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The Genetic Background of Iris Melanomas and Iris Melanocytic Tumors of Uncertain Malignant Potential

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List of abbreviations: DFS = disease-free survival, FISH = fluorescent in situ hybridization, H&E = haematoxylin and eosin, IHC = immunohistochemistry, IMTUMP = iris melanocytic tumors of uncertain malignant potential, n.a. = not available, MAPK = mitogen-activated protein kinase, NGS = next-generation sequencing, ROMS = Rotterdam Ocular Melanoma Studygroup, SNP = single nucleotide polymorphism, UM = uveal melanoma, VCF = variant call format


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Running head: Mutations in iris melanoma and nevi.

Supplementary table: This manuscript contains additional online-only material. Table S1 should appear online-only.

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Purpose: Uveal melanoma is the most common primary intraocular malignancy in adults. Iris melanoma comprises 4-10% of all uveal melanomas and have a lower mortality rate. The genetic changes in iris melanoma are not as well characterized as ciliary body or choroidal melanoma. The aim of this study was to gain more insight into the genetic background of iris melanoma and iris nevi.

Design: Multicenter, retrospective case series.

Participants: Patients diagnosed with iris melanoma or iris nevi who underwent surgical intervention as primary or secondary treatment.

Methods: Next-generation sequencing of GNAQ, GNA11, EIF1AX, SF3B1, BAP1, NRAS, BRAF, PTEN, c-Kit, TP53 and TERT was performed on thirty iris melanomas and seven iris nevi. Copy number status was detected using single nucleotide polymorphisms (SNP’s) included in the NGS panel, SNP-array and/or FISH. BAP1 immunohistochemistry was performed on all samples.

Main Outcome Measures: Mutation and copy number status were analyzed. Results of BAP1 immunohistochemistry were used for survival analysis.

Results: In 26 of the 30 iris melanoma and all iris nevi at least one mutation was identified. Multiple mutations were detected in 23 iris melanoma and 5 nevi as well as mutations in GNAQ and GNA11. Furthermore, 13/30 BAP1, 5/30 EIF1AX and 2/30 SF3B1 mutations were identified in iris melanoma. No correlation between BAP1 status and disease free survival was found. The iris nevi showed one EIF1AX and three BAP1 mutations. Two of the nevi, with a BAP1 mutation, were histologically ‘borderline malignant’. Mutations in NRAS, BRAF, PTEN, c-KIT and TP53 were detected in six iris melanomas and four iris nevi.

Conclusions: Mutations that are often found in uveal and cutaneous melanoma were identified in this cohort of iris melanomas and iris nevi. Therefore, iris melanomas harbor a molecular profile comparable to both choroidal melanoma and cutaneous melanoma. These findings may offer adjuvant targeted therapies for iris melanoma. There was no prognostic significance of BAP1 expression as seen in choroidal melanoma. Consequently, iris melanoma is a distinct molecular subgroup of uveal melanoma.
Histologic ‘borderline malignant’ iris nevi can harbor BAP1 mutations and may be designated Iris Melanocytic Tumors of Uncertain Malignant Potential (IMTUMP).
INTRODUCTION

Uveal melanoma is the most common primary intraocular malignancy in adults with an incidence of 7:1,000,000 people in the Western World. Iris melanomas comprise 4-10% of all UM. The observed and relative survival is higher compared to UM in general. There is no difference in incidence between men and women but they occur more often in the Caucasian population. Treatment includes surgical resection, enucleation, brachytherapy and proton beam irradiation. Currently no studies on targeted adjuvant therapies in primary or metastatic iris melanoma exist. The choice of treatment depends on tumor size, localization and patient preference. Diffuse iris melanomas are difficult to recognize causing a delay in diagnosis. Moreover, they have a greater risk of metastasis than nodular iris melanoma. Other clinical risk factors for metastasis include elevated intraocular pressure, iris root or angle involvement, increased tumor thickness, older patient age and extraocular tumor extension. The metastatic rate of iris melanoma is quoted as 1-10% at 5 years, 2-10% at 10 years and 10% at 20 years of follow up. A metastatic rate of 11% at 5-years was described in a series of biopsied iris melanoma. However, gene expression profiling of iris melanoma showed that 67% of iris melanoma exhibit a class I (low metastatic risk) gene expression profile and 33% a class II profile (high metastatic risk).

