**Title Page**

**Genetic findings in treatment-naïve and proton beam-radiated iris melanomas.**

**Short title:** Genetic findings in non-treated and treated iris melanomas

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**Subtitle**

Iris melanomas show similar genetic alterations to that seen in choroidal melanomas, in both treatment naïve and post proton beam radiotherapy-treated eyes. Iris melanomas were also found to display a low-metastatic risk chromosomal profile.

**ABSTRACT**

**Background/aims:** Iris melanomas (IM) are rare and have a lower mortality than posterior uveal melanomas (UM). Our aims were to determine the prevalence of genetic changes associated with prognosis of posterior UM, in both treated and non-treated IM.

**Methods:** Retrospective database review and molecular analysis of all patients diagnosed with IM at the Liverpool Ocular Oncology Centre (LOOC) between 1993 and 2015. Archival pathology specimens of confirmed IM cases were analysed for chromosomal alterations, using multiplex-ligation-dependent-probe-amplification (MLPA) or microsatellite-analysis (MSA) depending on DNA yield, and *BRAF* mutation status.

**Results:** 5189 patients were diagnosed with intraocular melanoma at LOOC from 1993-2015. Of these, 303 (5.8%) patients were diagnosed with IM. Tissue samples were available for 26 IM cases. Twelve of these cases had biopsies taken post-proton beam radiotherapy (PBR). Histological subtyping showed 14 IM being spindle, 2 epithelioid, and 10 were of mixed-cell type. Twenty of the 26 IM cases (77%) analysed genetically were classified as either disomy 3 (n=16) or monosomy 3 (n=4). Chromosome 6p gain was detected in 4/18 (22%) IM, and polysomy 8q in 6%. *BRAF* mutations were not detected in any of the four IM cases examined. One IM patient died from metastatic disease: this tumour was disomy 3 with 6p and 8q gains. All other patients were alive with no evidence of metastases at study closure.

**Conclusion:** Chromosomal aberrations seen in posterior UM can also be demonstrated using MLPA or MSA in both treatment naïve and PBR-treated IM. Most IM display a low-metastatic risk chromosomal profile.

**INTRODUCTION**

Uveal melanoma (UM) is the most common primary intraocular malignancy in adults. Iris melanomas (IM) represent a rare subset of UM, comprising only 5% of all UM. They are considered to have the best prognosis of all UM, with reported metastatic spread of 0.5% at 3 years and 7% at 10 years; and overall 5-year mortality rate reported to be 2-3%.[1-10]

Clinical risk factors for metastasis in IM include: size (>3mm diameter, 1mm thickness), diffuse growth pattern; indistinct ‘feathery’ edges; rapid growth; prominent intrinsic vascularity; pigment dispersion; raised intraocular pressure; hyphaema; pupillary ‘peaking’ or ‘splinting’; ectropion uveae, angle involvement (inferior clock hours); ciliary body involvement; or extrascleral extension and increasing patient age.[1,2] Histopathologically IM comprise spindle cells alone or spindle cells with benign naevus cells; less frequently, they are of epithelioid and mixed cell type.[1,2] Previous explanations for the relatively less aggressive behaviour of IM compared to other UM include: earlier detection due to physical location (i.e., lead-time bias); host-immune responses causing greater suppression of metastatic potential of IM than with ciliary body or choroidal melanoma; and/or less aggressive nature of IM compared with posterior UM.[5,7,9-15]

Posterior UM show distinct chromosomal alterations, which clearly correlate with the risk of metastatic death.[12] Hence, ocular oncology centres are offering molecular prognostic testing using various cytogenetic techniques, such as fluorescence in situ hybridisation (FISH), comparative genomic hybridisation (CGH), spectral karyotyping, microsatellite analysis (MSA), multiplex ligation dependent probe amplification (MLPA) and single nucleotide polymorphisms (SNPs).[11-14,16-23]

Only limited studies have been performed on the molecular genetic alterations in IM, because of their rarity. Because IM are so different from their posterior counterparts, we investigated whether the genetic changes associated with posterior UM (mainly chromosomes 1, 3, 6 and 8) were also detected in IM. Our second aim was to determine whether these changes could also be detected in IM post-PBR, as demonstrated in our previous data of genetic alterations in choroidal melanomas following PBR.[24]

**MATERIALS AND METHODS**

**Setting:** Liverpool Ocular Oncology Centre (LOOC), Royal Liverpool University Hospital (RLUH) and the Liverpool Ocular Oncology Research Group, Department of Molecular and Clinical Cancer Medicine, University of Liverpool. The study was approved by the local RLUH audit committee and the Health Research Authority (REC Ref 15/SC/0611). The study conformed to the principles of research in accordance with the Declaration of Helsinki.

