Genotypic Profiling of 452 Choroidal Melanomas with Multiplex Ligation-Dependent Probe Amplification

Bertil Damato¹, Justyna A. Dopierala², and Sarah E. Coupland²

Abstract

Purpose: Metastasis from uveal melanoma occurs almost exclusively with tumors showing chromosome 3 loss. We used multiplex ligation-dependent probe amplification (MLPA) to detect chromosome 1p, 3, 6p, 6q, 8p, and 8q abnormalities in uveal melanomas. The purpose of this study was to correlate our MLPA results with other risk factors and metastatic death.

Experimental Design: Patients were included if they had a uveal melanoma involving choroid. Correlations between baseline risk factors were analyzed using the chi-square test (without Yates’s adjustment) and the Mann–Whitney test, with log-rank analysis for associations with metastatic death.

Results: The patients (194 female; 258 male) had a median age of 59.4 years and a median follow-up of 1.89 years. MLPA abnormalities occurred in a wide variety of combinations. Ten-year disease-specific mortality was 0% in 133 tumors with no chromosome 3 loss, 55% in tumors with chromosome 3 loss but no chromosome 8q gain, and 71% in 168 tumors showing combined chromosome 3 loss and 8q gain. In tumors with both these abnormalities, epithelioid melanoma cytomorphology, closed loops, and high mitotic rate correlated with poor survival as did lack of chromosome 6p gain.

Conclusions: These results support the use of MLPA for routine clinical prognostication, especially if the genetic data are considered together with clinical and histologic risk factors. We showed a wide variety of MLPA results, which suggests that chromosomal abnormalities in uveal melanoma accumulate in a variable sequence.

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Approximately 90% of all uveal melanomas involve the choroid. Without timely treatment, these tumors result in a blind and painful eye. Despite successful ocular treatment, about 50% of patients die of metastatic disease, which usually involves the liver (1).

Estimation of survival probability tends to be based on clinical features, particularly largest basal tumor diameter, tumor thickness, ciliary body involvement, and extracocular spread (2–4). These tumor characteristics form the basis of the 7th edition of the UICC/AJCC TNM staging system (5). Several pathologic predictors are recognized, the most widely used comprising: presence of epithelioid cells; high mitotic count; low HSP27 staining score; and the presence of PAS+ closed connective tissue loops (6–9). In 1996, Prescher et al. showed a strong correlation between chromosome 3 loss and metastatic death (10). Since then, other chromosomal abnormalities have been shown to correlate with poor prognosis and these include: 8q gain; 8p loss; 1p loss; 6q loss and lack of 6p gain (11–13). White et al. suggested that chromosome 3 loss correlated with metastatic death only if combined with chromosome 8 gain (henceforth, summarized by us as “C38A” for “chromosome 3 and 8 abnormality”; ref. 11) Gene expression profiling has identified class 2 tumors with high risk of metastasis (14).

In 1999, we started offering genetic tumor typing to all patients treated by local resection or enucleation, using fluorescence in situ hybridization (FISH; 15). This method lacked sensitivity, however, so that small chromosomal abnormalities were missed (16). To some extent, this problem was alleviated by neural networks we developed, which analyzed clinical, histologic, and genetic risk factors (17).

In 2006, we replaced FISH with multiplex ligation-dependent probe amplification (MLPA), which examines for genetic gains and losses by means of a multiplex polymerase chain reaction (PCR refs. 18, 19). We used a kit specifically designed for uveal melanoma (SALSA P027 (B1; MRC-Holland). This comprises 12 control probes and test probes directed at 7 loci on chromosome 1, 13 loci on chromosome 3, 6 loci on chromosome 6, and 5 loci on chromosome 8. We previously evaluated MLPA with 73 choroidal melanomas and found good correlations with survival (19). Important findings from that study were that chromosome 3 loss and chromosome 8q gain
Patients and Methods

Patients

Patients were included in the main analysis if treated for uveal melanoma and if MLPA data were available. They were excluded if: (1) the patient was not resident on mainland Britain; (2) both eyes had melanoma; (3) the tumor did not involve choroid or (4) the patient received primary treatment elsewhere or was not initially treated. We also included 68 patients treated between January 1998 and February 2000 and who were included in previous studies (19).