Chromosomal abnormalities of iris melanoma are poorly characterized. Partial or complete loss of chromosome 3 was found in 41-45% and 15-29% respectively. Monosomy 3 was correlated with increasing patients’ age. While chromosome 3 loss is described in uveal melanoma as a risk factor for metastatic disease, in iris melanoma this was only associated with a progressive disease in a univariate analysis. Chromosome 9p loss was reported in 35%. Furthermore, loss of 1p and 6q, and gain of 6p, 8 and 8q was described. Also abnormalities of chromosomes 5 and 18 have been reported.

Mutations in genes encoding the guanine nucleotide-binding protein G subunit alpha q and 11 (GNAQ and GNA11) and the genes BAP1, SF3B1 and EIF1AX are typical for uveal melanoma. GNAQ mutations are more common in ciliary body and choroid UM compared to iris melanoma. The aim of this study was to elucidate the genetic background of iris melanoma and iris nevi and to ascertain whether iris melanoma constitutes a distinct molecular group amongst uveal melanoma. Next-generation sequencing
(NGS) and immunohistochemistry was used to identify mutations in genes that are involved in both uveal as well as cutaneous melanoma.

**MATERIALS AND METHODS**

**Inclusion**

Tissue was collected from patients with iris melanoma or iris nevi from The Royal Hallamshire Hospital (Sheffield, UK) and the Rotterdam Ocular Melanoma Studygroup (ROMS) database. The ROMS is a collaboration between the Erasmus MC (Rotterdam, The Netherlands) and The Rotterdam Eye Hospital (Rotterdam, The Netherlands). Patients with an iris melanoma or suspect iris nevi who underwent biopsy or enucleation between 1992 and 2016 were included. The study conformed to the tenets of the Declaration of Helsinki and was approved by the respective local ethics committees. Informed consent was obtained prior to treatment. All samples were reviewed by one of two ophthalmic pathologists (HM and RV) to ensure that all tumors were primary iris lesions. Patient charts were reviewed to ascertain diagnosis as primary iris melanoma, clinical and follow up data.

**Immunohistochemistry**

Immunohistochemical staining was performed with a BAP1-antibody (clone sc-28383, 1:50 dilution, Santa Cruz Biotechnology, Dallas, Texas, USA) on 4µm sections of formalin fixed paraffin embedded tissue (FFPE). An automated staining system (VENTANA BenchMark ULTRA, Ventana Medical Systems, Tuscon, Arizona, USA) was used following the protocol as described previously. Only nuclear expression was scored since nuclear expression is prognostic relevant in uveal melanoma. Loss of expression was defined as absent BAP1 expression in the nucleus.

**DNA isolation**

DNA was extracted from fresh and FFPE tumor tissue. DNA isolation from fresh material was performed using the QIAmp DNA mini kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions. DNA extracted from FFPE tissue was performed using lysisbuffer (Promega, Madison, Wisconsin, USA) and 5% Chelex (Bio-Rad, Hercules, California, USA) following the protocol as described before. Combined mutation and CNV detection by targeted next-generation sequencing in uveal
melanoma, Modern Pathology, in press). Tumor tissue was confirmed with flanking H&E-slides. DNA samples were stored at -20°C.

**Targeted next-generation sequencing**

Targeted NGS was performed using the Ion Personal Genome Machine (PGM) and the Torrent Server (Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to the manufacturers’ protocol. A panel including amplicons covering GNAQ, GNA11, BAP1, SF3B1 and EIF1AX was used. Moreover, NRAS, BRAF, PTEN, c-Kit, TP53 and TERT, genes that harbor mutations in cutaneous melanoma, were included. On chromosome 1, 3 and 8, amplicons that cover highly polymorphic regions were used to identify allelic imbalances (Smit KN, van Poppelen NM, Vaarwater J et al. Combined mutation and CNV detection by targeted next-generation sequencing in uveal melanoma, manuscript submitted).