**Inclusion and exclusion criteria:** A retrospective database review of all IM patients attending the LOOC between January 1993 and March 2015 was performed. All patients had undergone full systemic enquiry and ophthalmic examination including: slit-lamp examination, gonioscopy, ocular coherence tomography (OCT) and echographic measurements of the iris tumours using a water-bath filled ultrasound probe with 2% hypromellose (EyeCubed Ultrasound, Ellex Ltd.). Clinical, histopathological, genetic and follow-up data for the identified IM cases were recorded. All samples had been examined and classified by an Ophthalmic Pathologist according to the modified Callender’s classification of UM.[20] Based on clinical and histopathological examinations, cases were only included if they were only IM or IM with secondary anterior ciliary body involvement. Cases were excluded if iris involvement was judged to be secondary to invasion from a ciliary body melanoma.

**Genetic Analyses:** Where available, archival formalin fixed paraffin wax embedded (FFPE) specimens of IM were collated from six iridocyclectomies (one following a recurrence, post PBR) and six enucleations (three following a tumour recurrence after PBR) for DNA analysis. DNA extraction, quality assessment and quantification were performed as previously reported.[16] DNA was already available from an additional fourteen intraocular IM biopsies.

*MLPA*

Samples yielding ≥100ng DNA were analysed for chromosomal aberrations by MLPA using the Salsa P027 kit according to the manufacturers’ instructions and as previously described (MRC-Holland BV, Amsterdam, The Netherlands).[16]

*MSA*

IM samples yielding <100ng DNA were analysed for chromosome 3 aberrations by MSA as previously described.[21] In brief, primer pairs flanking eight microsatellites on chromosome 3 (four on 3p and four on 3q) were optimised for use in two separate multiplex PCR reactions. The PCR products were then analysed using the ABI 3500 genetic analyser and DNA sizing was performed with Genemapper™. The allele peak height in the tumour DNA was compared with normal DNA obtained from the same patient to determine the allele ratio and hence the presence or absence of loss of heterozygosity (LOH) in the tumour DNA.

*BRAF*

Qualitative measurement of *BRAF* codon 600 mutations was performed by quantitative real time PCR (qPCR) using the Qiagen™ *therascreen* *BRAF* RGQ PCR *CE/IVD* kit on the RotorGene Q5plex HRM platform according to the manufacturer’s instructions. In brief, 150ng DNA at a concentration of 5ng/µl was examined in five separate PCR reactions for the presence or absence of the following *BRAF* codon 600 mutations; V600E/Ec, V600D, V600K, V600R.

**Statistical analysis:** Data were analysed independently using MatLab 2006a (Math Works Ltd.). The McNemar test for association was used and statistical significance was taken when p<0.05.

**RESULTS**

**Patients and samples:**

A total of 5,189 patients were diagnosed with UM at LOOC between 1993 and March 2015. Of these, 311 patients were listed as IM in the database. Eight cases, however, were found to be predominantly ciliary body melanoma with secondary iris invasion, and were therefore excluded from this study. The remaining 303 patients had convincing primary IM (5.8%), consistent with previous reports of the frequency of these tumours.[8] There were 136 males and 167 females, with a mean age of 54 years (range: 10 - 91). The mean largest basal tumour diameter (LBD) was 4.3mm (range: 1.2 – 12.3mm) and mean tumour thickness was 1.5mm (range: 0.4 – 6.5mm).

Primary treatment of the IM consisted of: PBR (+/-biopsy) in 223 patients; 32 patients underwent local resection/iridocyclectomy; 16 patients required enucleation; 3 declined treatment; 24 patients chose observation alone, and 5 had treatment at their local hospital. The mean follow up was 56.5 months (range: 0 – 264.5 months; median follow up was 36.5 months). There were 7 deaths from metastatic disease.

