**The Effects of Plaque Brachytherapy and Proton Beam Radiotherapy on Prognostic Testing: a Comparison of Uveal Melanoma Genotyped by Microsatellite Analysis.**

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**Synopsis:**

In this cohort of 407 UM patients genotyped by microsatellite analysis (MSA), radiotherapy neither adversely affected genetic testing of UM by MSA nor increased the risk of metastatic death when UM is sampled before radiotherapy.

**ABSTRACT:**

**Background/Aims:** Proton beam radiotherapy and plaque brachytherapy are commonly applied in primary uveal melanoma (UM); however, their effect on chromosome 3 classification of UM by microsatellite analysis (MSA) for prognostication purposes is unknown, where the tumour is sampled post-irradiation. This study examined the prognostic accuracy of genotyping UM biopsied before or after administration of radiotherapy, by MSA.

**Methods:** 407 UM patients treated at the Liverpool Ocular Oncology Centre between January 2011 to December 2017, were genotyped for chromosome 3 by MSA; 172 and 176 primary UM were sampled prior to-and post irradiation, respectively.

**Results**: Genotyping by MSA was successful in 396/407 (97%) of UM samples (196 males, 211 females; median age of 61 years (range, 12 – 93) at primary treatment). There was no demonstrable association between a failure of MSA to produce a chromosome 3 classification and whether radiation was performed pre- or post-biopsy with an odds ratio of 0.96 (95% CI, 0.30-3.00; p=0.94). There was no evidence of association (measured as hazard ratios, h.r.) between risk of metastatic death and sampling of a primary UM before administration of radiotherapy (h.r. 1.1 [0.49, 2.50] p=0.81). Monosomy 3 (h.r. 12.0 [4.1, 35.0] p<0.001) was significantly associated with increased risk of metastatic death.

**Conclusions and Relevance:** This study revealed that successful genotyping of UM using MSA is possible, irrespective of irradiation status. Moreover, we found no evidence that biopsy prior to radiotherapy increases metastatic mortality.

**INTRODUCTION:**

In the management of primary uveal melanoma (UM), patients are increasingly being offered prognostic biopsy for genetic analysis of their UM to estimate metastatic risk. Because of the low DNA yield from some small tumour samples, microsatellite analysis (MSA) is often used to determine the copy number variation (CNV) of chromosome 3 (Chr3) for prognostic purposes. MSA was first successfully carried out in UM by Tschentscher et al. in 2000 (1), and several studies have since confirmed its efficacy in accurately stratifying patients as having high- or low metastatic risk (2-4).

Most patients with uveal melanoma are treated with some form of radiotherapy (5-7). The most common radiotherapy modalities employed in UM are plaque brachytherapy (PRXT) and proton beam radiotherapy (PBR) (7-10). Brachytherapy is most effective when used to treat small-to-medium sized UM where the thickness is ≤7mm (11), whereas PBR can be used to treat UM that are larger or closer to the optic disc and the fovea, taking advantage of the Bragg peak and utilising a modified beam structure (12). Newer techniques, such as stereotactic radiosurgery with CyberKnife or GammaKnife, achieve similar local control rates with eye retention to PBR but have a poorer visual prognosis post-treatment (13).

The purpose of this study was to: a) examine the mortality of patients with UM where Chr3 CNV was determined by MSA; b) assess the effect, if any, of radiotherapy on successful Chr3 tumour classification; and c) establish whether sampling tumours before the administration of radiotherapy affects metastasis free survival (MFS). Herein, we report MSA data from UM genotyped between 2011 and 2017, including the largest cohort to date of UM samples obtained after PBR and PRXT, correlating these findings with genetic, histopathological and clinical data in addition to mortality.

**MATERIALS AND METHODS:**

**Tumour Samples**

A database query was carried out to identify all UM patients who were examined and treated at the Liverpool Ocular Oncology Centre (LOOC), Royal Liverpool and Broadgreen University Hospital Trust between January 2011 and December 2017, and who had genetic testing of their UM performed by MSA. All patients underwent a full systemic and ophthalmic examination at the LOOC and clinical, histopathological, genetic and follow-up data were collected. PBR was administered at the Clatterbridge Cancer Centre (CCC) at a dose of 56Gy over 4 consecutive days. Ruthenium PRXT delivered an apical tumour dose of a minimum of 80-90Gy. Biopsies were performed either before or soon after PBR, or within 4 weeks of completion of PRXT. Trans-retinal and trans-scleral tumour biopsy samples were obtained using methods described previously (14). A single histology cytospin was produced for each biopsy and stained with May Grunewald Giemsa (MGG), as previously described (15). Confirmation of the presence of UM cells in the biopsy sample was undertaken by an experienced ophthalmic pathologist (SEC). All surgical resection samples (e.g. enucleation, local resection, endoresection and iridocyclectomy) were processed using methods described previously (16-19). Peripheral blood samples were collected at the time of surgical procedures to provide constitutional DNA used as a control in the MSA analysis.