**Mutation analysis**

Results from Ion Torrent next-generation sequencing were analyzed using Torrent Suite Software Version 4.4.3 (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and Integrative Genomics Viewer (IGV) Version 2.3.68 (97) (Broad Institute, Cambridge, Massachusetts, USA). All data was manually analyzed using IGV for the selected ten genes by two individuals. Mutations that occurred in more than 20% of the reads and with a minimal read count of 50 reads, were called. When there was a low DNA concentration or when one of the hotspot mutations was present in less than 20% of the total read count, mutations with a percentage between 10-20% were called. Intronic, non-coding regions and synonymous mutations were excluded. These results were compared with the mutations from the Variant Call Format (VCF) files. Mutations were validated using Sanger sequencing following a standardized protocol for FFPE material if material was available.

**Copy number variation**

Allelic imbalances were detected using the highly polymorphic regions on chromosome 3. This data was used to estimate the copy number variation. Furthermore, Nexus Copy Number software (BioDiscovery Incorporated, El Segundo, California, USA) was used to display copy number variations. Additional single nucleotide polymorphism (SNP) array and/or fluorescence in situ hybridization (FISH) data was used
when available. SNP-array and FISH results were obtained as described before. If there was loss of chromosome 3p, this was defined as loss of chromosome 3.

Statistical analysis

For statistical analysis IBM SPSS Statistics Version 21 (SPSS for Windows, International Business Machines Corporation (IBM), North Castle, New York, USA) was used. Kaplan-Meier analysis with log rank test was used for survival analysis. A P-value <0.05 was considered significant.

RESULTS

Patient characteristics

Iris melanomas

Between 1992 and 2016, from 31 patients that were treated for iris melanoma at Erasmus MC, The Rotterdam Eye Hospital and by the Ocular Oncology Service at the Royal Hallamshire Hospital, tissue material was available. From the Royal Hallamshire Hospital Sheffield 20 patients were included and 11 patients from the Erasmus MC and The Rotterdam Eye Hospital. One patient who developed liver metastasis after 34.3 months was excluded because of low tumor DNA concentrations, which made genetic analysis unreliable. There were 17 males (57%) and 13 females (43%) with a mean age at diagnosis of 47.1 years (range from 16.7 to 70.4 years). Fourteen patients were treated with iridocyclectomy (47%). All ten patients from Erasmus MC and The Rotterdam Eye Hospital and one patient from the Royal Hallamshire Hospital underwent enucleation (37%). Three patients were treated with local iris resection (10%), one with iridectomy (3%) and one with proton beam therapy (3%). This latter patient was treated with cryotherapy for raised intraocular pressure 47.8 months after primary treatment, followed by enucleation because of a blind painful eye.

Two patients (7%) received additional treatment with ruthenium plaque and proton beam therapy because of incomplete excision of iris melanoma. One patient received additional treatment (stereotactic radiotherapy) although the resection was histologically complete. In two patients (7%) recurrent iris melanoma developed after 28.6 and 15.5 months after the primary treatment, necessitating proton beam
therapy and enucleation respectively. In one patient, 37.0 months after additional treatment, diffuse recurrent iris melanoma with raised intra ocular pressure developed and the eye was enucleated.

Three patients (10%) underwent trabeculectomy because of glaucoma, (five, five and eleven years) prior to the diagnosis of iris melanoma. Two patients were clinically diagnosed to have an iris nevus at the time of trabeculectomy. In the third patient, pigment was seen preoperative. Biopsy of the iris four years later revealed a borderline malignant nevus and iris melanoma was diagnosed after seven years. In this patient, metastatic disease developed 21.3 months after primary treatment of iris melanoma. The other two patients who underwent trabeculectomy did not develop metastatic disease. One patient was clinically diagnosed with a nevus and receive a Baerveldt Glaucoma Implant (BGI) because of glaucoma almost 1.5 year before the diagnosis iris melanoma was made. Because of the iris melanoma diagnosis, the BGI was surgically closed and the eye was enucleated three weeks later. See Table 1 for an overview of patient characteristics.