DNA was available for analysis from 26 IM patients (19 men and 7 female), who had a mean age of 52.8 years (range: 22 - 84) (Table 1). For 22 patients, DNA was obtained from samples taken at primary treatment: 12 as biopsies taken immediately following PBR on the last day of therapy (Table 1). The remaining 4 DNA samples were obtained following a second procedure (i.e., enucleation or iridocylectomy after PBR because of tumour recurrence) (Table 1). Eight of the 26 tumours had anterior ciliary body involvement although the primary tumour site was judged to be iridal. The tumours had a mean LBD of 4.8 mm (range: 1.2 - 12.3mm) and a mean thickness of 1.8 mm (range: 0.5 – 5.0mm).

Histological examination showed the IM to be of spindle cell type in 14 cases, epithelioid cell type in 2, and mixed cell type in 10 tumours (Table 1). Mean follow up of the patients from the date of primary diagnosis was 29.6 months (range: 0.2 – 135.4; median follow up was 14.1 months). There were two deaths – one from metastatic disease, and one from sepsis, which developed a year post treatment. The other patients (n=24) were alive with no evidence of metastasis at the time of data analysis.

**Table 1:** Clinical, histopathological and genetic data from the 26 cases of IM analysed.

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Patient** | **Gender** | **Age at diagnosis (years)** | **Diagnosis** | **Primary management** |  | **LBD (mm)** | **UH (mm)** | **Cell type** | **Chromosomal data** | | | | | | | ***BRAF*** | **Death; cause** | **Follow-up time (months)** |
| **Secondary treatment** | **Chr 1p** | **Chr 3p** | **Chr 3q** | **Chr 6p** | **Chr 6q** | **Chr 8p** | **Chr 8q** |
| 1 | F | 32 | Iris melanoma | Iris excisional biopsy§ | - | 2.3 | 2.2 | Spindle | N | U | N | N | N | N | N | - | No | 4.7 |
| 2 | M | 33 | Iris melanoma; ant CB spread | Iridocyclectomy§ | - | 1.5 | 1.1 | Mixed | N | L | N | N | N | N | N | - | No | 28.3 |
| 3 | M | 55 | Iris melanoma | Iridocyclectomy§ | - | 7.5 | 1.1 | Mixed | N | N | N | N | N | N | N | - | No | 39.7 |
| 4 | M | 58 | Iris melanoma | PBR + biopsy§ | - | 6.5 | 2.9 | Spindle | N | L | L | G | N | N | N | NMD | No | 35.2 |
| 5 | M | 48 | Iris melanoma | PBR | Iridocyclectomy§ | 3.3 | 1.8 | Mixed | N | N | N | N | N | N | N | NMD | No | 124.9 |
| 6 | M | 51 | Iris melanoma; CB spread | PBR | Enucleation§ | 2.9 | 1.3 | Mixed | N | L | L | N | N | L | N | - | No | 26.8 |
| 7 | M | 57 | Iris melanoma | PBR + biopsy¥§ | - | 4.2 | 1.3 | Spindle | N | N | N | N | N | N | N | - | No | 8.7 |
| 8 | M | 53 | Iris melanoma | PBR + biopsy§ | - | 5.4 | 3.1 | Mixed | N | N | N | N | N | N | N | - | No | 0.7 |
| 9 | M | 79 | Iris melanoma; ant CB spread | Enucleation§ | - | 7.2 | 3.3 | Mixed | N | L | L | L | N | N | N | - | No | 3.23 |
| 10 | M | 57 | Iris melanoma; angle & ant CB spread | PBR | Enucleation§ | 6.7 | 5.0 | Mixed | N | N | N | G | L | L | G | NMD | Yes; metastatic disease | 67.1 |