This study was conducted in accordance with the tenets of the Declaration of Helsinki and Good Clinical Practice Guidelines. Approval for the study was obtained from the Health Research Authority

South Central - Hampshire B Research Ethics Committee(RECref 15/SC/0611). All samples and data were provided by the Ocular Oncology Biobank (REC Ref 16/NW/0380). All patients had provided informed consent for the use of their samples and data in research.

**DNA Extraction and Genotyping by Microsatellite Analysis**

DNA extraction was performed on UM tissue and blood samples as described by Lake et al. (modified QIamp and DNeasy Blood and Tissue kit; Qiagen, Crawley, UK) (20). DNA was resuspended in TE buffer (10mM Tris-HCl pH 8.0, 0.1mM EDTA) (Life Technologies Ltd. Carlsbad) and quantified using fluorometric methods (Invitrogen Qubit fluorometer and broad-range DNA quantification assay; Life Technologies, Carlsbad, CA; Glasgow, UK). All samples tested by MSA had a DNA yield between 2 - 20ng/μl. MSA was performed using a modified protocol from Thomas et al. (4). Briefly, genetic analysis was carried out using a polymerase chain reaction (PCR)-based technique assessing 8 polymorphic microsatellite markers on Chr3; 4 microsatellite loci on 3p and 4 microsatellite loci on 3q (supplementary Table 2). All PCR steps were carried out on a G-Storm GS1 Thermal Cycler (GRI - Genetic Research Instrumentation Ltd, Essex) using the following conditions: initial activation step at 95°C for 15 minutes; and then 35 cycles of 94°C for 30s, 56°C for 90s and 72°C for 60s with a final extension for 30 minutes at 60°C, cooling at 10°C. PCR products were loaded in 8.5μl Hi-Di Formamide containing 1μl LIZ500 size standard analysed using the ABI 3500 Genetic Analyzer. Fragment analysis was completed using GeneMapper v3.5 software (Applied Biosystems). An allelic ratio was calculated by normalising the allele peak area of the tumour against the corresponding blood sample (the control sample). The genotype of a locus was assigned based on the calculated AR: AR≥2.5, loss of heterozygosity (LOH); AR 2.49 - 1.4, allelic imbalance (AI) or AR≤1.39, no loss of heterozygosity (NLOH).

**Chr3 classification**

A UM was classified as monosomy 3 (M3) when two or more microsatellites on chromosome arm 3p and two or more microsatellites on chromosome arm 3q showed LOH. UM were classified as disomy 3 (D3) when two or more microsatellites on chromosome arm 3p and two or more microsatellites on chromosome arm 3q showed NLOH. Partial loss of Chr3 (PL) was assigned if at least 2 markers were lost on one chromosome arm. UM, in which two or more microsatellites on chromosome arm 3p and two or more microsatellites on chromosome arm 3q showed AI, were classified as AI. When UM had 2 NLOH and 2 LOH on each arm, a tumour was classified as M3. UM were considered as ‘unclassifiable’, if none of the above conditions could be met (15) (Figure 1).

**Statistical Analyses**

Follow-up (years) was calculated from the date of the first diagnosis of the primary UM until development of metastasis or study closure on 17th January 2019. Statistical analyses were carried out using SPSS Statistics v.24 (IBM, city, country), Microsoft R 3.5.1 and the packages rms, cmprsk and mstate (manufacturer, city, country). Because of the small sample size, to control the false discovery rate, the statistical significance was defined as p <0.001 (2-sided).

**RESULTS**

**Patient Demographics**

From January 2011 to December 2017, 407 UM patients receiving treatment at the LOOC underwent genotyping for Chr3 using MSA. The study cohort comprised 196 males and 211 females with a median age of 61 years at primary management (range, 12 – 93 years) (S1). The median follow-up was 54 months (range; 5 – 368 months). The UM had a median largest basal diameter of 11.2 mm (range; 1.8 – 20.8 mm) and a median ultrasound height of 3.1 mm (range; 0.9 – 18.5 mm). 53/407 (13%) UM involved the ciliary body and 5/407 (1%) had extraocular extension. The tumour size category according to the 8th AJCC TNM classification system was: T1 in 186 cases (46%), T2 in 146 cases (36%), T3 in 59 cases (14%), and T4 in 16 cases (4%). On histological examination, 116/407 (29%) UM samples contained epithelioid cells.