The mean disease free survival (DFS) was 114.5 months with a range from 13.8 to 239.3 months. Metastasis in the liver developed in two patients (7%) after 21.3 and 31.9 months. Kapan Meier analysis showed no significant difference in disease free survival between patients with a BAP1 positive tumor compared to a BAP1 negative tumor ($P = 0.470$), (Figure 1).

Iris nevi

The seven patients with iris nevi from the ROMS-database comprised five females (42%) and two males (29%) with a mean age at diagnosis of 58.5 years (range 0.2 – 78.3 years). One patient underwent enucleation (14%), in three patients the nevi was excised in toto (43%) and three were biopsied (43%). None of these patients developed metastasis during follow-up (35.8-64.7 months). Six nevi were histologically classified as ‘borderline malignant’ according to the Jakobiec and Silbert classification.

Genetic analysis

Ion Torrent data (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was analyzed for GNAQ, GNA11, BAP1, SF3B1, EIF1AX, NRAS, BRAF, PTEN, C-KIT, TP53 and TERT promoter mutations. TERT promoter results were excluded for further analysis due to a read count lower than fifty. An overview of
the results is displayed in Figure 2. A GNAQ mutation was found in 15 iris melanomas (50.0%) in which 11 tumors harbored a c.626A>T:p.Gln209Leu mutation (37%), two a c.626A>C:p.Gln209Pro mutation (7%), one a c.548G>A:p.Arg183Gln (3%) and one both a c.619G>A:p.Gly207Arg as well as a c.620G>A:p.Gly207Glu mutation (3%). GNA11 was mutated in nine iris melanomas (30%) which consisted of six c.626A>T:p.Gln209Leu (20%) and three c.547C>T:p.Arg183Cys mutations (10%). An EIF1AX mutation was identified in five tumors (17%); three c.5_6TT:p.Pro2Leu mutations (10%), one c.22G>A:p.Gly8Arg mutation (3%) and one c.44G>A:p.Gly15Asp mutation (3%). A c.1873C>T:p.Arg625Cys mutation in SF3B1 was seen in one iris melanoma (3%) and a c.1858A>G:p.Met620Val mutation in another tumor (3%). One or more BAP1 mutations were found in 13 iris melanomas (43%).

For three iris melanomas no mutation status of NRAS, BRAF, PTEN, c-KIT and TP53 was available. A TP53 mutation was detected in four (13%), a NRAS mutation in three (10%), a PTEN mutation in three (10%), a c-KIT mutation in two (7%) and a c.1781A>G:p.D594G BRAF mutation in one iris melanoma (3%). The exact mutations are described in Supplementary Information, Table S1 (available at www.aaojournal.org). Four iris melanomas did not have a mutation in any of the tested genes, BAP1 IHC was positive for all four of these samples.

In the iris nevi (n=7), four GNAQ c.626A>T:p.Gln209Leu mutations (57%) and one GNA11 c.626A>T:p.Gln209Leu (14%) were found. Three nevi, of which two borderline malignant, harbored one or more BAP1 mutations (43%), one an EIF1AX c.16G>A:p.Gly6Ser mutation (14%). Mutations in NRAS were found in four nevi (57%), c-KIT in three (43%), PTEN in one (14%) and TP53 in one nevus (14%). An overview of the mutations in iris melanoma and nevi are shown in Figure 2. See supplementary information Table S1 for a detailed overview of the mutations that were detected.