This study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice Guidelines. Consent for the use of tissues and data for research was obtained from all patients. Institutional Review Board/
samples and controls consisting of normal choroidal tissue were collected in microfuge tubes. Tissue lysis and protein digestion were conducted. Samples were incubated overnight at 56°C on Thermomixer comfort (Eppendorf). Fresh Proteinase K was added after 16 hours of incubation and samples were incubated for additional 4 hours. Genomic DNA was isolated using a high salt concentration and ethanol precipitation. DNA was dissolved in 20–100 μL of TE buffer, depending on pellet size. The DNA concentration and absorbance were measured with the NanoDrop Spectrophotometer (Thermo Scientific). Multiplex PCR was adapted from Dongen et al. (22). It was carried out on samples with a concentration exceeding 40 ng/μL to assess DNA quality using a Technne TC-412 thermal cycler (Technne). PCR products were visualized on a 2% agarose gel (150 mA for 30 minutes) stained with 1× SYBR Safe (Invitrogen), using the Bio Doc-It Imaging System (Ultra-Violet Products Ltd). The MLPA procedure and capillary electrophoresis were conducted as previously reported using the SALSA P027.B1 Uveal Melanoma Kit for 432 tumors. Briefly, 6 nontumor controls were used in each MLPA assay. MLPA reactions were conducted using a G-Storm GS1 thermal cycler (Gene Technologies Ltd) with fragment detection being conducted using the ABI-3130XL Genetic Analyzer and GeneMapper software (Applied Biosystems). Raw data were received as peak heights, as a measure of peak intensity, for each of the 43 probes (31 test probes and 12 control probes). MLPA was conducted in triplicate for each UM sample.

The analysis was conducted using an adapted version of the Excel spreadsheet designed by the National Genetics Reference Laboratory (NGRL; http://www.ngrl.org.uk/Manchester/mlpapubs.html), as described previously (19). We modified this method to exclude UM control loci that seemed abnormal (1920). The MLPA data were considered as reliable if 7 or more control probes were within the normal range. The dosage quotient (DQ) was categorized as suggested by the NGRL as: deletion (D), ≤0.65; borderline loss (B), 0.65–0.84; normal = diploid (N), 0.85–1.14; borderline amplification (Q), 1.15–1.35; and amplification (A) ≥1.35. Chromosomes were considered to be abnormal if any loci showed reproducible borderline or definite gain or loss, with such abnormality being described as partial or total according to whether any or all of the loci were abnormal.

Statistical analyses

Clinical, pathologic, and cytogenetic data were computerized into a customized database prospectively. Tumors were categorized as involving ciliary body if they extended anterior to ora serrata and were recorded as having epithelioid cells irrespective of the proportion of such cells in the tumor. Extraocular extension was recorded as being present irrespective of whether this was noted clinically or histopathologically.

We notified the NHS Cancer Registry of all newly diagnosed patients with ocular melanoma. These were flagged at the Registry, which informed us automatically of the date and cause of any deaths. If we did not receive such information about any patient by the close of the study, we assumed that the patient was alive. Because of our reliance on the NHS Cancer Registry, patients from overseas were excluded from follow-up studies.

Depending on the wording of the death certificate, death from metastatic disease was coded as definitely or probably caused by uveal melanoma unless another primary malignancy was specified as the cause in the death certificate. Follow-up time was estimated from the time of treatment to the date of death or to the 18th March 2010, when the data were downloaded.

Data analysis was done using a statistical program (SPSS; SPSS Inc.). Correlations between baseline risk factors were analyzed using the chi-square test (without Yates’s adjustment) and the Mann–Whitney test.

The Kaplan–Meier method was used to compute metastatic mortality and groups were compared using the log-rank test. A P value of less than 0.05 was considered to be statistically significant. All statistical tests were 2-sided.

Results

Baseline data

Demographic and baseline characteristics are summarized in Table 1. The 452 patients had a mean age of 59.4 years (range, 7.3–93.8). The tumors had a mean largest basal diameter of 14.3 mm (range, 4.8–23.6) and a mean thickness of 6.7 mm (range, 1.0–16.8).

Primary treatment consisted of: enucleation (209 cases; 46.2%); proton beam radiotherapy (73, 16.2%); brachytherapy (69, 15.3%); transscleral resection (57, 12.6%); endoresection (40, 8.8%); and photodynamic therapy (4, 0.9%).

MLPA results

The various combinations of MLPA abnormality are shown in Figure 1. As mentioned before, chromosomes were considered abnormal if any loci showed borderline or definite gain or loss. There were 34 tumors without 1p loss, 3 loss, 6p gain, or 8q gain, with 18 of these showing no chromosomal abnormalities at all and the remaining 16 having 1p gain (2), 3 gain (11), 6q loss (1), 8p loss (1), and 8q loss (1).

