations in the UM genes do not differ between tumors with diploid and polyploid karyotypes, indicating a similar behavior and progression toward metastatic disease, suggesting polyploid UM are not a subclass in UM.

Caution should be taken in the interpretation of chromosomal abnormalities and using one technique only this could possible lead to misclassifications. Uveal melanoma are characterized by nonrandom recurring chromosomal losses and gains.3 Loss of chromosome 3 has been correlated to metastasis,3,5 but in polyploid UM with loss of one or multiple copies of chromosome 3 this does not automatically result in LOH. This is shown in our polyploid tumors, which all contain a relative loss of chromosome 3, while three tumors do not display a LOH (Figs. 1, 2). These three patients without LOH are still alive with a median DFS of 11 years (range, 76–192 months), which is comparable to the survival of patients with disomy 3 tumors.3 Onken et al.30 described that LOH of chromosome 3 is superior to quantitative loss of monosomy 3, and that is also the case in polyploid UM in our study. We emphasize the importance of SNP-array to investigate the zygosity of UM, to reduce false-negative (disomy 3 with LOH) and false-positive (relative monosomy 3 without LOH) prognostification. However, we cannot use the same reasoning for chromosome gain. Increase in copies of chromosome 8q is shown to be associated with shorter DFS.31 In polyploid UM, all tumors contain more than two copies of 8q, while four tumors do not have a relative gain based on the baseline of four copies. One of these four patients developed liver metastasis at 54 months and is still alive after a partial hepatectomy 20 months later, two died due to another cause at 38 and 149 months respectively, and one is alive and metastasis-free at 76 months. Because the survival of these patients is not homogenous we cannot draw conclusions regarding the pathogenicity of absolute gain without relative gain (tri- and tetrasomy) of chromosome 8q.

In conclusion, here we show that polyploid UM do not differ from diploid UM based on prevalence of mutations in the UM genes, and that similar to patients with diploid UM, BAP1 is the most significant prognostic predictor of metastasis in patients with polyploid UM (HR 15.90). Yet, the increased chromosome count and frequent losses in polyploid tumors can cause wrongful interpretations of chromosomal data and should therefore be analyzed for ploidy status.

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**References**


### Table 2. Continued

| Covariate | Univariate | | | | | | Multivariate | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 95% CI | *P Value* | HR | 95% CI | *P Value* |
| **EIF1AX** | | | | | |
| Wild-type | | | | * | 0.228 |
| Mutated | | | | - | - |

CI, confidence interval of survival (months/HR); HR, hazard ratio (expB). *P value of P ≤ 0.05 was considered significant. Log-rank test and Cox regression analyses were used to obtain univariate analyses for categorical and continuous data respectively. Multivariate analyses was conducted with Cox regression analyses with variables significantly associated with survival in the univariate analyses. Bold numbers indicate significant *P* values at *P* ≤ 0.05. * No statistics could be computed because all cases were censored.
Polyploidy in Uveal Melanoma


APPENDIX

ROMS: Rotterdam Ocular Melanoma Study Group

Serdar Yavuzyigitoglu, MD, PhD Student, Department of Ophthalmology, Department of Clinical Genetics, Erasmus Medical Center, The Netherlands.

Kyrı N. Smit, MSc, PhD Student, Department of Ophthalmology, Department of Clinical Genetics, Erasmus Medical Center, The Netherlands.

Natasha van Poppele, MD, PhD Student, Department of Ophthalmology, Department of Clinical Genetics, Erasmus Medical Center, The Netherlands.

Jolanda Vaarwater, Laboratory Technician, Department of Ophthalmology, Department of Clinical Genetics, Erasmus Medical Center, The Netherlands.

Dion A. Paridaens, Ophthalmologist, MD, PhD, Rotterdam Eye Hospital, Rotterdam, The Netherlands.

Hanneke W. Mensink, Ophthalmologist, MD, PhD, Rotterdam Eye Hospital, Rotterdam, The Netherlands.

Nicole C. Nau, Ophthalmologist, MD, PhD, Department of Ophthalmology, Erasmus Medical Center, The Netherlands.

Jackelen G. van Beek, Ophthalmologist, MD, Department of Ophthalmology, Erasmus Medical Center, The Netherlands.

Annelies de Klein, Clinical Cytogeneticist, PhD, Department of Clinical Genetics, Erasmus Medical Center, The Netherlands.

Emine Kiliç, Ophthalmologist, MD, PhD, Department of Ophthalmology, Erasmus Medical Center, The Netherlands.