At the time of study closure on 17th January 2019, 347/407 (85%) patients were alive, 35/407 (9%) patients had developed or died from metastatic disease, 20/407 (5%) patients died from other or unknown causes, and 5/407 (1%) patients were lost to follow-up.

Nine of the 407 UM samples (2%) analysed were diagnostic biopsies: 6/9 went on to have secondary enucleation; 1/9 had a subsequent endoresection with 2/9 receiving no further treatment.

The 407 examined UM samples consisted of: 359 intraocular biopsies, 31 enucleations, 4 local resections, 10 endoresections and 3 iridocyclectomies (Figure 2). Seven cases analysed were excluded from the time to biopsy analysis following radiotherapy due to sampling more than 2 years after radiotherapy treatment (n=4) and overseas patients who were lost to follow-up (n=3), leaving 345.

Of the 345 UM patients that received radiotherapy, 169 (49%) and 176 (51%) were sampled pre- and post-radiotherapy, respectively. The median time interval between biopsy and PBR was 32 days (post treatment?) with the range spanning from 66 days pre-operatively (including some diagnostic biopsies) to 284 days post-operatively. Similarly, the median time interval between biopsy and PRXT was 0 days with the range spanning from 49 days pre-operatively to 129 days post-operatively. Of the 176 samples analysed post-radiotherapy only 6 (3%) failed to provide a Chr3 classification. The median time to biopsy of these 6 cases was 36.5 days (range, 24 – 52)

**Microsatellite Analysis**

395/407 (97%) UM samples were successfully genotyped according to the MSA classifications described in Materials and Methods: 97 (24%) UM were M3 (20% of AJCC 1, 48% AJCC 2, 20% AJCC 3 and 9% AJCC 4); 256 (63%) UM were D3 (53% AJCC 1, 32% AJCC 2, 13% AJCC 3 and 2% AJCC 4); 16 (4%) UM were PL, all loss of 3q, (AJCC 1, 2, 3, 4); and 26 (6%) UM were AI (AJCC 1, 2, 3, 4). Further, 12 (3%) UM were considered ’unclassifiable’ (AJCC 1, 2, 3, 4). Of these, 6/12 (50%) were sampled pre-radiotherapy and 6/12 (50%) post-radiotherapy. Of the 35 UM patients who died of metastatic UM, 27 (77%) had M3 UM; 2 had loss of 3q; 5 were D3; and 1 was ‘unclassifiable’.

For samples taken pre- and post-radiotherapy, the genetic results were comparable and there was no significant difference in the number of UM cases for which Chr3 data (either M3, D3, PL or AI) was obtained (Chi-squared p=0.099).

**Cox Analysis**

The dataset comprised of 345 complete observations. The variables used in the analysis are shown in Table 1.

A Cox proportional hazards model (21) was fitted to assess the impact on metastatic death hazard rate of biopsy sampling before or after radiotherapy and Chr3 classification. Contrasts were specified so D3 was the baseline level, and the hazard rate for the other four Chr3 classes are specified in relation to D3.

The hypothesis of the proportionality of hazards was assessed (21). Table 2 shows the hazard ratios, p-values of the z statistics and proportionality of hazards test for each factor. There is evidence only of a difference between hazard rates associated to M3 and D3.

Because of the small number of metastatic deaths, the 95% confidence intervals on the hazard ratios of Chr3 classes were wide; in the case of AI, no metastatic events occurred, so the model parameters could not be identified.

The C-index (21) with 95% confidence interval was 0.78 [0.69, 0.87], denoting good discrimination performance.

**Logistic Regression**

A logistic regression model (21) was fitted to assess the impact of biopsy sampling before or after radiotherapy on the success of genotyping. There was no evidence of this factor affecting the success of genotyping by MSA, with odds ratio of 0.96 (95% CI, 0.30, 3.0; p=0.94)

**Cumulative Incidence Analysis**

Figure 3 shows the cumulative incidence (c.i.) of metastatic death by Chr3 levels. No metastatic death events were observed in the AI group, so the cumulative incidence was zero. It should be noted that the Loss 3q c.i. and Unclassifiable c.i. were essentially the same, overlapping with M3 c.i. up to about 3 years. Furthermore, the estimated 95% confidence limits on Loss 3q c.i. and Unclassifiable c.i. (not shown to avoid clutter) were so large that they enclosed both the M3 and D3 c.i.