Table 1 shows the prevalence of the chromosome 1p loss, 3 loss, 6p gain, and 8q gain, according to the patient demographics. Chromosome 3 loss and chromosome 8q gain correlated with all clinical and histologic risk factors for metastasis, both individually and in combination (Table 1). Chromosome 1p loss correlated with large tumor size, ciliary body involvement, and increased mitotic rate. This chromosomal abnormality was more common in melanomas affecting the right eye. Chromosome 6p gain correlated strongly with lack of ciliary body involvement and weakly with lack of epithelioid cellularity. The prevalence of C38A increased from 33.3% to
<table>
<thead>
<tr>
<th>Variable</th>
<th>Category Number</th>
<th>Chromosome Loss</th>
<th>Chromosome Gain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Female 194</td>
<td>70 (36%)</td>
<td>0.43</td>
</tr>
<tr>
<td></td>
<td>Male 258</td>
<td>84 (33%)</td>
<td>0.57</td>
</tr>
<tr>
<td>Eye</td>
<td>Left 220</td>
<td>61 (28%)</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>Right 232</td>
<td>93 (40%)</td>
<td>0.35</td>
</tr>
<tr>
<td>Ciliary body involvement</td>
<td>No 308</td>
<td>92 (36%)</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>Yes 143</td>
<td>62 (43%)</td>
<td>0.13</td>
</tr>
<tr>
<td>Extraocular spread</td>
<td>No 133</td>
<td>133 (33%)</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td>Yes 53</td>
<td>21 (40%)</td>
<td>0.08</td>
</tr>
<tr>
<td>Basal tumor diameter, mm</td>
<td>&lt;10 36</td>
<td>5 (14%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>10–12 112</td>
<td>30 (27%)</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td>13–15 136</td>
<td>49 (36%)</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>16–18 111</td>
<td>43 (39%)</td>
<td>0.63</td>
</tr>
<tr>
<td>Tumor thickness, mm</td>
<td>&lt;3 43</td>
<td>12 (28%)</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>3–5 134</td>
<td>40 (30%)</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>6–8 147</td>
<td>47 (32%)</td>
<td>0.36</td>
</tr>
<tr>
<td>Mitoses/40 HPFb</td>
<td>&lt;5 141</td>
<td>47 (33%)</td>
<td>0.498</td>
</tr>
<tr>
<td></td>
<td>&gt;4 118</td>
<td>62 (53%)</td>
<td>0.001</td>
</tr>
<tr>
<td>Chromosome 1p loss</td>
<td>Absent 298</td>
<td>151 (51%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Present 154</td>
<td>126 (42%)</td>
<td>0.16</td>
</tr>
<tr>
<td>Chromosome 3 loss</td>
<td>Absent 175</td>
<td>126 (46%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Present 277</td>
<td>126 (46%)</td>
<td>0.03</td>
</tr>
<tr>
<td>Chromosome 6p gain</td>
<td>Absent 208</td>
<td>153 (74%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Present 244</td>
<td>67 (31%)</td>
<td>0.36</td>
</tr>
<tr>
<td>Chromosome 6q23.1 loss</td>
<td>Absent 352</td>
<td>213 (61%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Present 100</td>
<td>64 (64%)</td>
<td>0.38</td>
</tr>
<tr>
<td>Chromosome 8p loss</td>
<td>Absent 371</td>
<td>126 (34%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Present 81</td>
<td>28 (25%)</td>
<td>0.36</td>
</tr>
<tr>
<td>Chromosome 8q gain</td>
<td>Absent 168</td>
<td>72 (43%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Present 284</td>
<td>116 (41%)</td>
<td>0.30</td>
</tr>
<tr>
<td>Chromosome 3 loss and 8q gain</td>
<td>Absent 247</td>
<td>59 (24%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Present 205</td>
<td>95 (46%)</td>
<td>0.36</td>
</tr>
</tbody>
</table>

*Mann–Whitney test.

*40× objective.
87.5% as the basal tumor diameter increased from under 10 mm to more than 18 mm (Table 1). C38A correlated with chromosome 1p loss and inversely with chromosome 6p gain. With regard to TNM stage (7th edition), C38A were present in 25% of T1 uveal melanomas, 33% of T2 tumors, 46% of T3 tumors, and 78% of T4 tumors (Mann–Whitney, $P < 0.001$).

**Survival**

The follow-up times had a median of 1.89 years in 395 patients who were alive at the close of the study. A total of 57 patients died, the diagnosed cause of death being metastasis. With tumors having more than 1 abnormal chromosome, only data for 1p−, 3, 6p+, and 8q− are shown.