Reliable Sanger sequencing results were obtained from three patients with a mutation in PTEN, BRAF and NRAS. The mutations in BRAF and PTEN were confirmed. Surprisingly, besides the known PTEN mutation, another mutation in PTEN was detected with Sanger sequencing, a c.703G>A:p.Glu235Lys mutation.
Immunohistochemistry

Immunohistochemical staining for BAP1 was performed on all iris melanoma and iris nevus sections. None of the iris nevi showed loss of BAP1 expression (Figure 3) BAP1 expression was positive in 21 iris melanoma samples (70%) and negative in 9 samples (30%). Six iris melanomas showed no BAP1 expression in >90% of the tumor cells, in two cases loss of BAP1 expression was observed in 80% and 50% of the tumor cells, respectively. In the remaining BAP1 negative iris melanoma, part of the tumor (40%) consisted of epithelioid cells which lacked BAP1 expression and whereas the spindle tumor cells did show BAP1 expression, see Figure 4.

Copy number status

Copy number loss of chromosome 3 was detected in 13 samples consisting of 12 iris melanoma and one borderline nevus. SNP-array data was available for four samples and FISH was performed in ten samples. The results from copy number detection using the SNP’s from the NGS panel, SNP-array and FISH were consistent whenever more than one technique was available for analysis. The copy number status of cases 21-29 and 31 were evaluated by more than one technique. An overview of the copy number status, BAP1 IHC and BAP1 mutations is given in Figure 2.

DISCUSSION

To our knowledge, this is the largest study of genetic mutation analysis in iris melanoma and iris nevi for genes that are involved in either uveal or cutaneous melanoma. Iris melanoma and nevi harbor mutations that are found in primary choroidal and cutaneous melanoma. In UM, prognosis is related to nuclear BAP1 expression\textsuperscript{20,21} while in this study, no significant association was found between nuclear BAP1 expression and disease free survival in iris melanoma. Knowledge of the molecular profile is fundamental since potential therapies targeting the cutaneous melanoma signature could have clinical implications in iris melanoma.

Thirty iris melanomas and seven iris nevi were analyzed for mutations in GNAQ, GNA11, EIF1AX, SF3B1, BAP1, NRAS, BRAF, PTEN, c-KIT and TP53 using NGS and BAP1 immunohistochemistry. In this cohort, more GNAQ mutations were detected compared to GNA11 mutations, which is in line with
previous reported mutations in iris melanoma. A hotspot GNAQ or GNA11 mutation was found in 23 (77%) iris melanomas and five iris nevi (72%). These mutations are the same hotspot mutations as described in uveal melanoma. However, the mutation rate is lower compared to uveal melanoma in which a rate up to 93% is described. Other genes that have been described in 3.0-7% of uveal melanoma involving the Gs\textsubscript{a} activating or Gi\textsubscript{a} inhibitory adenylyl cyclase pathway, such as CYSLTR2 and PLCB4, could be involved in iris melanoma as well. It would be interesting to investigate whether CYSLTR2 and PLCB4 are mutated in iris melanoma with a GNAQ or GNA11 wildtype profile, although no mutations in CYSLTR2 have been found in an earlier study of nineteen iris melanomas. GNAQ and GNA11 upregulate the mitogen-activated protein kinase (MAPK) pathway as well as activating BRAF and NRAS mutations. However, the mutation in BRAF (D594G) in our cohort did co-exist with a GNA11 mutation. Mutations in BRAF have been described previous in 9/19 iris melanomas, but these mutations were located at a different position than in our cohort.

NRAS mutations were detected both with and without mutations in GNAQ and GNA11. Inhibition of MEK, a kinase in the mitogen-activated protein kinase (MAPK), is an accepted treatment in specific metastatic cutaneous melanoma cases. In contrast, response rates are lower in patients with metastatic uveal melanoma. Since iris melanomas harbor mutations in genes that are present in cutaneous melanoma as well, unlike uveal melanoma, a study to elucidate the effect of MEK-inhibitors in this specific patient group may be warranted.