Gray’s K-sample test statistic (22) for comparing the c.i. across the Chr3 levels is 34 (p<0.001). This result qualitatively agreed with the Cox analysis, and the difference could be attributed mostly to M3 and D3.

**DISCUSSION**

To our knowledge, this is the largest series of post-radiotherapy UM samples genotyped for Chr3 status to date. We have shown that MSA can be used to establish Chr3 status in 97% of cases. Taking a biopsy before administration of radiotherapy did not increase the risk of metastatic death. Furthermore, neither PBR nor PRXT affected genotyping classification.

MSA is a molecular technique that can accurately determine Chr3 status in small UM biopsy samples with low DNA concentrations (23). In our study, genotyping was successful in 97% of all cases examined resulting in classification into two main groups, M3 and D3. Of interest, a large proportion of M3 classifications by MSA were observed in AJCC stages 1 & 2 highlighting that smaller tumours are not immune from being at high risk of developing metastases. Cases with PL of Chr3 and AI were also observed. PL of Chr3 in this study occurred exclusively as loss of 3q in 4% of the UM genotyped. Thomas et al. also reported PL of Chr3 in 4% of UM cases analysed by MSA, whilst in the study by Shields et al. PL of Chr3 was detected in 27% of UM cases (4, 23). The incidence of PL of Chr3 varies greatly in the literature, with some studies reporting it to be between 0% and 48% by MSA and other techniques (24). It is suggested that PL of Chr3 is caused by tumour heterogeneity. Cytomorphological heterogeneity is well documented in posterior UM and has led to concerns that extracting a biopsy from a single site may not be representative of the whole tumour (25). Previous studies examining this have shown heterogeneity of gene loci dosage quotients in studies utilising MLPA; however, this did not affect the overall copy number variation classification (26). In the present study, 8 UM patients underwent a subsequent enucleation following biopsy, and the prognostic results were concordant for each of these. Similarly, Coupland et al. reported concordant Chr3 data for patient-matched samples in 28 UM cases that were initially biopsied and subsequently resected (27).

In this study, no patients with AI developed metastatic disease; however, because of the small number of cases with this classification, no conclusive association of AI with mortality could be made. AI was first reported by Tschentscher et al. who consistently observed allele ratios that fell just below the cut off thresholds for gain or loss (1). They reasoned that this may be the result of clonal heterogeneity or more focal dosage changes. Thomas et al. demonstrated that UMs with AI was showed survival similar to those with M3 UM, and thus were associated with a poor prognosis (4). This may be related to differences in the classification of AI between their study and ours. For example, in their study, UM were defined as ‘AI’ if at least 2 markers showed AI even if the remaining markers were LOH, which in the current study would have been classified as M3.

The impact of taking a biopsy before or after radiotherapy on the success of genotyping was also assessed, using a logistic regression model. The odds ratio shows no evidence of an effect, but it should be considered the small number of failed genotyping entries, which tends to bias the classification towards the class with the largest number of entries (in this case, successful genotyping). This is consistent with the results of Hussain et al. who demonstrated that genetic analysis of UM by MLPA and MSA after treatment with PBR provided Chr3 classifications predictive of metastasis free survival (14). Similarly, in a study by Coupland et al. four UM samples obtained both pre- and post-radiotherapy showed concordant genetic results, demonstrating successful genotyping of irradiated specimens (27). Another analysis by Wackernagel et al. utilised array CGH to test samples pre- and post-radiotherapy; 5 patients had genetic analysis done before and after radiotherapy, and their results were also completely concordant, thus confirming the suitability of these samples for genotype analysis (28). This was not the case in the study by Dogrusöz et al. who utilised karyotyping and FISH to genotype irradiated tumours (29). Analysis of these samples was largely unsuccessful mainly due to tumour shrinkage and necrosis associated with irradiation, and in comparison to other studies, there was a significantly longer time from irradiation until enucleation (5-146 months). Most recently, Matet et al. demonstrated genetic concordance of clinically relevant chromosomes in 94% of matched biopsies taken before PBR and a subsequent endoresection taken less than 3 months following radiotherapy (30).