Figure 1 shows the disease-specific mortality rates according to histopathologic predictors of metastasis. Only 40 of these 162 (24%) uveal melanomas with available cytomorphic analysis and with this lethal combination of chromosomal abnormalities showed no apparent epithelioid cells, and of these only 2 (1%) were fatal. Closed loops and higher mitotic count also indicated poorer survival. The number of pathologic predictors inversely correlated with survival (log rank, $P = 0.003$).

**Discussion**

**Main findings**

The MLPA results correlated strongly with metastatic death, especially when considered with clinical and histopathologic predictors of metastasis. These findings support the use of MLPA for uveal melanoma prognostication in routine clinical practice.

**Strengths and weaknesses**

The main strengths of this study were the large number of cases and the abundant clinical and histologic data.
Fig. 2. Kaplan–Meier survival curves correlating disease-specific mortality with chromosome 1p loss, 3 loss, 6p gain, and 8q gain, according to whether borderline MLPA results were considered normal or abnormal and whether abnormality was detected in none, some, or all loci. The prognostic significance of borderline and partial MLPA abnormality varied between chromosomes.
Correlations between chromosomal abnormalities and with metastatic risk factors

The correlations in Table 1 are very much in keeping with the literature, with chromosome 3 loss, 8q gain, and lack of 6p gain being associated with clinical and histologic risk factors for metastasis. Interestingly, despite their opposite correlations with prognosis, chromosome 6p gain correlated with chromosome 8q gain, possibly indicating the purposeless nature of chromosomal instability. Surprisingly, 1p loss seemed to be significantly more common in the right eye, but further studies are required to determine whether this association is genuine or the result of chance.

Survival according to chromosomal abnormalities

The Kaplan–Meier curves in Figure 2 show that reliance purely on "definite" chromosome 3 loss would be misleading, as some patients would die of metastasis despite being reassured of a good prognosis. Metastatic death occurred in patients having only borderline loss of 1 or 2 loci on chromosome 3. It is therefore necessary to consider borderline chromosome 3 loss as significant even if only a single locus is abnormal. Our previous studies suggest that borderline MLPA results occur when there is chance.

Our data confirm that metastatic disease also occurs in uveal melanoma with partial chromosome 3 deletion, which is not specific to any individual locus tested with MLPA. None of 133 uveal melanomas without chromosome 3 loss were fatal as compared with 70% of tumors showing C38A. Many of the "nonmetastasizing" tumors were large, indicating that lethal genetic mutations can often occur late or not at all. Metastatic death occurred with only 3 (5.4%) of 56 tumors showing chromosome 3 loss without 8q gain. There is scope for further studies investigating chromosome 8q using more sensitive techniques (e.g., array CGH or SNP arrays) to determine whether subtle abnormalities were missed by MLPA. These studies would determine whether
C38A is essential for metastasis. It would also be interesting to determine the gene expression profiles of these 3 patients.

In the 168 patients with C38A, almost all of whom are expected to die of metastatic disease, survival was worse if 6p gain was absent. Survival was also worse in the presence of epithelioid cells, PAS+ closed loops, and a high mitotic count, correlating inversely with the number of pathologic predictors present. Despite its relative subjectivity, melanoma cytomorphology correlated with survival in patients with C38A. It would be interesting to determine the GEP class of spindle-cell, C38A melanomas.

Prevalence of chromosome abnormalities

It has previously been proposed that all uveal melanomas are derived from tumors with chromosome 3 loss and chromosome 6p gain, respectively (23). Such a view is contradicted by our finding that each of the 4 chromosomes tested by MLPA showed isolated abnormalities, the other 3 chromosomes being apparently normal (Fig. 1). In 18 uveal melanomas, all loci tested with MLPA were normal. Further studies using higher-resolution molecular genetic methods, such as array CGH, are required to identify subtle abnormalities that might have been missed by MLPA. It would also be interesting to determine the GEP of tumors with only chromosome 3 loss or chromosome 8q gain but not both of these abnormalities.

Clinical implications

The results presented in Figure 3 support the application of MLPA for genetic tumor typing for prognostication, but further, long-term audit is required. This is because some patients without C38A may develop metastatic disease after several years.

Figure 5 indicates that prognostication is enhanced by considering genetic abnormalities together with histologic predictors of metastasis. We have designed neural networks that do this, also taking clinical stage and competing risks into account. Figures 3 and 4 suggest that prognostication might be improved by considering not only chromosome 3 loss but also abnormalities on the other chromosomes tested with MLPA. We hope to train our neural networks with such data once a sufficient number of events have occurred.