Mutations in SF3B1 and EIF1AX were detected in 7% and 17% cases respectively. Considering the sample size, this is comparable to uveal melanoma in which mutations in SF3B1 vary between 10% to 24% and EIF1AX mutated tumors are reported around 20%. A recent study of 19 iris melanomas showed mutations in EIF1AX, but no mutations in SF3B1, BRAF, NRAS and c-KIT. However, mutations in NRAS, BRAF, PTEN, c-KIT and TP53 were found in both iris melanoma and nevi in our series. In The Cancer Genome Atlas, only one deletion in c-KIT has been described before. This supports our hypothesis that iris melanoma should be treated as a distinct subgroup of uveal melanoma. An extra mutation in 50% of the alleles of PTEN was detected at confirmation testing with Sanger sequencing. Possibly, only one allele was covered with NGS so that this mutation was not detected. In four iris
melanoma no mutations were detected which supports our hypothesis of iris melanoma as a distinct subgroup. Possibly, other driver genes are involved in the development of iris melanoma. These samples are subject for additional investigations.

Some studies suggest that mutations in uveal and iris melanoma might be associated with ultraviolet exposure. However, in a whole-genome sequencing study of uveal melanoma, no UV-induced mutation signature was found. In the current study, it is doubtful whether the mutations that we identified in NRAS, BRAF, PTEN, c-KIT and TP53 are related to ultraviolet light exposure since the primary tumors were located in different quadrants of the eye. Furthermore, the mutations that were found in the cutaneous melanoma associated genes were not predominantly C>T or CC>TT mutations, which are known to be caused by ultraviolet light damage. Neither relations between the mutations and geographical differences or regional effects could be observed. Future studies are needed to validate the prevalence of mutations in NRAS, BRAF, PTEN, c-KIT and TP53 and their clinical relevance in iris melanoma.

It is known that chromosome 3 loss is correlated with BAP1 mutations in uveal melanoma. Therefore, copy number status was compared to BAP1 mutations detected with NGS and BAP1 IHC. Loss of chromosome 3 was detected in 13 samples, including one iris nevus. Chromosome 3 loss is described in iris melanoma as well as abnormalities in chromosome 1, 5, 6, 8, 9 and 18. Loss of expression of BAP1 using immunohistochemistry is described in 43% to 50% of uveal melanomas and in 1/3 iris melanomas. In our study immunohistochemistry for BAP1 was negative in 30% of iris melanomas but a BAP1 mutation was found in 43% using Ion Torrent next generation sequencing (Thermo Fisher Scientific, Waltham, Massachusetts, USA). In four tumors with BAP1 expression, a mutation was detected with the sequencing results. Two of these iris melanomas had two copies of chromosome 3 which means that the wildtype allele can produce the BAP1 protein. For the other two cases with monosomy 3, it is possible that the mRNA is not degraded by nonsense-mediated mRNA decay. Probably, a non-functional BAP1 protein is expressed in these tumors. In all tumors with loss of BAP1 expression, mutations were detected with NGS.
In general, iris melanomas have a favorable prognosis compared to posterior uveal melanoma.\textsuperscript{5} \textit{BAP1} mutations and chromosome 3 loss are correlated with a poor prognosis in posterior uveal melanoma.\textsuperscript{15,20} Metastatic disease to the liver developed in two patients with iris melanoma (6.7%), one of them underwent trabeculectomy prior to the diagnosis. Both tumors harbored a \textit{BAP1} mutation and had no \textit{BAP1} expression in the tumor cells. Nevertheless, this study demonstrates that there is no relation between \textit{BAP1} and prognostic outcome in iris melanoma (Figure 1). Therefore, the prognostic value of chromosome 3 and \textit{BAP1} status for iris melanoma is equivocal.

In the iris nevi, mutations in \textit{GNAQ} and \textit{GNA11} were identified. This is in line with the concept that mutations in these genes are an early event in tumorigenesis.\textsuperscript{18} Moreover, a \textit{GNAQ} mutation in an iris nevus is described before.\textsuperscript{25} Interestingly, mutations in \textit{BAP1} were detected in three nevi, two of which were classified histologically as ‘borderline malignant’ prior to knowing the \textit{BAP1} status. One of these ‘borderline malignant’ nevi was from an enucleated eye and the other two were excised because they were also clinically suspect. Since these ‘borderline malignant’ nevi were completely removed, it is uncertain if they would have developed into iris melanoma. Because most nevi showed borderline characteristics, the mutation status of typical nevi might be different. All ‘borderline malignant’ iris nevi showed retained \textit{BAP1} expression. It is possible that the \textit{BAP1} expressing nevus cells obscured the small number of malignant subclones to confidently identify loss of \textit{BAP1} expression in these lesions. Further single cell analysis is warranted to resolve this issue. In case of a heterozygous mutation, the other allele can produce \textit{BAP1}.