To our knowledge, this is the first study of its kind to examine whether taking a biopsy before treatment by radiotherapy is associated with death from metastatic UM. An *ex vivo* study performed by Glasgow et al. demonstrated iatrogenic dissemination of tumour cells following transvitreal biopsy (31). There have also been other case reports and series of suspected dissemination, which has contributed to the reluctance of some ophthalmologists to take diagnostic and prognostic biopsies (32-35). In this study, Cox analysis shows no evidence that a biopsy taken before or after radiotherapy affects metastatic event hazard rate. This is consistent with the findings of a recent study by Bagger et al. where a retrospective nationwide audit of 1637 UM patients demonstrated that melanoma-specific mortality was not increased in biopsied patients as compared with non-biopsied patients (36). The Chr3 classifications show an evidence of an increased (with respect to D3) hazard of metastatic death in the case of M3, but no evidence for the other classes. Effects could not be reliably estimated in the case of AI because of a lack of associated metastatic death events. In all cases, the small number of events translates into large uncertainties in the estimation of hazard ratios. Analysis of cumulative incidences provides qualitatively similar results.

One of the limitations of this study was the relatively short follow-up for some of the cases included in analyses, with 5-months being the shortest interval; however, it was still possible to show statistically significant differences between genotype results.

Although, MSA is a highly successful genotyping technique at our centre for UM samples yielding a small amount of DNA, next-generation sequencing (NGS) panels are increasingly being used for this type of analysis. This comprises of broader pan cancer panels (37, 38), whole exome sequencing (39) and targeted NGS panels (40), including a bespoke NGS UM panel quite advanced in its development at our own centre (41), based on The Cancer Genome Atlas mutational data (42), which also successfully obtains reliable genotypes in previously irradiated tumours.

In summary, we have shown that MSA is a reliable genotyping technique that can provide Chr3 classification in irradiated and non-irradiated UM. In addition, melanoma-specific mortality is not increased when UM are biopsied prior to radiotherapy. UM that have undergone radiotherapy are not less likely to yield a Chr3 classification than non-irradiated samples.

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**Figure Legends**

**Figure 1. Figure 1: Examples of chromosome 3 microsatellite analysis classifications in UM. UM are classified as: Monosomy 3 (M3) when two or more microsatellites on chromosome arm 3p and two or more microsatellites on chromosome arm 3q showed LOH; disomy 3 (D3) when two or more microsatellites on chromosome arm 3p and two or more microsatellites on chromosome arm 3q showed NLOH (when UM had 2 NLOH and 2 LOH on each arm, a tumour is classified as M3); Partial loss of Chr3 (PL) was assigned if at least 2 markers were lost on one chromosome arm; Allelic imbalance (AI) if two or more microsatellites on chromosome arm 3p and two or more microsatellites on chromosome arm 3q showed AI; Unclassifiable (U) if none of the above conditions could be met.**

**Figure 2. Flowchart of specimens examined in the present study;** n=407 samples examined, n=353 received radiotherapy either n=136 proton beam radiotherapy or n=217 ruthenium plaque radiotherapy. n=174 samples were taken before administration of either proton beam or plaque radiotherapy of which n=6 failed genotyping. n=179 samples were taken after administration of either proton beam or plaque radiotherapy of which n=6 failed genotyping.

**Figure 3.** Cumulative incidence functions and number at risk for each time interval, by Chr3 level (M3=Monosomy; U=Unclassifiable; PL=Loss 3q; D3=Disomy; AI=Allelic imbalance.).

**Tables**

**Table 1: Characteristics of 345 UM cases genotyped either before or after RXT**

|  |  |
| --- | --- |
| **Variable** | **Values** |
| Chr3 classification | D3: 216 [[add percentages?]]AI: 23Loss 3q: 14M3: 80Unclassifiable: 12 |
| Genotyping | Successes: 333Failures: 12 |
| Biopsy before/after RXT | Before: 169After: 176 |
| Survival status | Censored: 305Metastasis death: 24Other causes death: 16 |
| Follow-up (years) | Min: 0.41Max: 8 |

**Table 2: Cox Model Statistics**

|  |  |  |  |
| --- | --- | --- | --- |
| **Factor** | **Hazard ratio [95% CI]** | **z test** **p-value** | **PH χ2 test** **p-value** |
| BXbRXT | 1.1 [0.49, 2.5] | 0.81 | 0.56 |
| Chr3 – Unclassifiable | 5.5 [0.61, 49] | 0.13 | 0.65 |
| Chr3 – Monosomy | 12 [4.1, 35] | <0.001 | 0.39 |
| Chr3 – Loss 3q | 7.4 [0.82, 66] | 0.075 | 0.65 |
| Chr3 – Allelic Imbalance | - | - | - |