Gene expression profiling of uveal melanomas has been shown to correlate with metastatic death with high sensitivity and specificity. Because of its high cost, it may be unaffordable in some centers. In such situations, GEP may be reserved for patients with inconclusive MLPA results, that is, in tumors with chromosome 3 loss but without 8q gain.

It has been suggested that metastasis commences years before the primary ocular tumor is detected so that ocular treatment does not influence survival (24). The wide diversity of chromosomal abnormalities shown in Figure 1 suggests that such abnormalities accumulate in a variety...

Fig. 5. Kaplan–Meier survival curves showing disease-specific mortality in 168 C38A melanomas, according to: (A) epithelioid cells; (B) closed loops; (C) high mitotic rate; and (D) the sum of these histologic predictors of metastatic death. Metastasizing tumors with histologic features indicating a higher grade of malignancy were associated with poorer survival.

<table>
<thead>
<tr>
<th></th>
<th>No metastatic death</th>
<th>Survival time (Y)</th>
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<tbody>
<tr>
<td></td>
<td>Present</td>
<td>Absent</td>
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<tr>
<td>N</td>
<td></td>
<td>Absent</td>
</tr>
<tr>
<td>(n)</td>
<td></td>
<td>Present</td>
</tr>
<tr>
<td>162</td>
<td></td>
<td>(n = 40)</td>
</tr>
<tr>
<td>117</td>
<td></td>
<td>(n = 71)</td>
</tr>
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<td>118</td>
<td></td>
<td>(n = 40)</td>
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<td>117</td>
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<td>(n = 71)</td>
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<td>118</td>
<td></td>
<td>(n = 25)</td>
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<tr>
<td>117</td>
<td></td>
<td>(n = 40)</td>
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</table>

Kaplan–Meier survival curves showing disease-specific mortality in 168 C38A melanomas, according to: (A) epithelioid cells; (B) closed loops; (C) high mitotic rate; and (D) the sum of these histologic predictors of metastatic death. Metastasizing tumors with histologic features indicating a higher grade of malignancy were associated with poorer survival.
of sequences. For example, in tumors with C38A, chromosome 3 loss may precede chromosome 8q gain and vice versa and either of these may theoretically occur at the tumor’s inception or in a longstanding melanoma without abnormality detectable with MLPA or in a tumor with 1p loss. With such variable tumor progression, lethal chromosomal abnormalities may perhaps develop later in some tumors than in others. The observation that metastatic disease occurs almost exclusively with tumors showing both chromosome 3 loss and 8q gain supports the hypothesis that these 2 chromosomal abnormalities constitute a lethal “double-hit” (or serve as biomarkers for closely linked lethal abnormalities). Whether either or both of these abnormalities causes metastatic spread or merely accelerates growth of metastases is unknown. In any case, if a “double-hit” is required for metastatic disease to occur and if the 2 genetic aberrations do not occur simultaneously, then a process of “crescendo malignancy” can occur in some tumors. Such stepwise progression is supported by our recent observation of a choroidal melanoma that suddenly grew dramatically after several years of apparent dormancy (25). The base of the tumor showed only spindle melanoma cells with borderline abnormality of only a single chromosome 3 locus whereas the apex of the recent tumor outgrowth showed epitheloid cells with definite abnormality of almost all loci on chromosome 3, 8q, and 6p.

It seems that there are 3 groups of patients with uveal melanoma: those whose tumor has already metastasized by the time they present with their ocular tumor; those whose tumor will never metastasize, even if left untreated; and those in whom metastasis is prevented by timely treatment. At present, it is not possible to predict which tumors without lethal chromosomal abnormality will subsequently gain metastatic potential, so that it is not known whether any asymptomatic uveal melanomas can safely be left untreated. Intuitively, deferred treatment would be less risky in the absence of chromosome 3 loss and chromosome 8q gain, especially if chromosome 6p gain was present. Further studies are needed, however, to test this hypothesis.

Conclusions

Our preliminary audit of our MLPA assessment of choroidal melanomas suggests that this investigation is highly reliable at predicting metastatic disease from uveal melanoma, at least in the early postoperative years.

Disclosure of Potential Conflicts of Interest

The authors declare no conflict of interest.

Authors’ Contributions

B. Damato treated the patients, collected the data, conducted the statistical analyses, and cowrote the manuscript. J.A. Dopierala collated and analyzed the MLPA data and was involved in fruitful discussions. S.E. Coupland conducted the tumor sampling, histomorphologic, and immunohistochemical analyses of the uveal melanomas and cowrote the manuscript.

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