| 11 | F | 52 | Iris melanoma | Iridocyclectomy§ | - | 1.2 | 1.1 | Spindle | N | U | N | G | N | N | N | - | No | 34.6 |
| 12 | F | 84 | Iris melanoma; angle & ant CB spread | Enucleation§ | - | 12.3 | 3.6 | Mixed | N | L | L | N | N | N | N | - | No | 0.6 |
| 13 | M | 25 | Iris melanoma; ant CB spread | Iridocyclectomy§ | - | 1.9 | 1.6 | Spindle | - | N\* | N\* | - | - | - | - | - | No | 31.9 |
| 14 | M | 64 | Iris melanoma; angle spread | PBR | Enucleation§ | 4.5 | 0.7 | Epithelioid | N | L | N | N | N | N | N | - | No | 41.1 |
| 15 | M | 35 | Iris melanoma | PBR + biopsy§ | - | 2.3 | 0.5 | Spindle | - | N\* | N\* | - | - | - | - | - | No | 14.1 |
| 16 | M | 61 | Iris melanoma | PBR + biopsy§ | - | 4.3 | 1.8 | Spindle | - | N\* | N\* | - | - | - | - | - | No | 7.3 |
| 17 | F | 44 | Iris melanoma | PBR + biopsy§ | - | 2.4 | 1.0 | Spindle | - | N\* | N\* | - | - | - | - | - | No | 6.6 |
| 18 | M | 73 | Iris melanoma | PBR + biopsy§ | - | 8.7 | 1.2 | Spindle | N | N | N | N | N | N | N | NMD | Yes; sepsis | 9.3 |
| 19 | M | 60 | Iris melanoma | PBR + biopsy§ | - | 8.6 | 3.3 | Spindle | N | N | N | G | N | N | N | - | No | 72.6 |
| 20 | F | 61 | Iris melanoma; ant CB spread | Iridocyclectomy§ | - | 4.6 | 2.0 | Spindle | N | N | N | N | N | N | N | - | No | 12.5 |
| 21 | M | 47 | Iris melanoma; ant CB & episcleral spread | Enucleation§ | - | 2.6 | 1.1 | Mixed | N | N | N | N | N | N | N | - | No | 0.2 |
| 22 | M | 54 | Iris melanoma | PBR + biopsy§ | - | 4.4 | 1.1 | Epithelioid | - | N\* | N\* | - | - | - | - | - | No | 135.4 |
| 23 | F | 62 | Iris melanoma | PBR + biopsy§ | - | 6.5 | 1.2 | Mixed | N | L | N | N | N | N | N | - | No | 13.1 |
| 24 | M | 22 | Iris melanoma | PBR + biopsy§ | - | 3.3 | 2.0 | Spindle | - | N\* | N\* | - | - | - | - | - | No | 13.2 |
| 25 | F | 64 | Iris melanoma | PBR + biopsy§ | - | 4.7 | 1.6 | Spindle | - | N\* | N\* | - | - | - | - | - | No | 23.0 |
| 26 | M | 43 | Iris melanoma | PBR + biopsy§ | - | 3.7 | 1.1 | Spindle | - | N\* | N\* | - | - | - | - | - | No | 14.1 |

Abbreviations: F = female; M = male; CB = ciliary body; PBR = proton beam radiotherapy; ¥ = biopsy taken prior to PBR; §= sample on which genetic testing was performed; LBD = largest basal diameter; UH = ultrasound height; N = normal; U = unclassifiable; L = loss; G = gain; \* = Chromosome 3 data determined by MSA; NMD = no mutation detected; - = not performed/not applicable.

**Genetic analysis:**

Analysis of chromosome 3 status demonstrated monosomy 3 in 4/26 (15%) cases and disomy 3 in 16/26 (62%) cases. Of the remaining 6 cases, 3 showed loss of chromosome 3p with a normal 3q, and 3 were reported as unclassifiable for chromosome 3p but had a normal 3q. MLPA data for chromosomes 1p, 6 and 8 were available for 18 cases, and showed gain of 6p in 4 cases and polysomy 8q in a single case (Table 1). As previously reported for choroidal melanoma, genetic testing in IM was successful in all samples analysed post-PBR.[24] No *BRAF* codon 600 mutations were found in the four IM cases for which there was sufficient DNA to perform this test.