To conclude, our study identified mutations in \textit{GNAQ, GNA11, BAP1, SF3B1, EIF1AX, BRAF, PTEN, c-KIT} and \textit{TP53} in iris melanoma and iris nevi. These mutations were found in a cohort composed of samples from different institutes, with an even distribution. ‘Borderline malignant’ iris nevi harbor mutations that confirm their clinical and histopathological borderline malignant status. We think it would be better to designate such cases as iris melanocytic tumors of uncertain malignant potential (IMTUMP), in line with the terminology used for uncertain cutaneous melanocytic lesions (e.g. MelTUMP-melanocytic tumor of uncertain malignant potential).\textsuperscript{38} This would be justified on a combination of histological and molecular findings presented in this study. Since \textit{BRAF, PTEN, c-KIT} and \textit{TP53} mutations are not typical
for uveal melanoma, iris melanoma and iris nevi should be considered a distinct subgroup, based not only on clinical and histopathological criteria, but also on molecular grounds.
The authors would like to thank Frank Magielsen for his help in validating the results using Sanger sequencing.
REFERENCES


**Legends figures**

**Figure 1.** Kaplan-Meier curve showing disease-free survival for iris melanoma with a positive BAP1 expression compared to iris melanoma with a BAP1 negative expression. There is no significant difference between the two groups ($P > 0.05$).

**Figure 2.** Overview of mutations, copy number variation and BAP1 immunohistochemistry in all iris melanomas. The numbers represents all iris melanoma and nevi samples. In the first row the known uveal melanoma hotspot mutations in \textit{GNAQ} and \textit{GNA11} detected with next-generation sequencing are displayed. The second and third row represents mutations that were identified with next-generation sequencing in \textit{GNAQ}, \textit{GNA11}, \textit{BAP1}, \textit{SF3B1}, \textit{EIF1AX}, \textit{NRAS}, \textit{BRAF}, \textit{PTEN}, \textit{c-KIT}, and \textit{TP53}. The fourth row indicates the copy number variation of chromosome 3 detected with SNP's included in the next-generation sequencing panel, fluorescent in situ hybridization and/or SNP-array data. The fifth row represents BAP1 expression using immunohistochemistry. Abbreviations: CNV = copy number variation; IHC = immunohistochemistry. * Metastasizing tumors; † borderline malignant.

**Figure 3.** Histopathological features of two iris nevi. A and B are the same nevus as well as C and D. Left nevus: monosomy 3, no \textit{BAP1} mutation was detected. Right nevus: disomy 3, a c.2146G>A mutation in \textit{BAP1} was identified. A, Haematoxylin and eosin (H&E) staining of an iris nevi (400x). B, H&E staining of an iris nevi (400x). This is an Iris Melanocytic Tumor of Uncertain Malignant Potential (IMTUMP). C, \textit{BAP1} staining of an iris nevus, there is nuclear expression (400x). D, Positive nuclear \textit{BAP1} expression in an borderline malignant iris nevus (400x).

**Figure 4.** Histopathological features and next-generation sequencing (NGS) results displayed in Integrative Genomics Viewer (IGV) of three iris melanoma samples. A, Haematoxylin and eosin (H&E) staining (200x). B, H&E-staining of mixed spindle and epithelioid tumor cells (100x). C, The tumor shows mixed spindle and epithelioid cells in a H&E staining (200x). D, Positive nuclear \textit{BAP1} immunohistochemical (IHC) expression in the tumor cells (400x). E, IHC revealed no \textit{BAP1} expression (100x). F, Positive \textit{BAP1} expression (IHC) in spindle cells, absent \textit{BAP1} expression in epitheloid cells.