The patient who died from metastatic disease was middle-aged with a diffuse IM involving the angle. The patient had been treated with PBR, followed by enucleation 4.8 years later when the tumour recurred (Table 1). The time from diagnosis to metastatic death was 5.6 years.

**DISCUSSION**

In this study we present clinical, histopathological and genetic data for the largest series of IM analysed to date, and show that chromosomal aberrations reported in choroidal melanomas (i.e. monosomy 3 and polysomy 8q) are also detected in IM. Most importantly, we also demonstrate for the first time in IM that, consistent with our previous data for choroidal melanomas [24], genetic testing is possible following PBR. In earlier studies, genetic testing was performed on untreated IM specimens only, taken either intraoperatively as fine needle aspirates prior to tumour removal or plaque brachytherapy, from IM resections or from enucleations.[6,8,23] Only one previous study included a single case of recurrent IM post plaque brachytherapy.[9]

With respect to the reported genetic changes in IM, there are inconsistent results on the frequency of chromosome 3 loss in these tumours. White et al. 1995 were the first to report chromosomal alterations in one IM demonstrating rearrangements in chromosomes 4, 8 and 12, but no aberrations in chromosomes 1, 3 and 6.[23] In a study of three IM, alterations of chromosomes 3, 6, 8 and Y were shown.[6] Shields et al. 2011 used MSA to detect LOH across chromosome 3 in 17 cases of IM.[8] They demonstrated mainly partial loss of chromosome 3 (41%), and equal numbers of cases classified as disomy 3 and monosomy 3 (29%), where the latter was associated with increasing patient age and epithelioid cell type. In an analysis of 20 IM by FISH, Mensink et al. 2011 found losses on both chromosome arms 3p and 3q in six cases (30%), and a loss of 3p alone in a further three cases (15%).[9] These authors also described metastatic spread in four IM patients, with all IM showing monosomy 3.[9] Interestingly, this contrasts our findings in the present study, in which only one patient died from metastasis, and this was not associated with chromosome 3 loss. Polysomy 8q, which is also associated with a poor prognosis in patients with choroidal melanoma, was identified in 50% of the cases analysed in the study by Mensink et al.[9] This result again differs to the current study, in which only a single case demonstrated polysomy 8q. Although the frequency of monosomy 3 and polysomy 8q detected in IM in our cohort is lower (15% and 6% respectively) than that described in the two above-mentioned papers, it is consistent with that of genetic alterations commonly reported in choroidal melanomas.[12,17-19] It is possible that the differing frequencies of chromosomal alterations observed in IM reflect varying genetic analytical techniques used in the studies.

With respect to mutations in the *BRAF* gene (a member of the RAF family that encodes a serine/threonine protein kinase) and ocular melanoma, they have been detected in 29–40% of conjunctival melanomas.[25,26] In contrast, most (if not all) studies have not found *BRAF* mutations in either primary or metastatic posterior UM.[12] Intriguingly, however, Henriquez et al. reported T1799A mutations in the *BRAF* gene in 9/19 (47%) cases of IM.[27] In the current study, we did not detect *BRAF* mutations in any of the four cases that we analysed. We do recognise that this is a small number of cases, and that examination of a larger IM cohort is required before making any definitive statement regarding *BRAF* mutational frequency in this tumour.

Similarly, we do acknowledge the other weakness in the current study – namely, the absence of information regarding the presence or absence of mutations recently described to be associated with posterior UM, i.e. *GNAQ/11*, *SF3B1 BAP1* and *EIF1AX*. At the time this study was conducted, the standard genetic technique applied for routine clinical care within the National Health Service setting was MLPA or MSA. These techniques only determine copy number variation or LOH, and do not detect any of the above mutations. Unfortunately, the DNA concentrations obtained from most of the samples did not allow for any additional mutational analyses. However, we do recognise the importance of determining the mutational status of these genes in IM, and endeavour to analyse them in these rare tumours in future collaborative studies.

In summary, IM show similar chromosomal aberrations to choroidal UM, in both PBR-treated and non-treated eyes. A higher proportion of IM demonstrate a low-metastatic risk profile, reflecting the indolent clinical course. Further high resolution genomic (and potentially proteomic) analyses are required to better understand the pathogenesis and molecular biology of these tumours.

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