Frequent *GNAQ*, *GNA11*, and *EIF1AX* Mutations in Iris Melanoma

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Purpose. The most common malignant intraocular tumors with a high mortality in adults are uveal melanomas. Uveal melanomas arise most frequently in the choroid or ciliary body (97%) and rarely in the iris (3%). Whereas conjunctival and posterior uveal (ciliary body and choroidal) melanomas have been studied in more detail genetically, little data exist regarding iris melanomas.

Methods. In our study, we genetically analyzed 19 iris melanomas, 8 ciliary body melanomas, 3 ring melanomas, and 4 iris nevi. A targeted next-generation sequencing approach was applied, covering the mutational hotspot regions of nine genes known to be mutated in conjunctival and uveal melanoma (*BRAF, NRAS, KIT, GNAQ, GNA11, CYSLTR2, SF3B1, EIF1AX*, and *BAP1*).

Results. Activating *GNAQ* or *GNA11* hotspot mutations were detected in a mutually exclusive fashion in 84% (16/19) of iris melanomas. *EIF1AX* gene mutations also were frequent, detected in 42% (8/19) of iris melanomas. In 4 iris nevi, one *GNAQ* mutation was identified. *GNAQ, GNA11, EIF1AX*, and *BAP1* mutations were identified at varying frequencies in ciliary body and ring melanomas.

Conclusions. In this most comprehensive genetic analysis of iris melanomas published to date, we find iris melanomas to be related genetically to choroidal and ciliary body melanomas, frequently harboring *GNAQ*, *GNA11*, and *EIF1AX* mutations. Future studies will need to assess if screening mutation profiles in iris melanomas may be of diagnostic or prognostic value.

Keywords: iris, malignant melanoma, genetics, *GNAQ*, *GNA11*, *EIF1AX*

The primary intraocular malignancy associated with a high mortality in adults is uveal melanoma. Uveal melanoma has an incidence of 6 cases per 1 million persons per year.[1] Nearly 97% of all uveal melanomas involve the choroid and ciliary body, whereas only 3% involve the iris. The incidence of iris melanomas is low: 0.4 to 0.6 cases per 1 million persons per year.[2,3] The prognosis in patients with uveal melanoma depends on tumor size, tumor location, and histopathologic and cytogenetic features.[4–7] Iris melanomas have a much lower 10-year metastatic rate (6.9%) than ciliary body melanomas (33.4%) and choroidal melanomas (25%).[5] The favorable prognosis of iris melanoma could be related to lower biological aggression and smaller tumor size.[8] Due to the visible tumor location, iris melanoma tumors are diagnosed earlier, and at a much smaller size than choroidal melanoma (55 vs. 300 mm^3).[5,9]

Little is known regarding the genetic pathogenesis of iris melanoma. In contrast, advances in recent years have yielded a fairly detailed understanding of the pathogenesis of ciliary body and choroidal melanoma. Varying gene expression and chromosome aberration profiles divide uveal melanoma into two classes of tumors correlating with patient prognosis. Poor prognosis is associated with monosomy 3, loss of chromosome 6p, and gain of chromosome 8q.[6,7,10–12] A more favorable prognosis is associated with tumors harboring gains of chromosome 6p.[12,13]

Activating mutations in *GNAQ* or *GNA11* are identified in 85% to 91% of uveal melanomas.[14,15] Both genes encode G-coupled protein receptor subunits, which are involved in proliferation, differentiation, and apoptosis pathways, and activate the protein kinase C (PKC), mitogen-activated protein kinase (MAPK) and YAP signaling pathways.[16–19] *GNAQ* and *GNA11* mutations probably are initial events in tumorigenesis, because in addition to being observed in the majority of uveal melanomas, they also are present in benign uveal nevi.[20–22] The mutation status of *GNAQ* and *GNA11* shows absent or limited correlation with patient survival. However, mutations in eukaryotic translation initiation factor 1A (*EIF1AX*), splicing factor 3b subunit 1 (*SF3B1*), and BRCA1-associated protein 1 (*BAP1*) are associated with patient prognosis.[23–25] BAP1 functionally removes ubiquitin molecules, thereby regulating the function of different proteins.[26] A loss of BAP1 leads to dedifferentiation of melanocytes, potentially associated with prometastatic behavior in uveal melanomas.[27] Mutation of *BAP1* generally is observed in conjunction with chromosome 3 monosomy in uveal melanoma and is associated with poor prognosis.[13,25] *EIF1AX* and *SF3B1* mutations, on the other hand, are associated with chromosome 3 disomy, a class 1 gene expression pattern and favorable prognosis.[12,23,28] It is not yet fully understood how these mutations promote cancer. Interestingly, all three genes are primarily mutated in a mutually exclusive fashion in uveal melanoma.

Existing data on genetic alterations in iris melanomas are limited. Recurrent *BRAF* mutations have been reported.[29] A later study described two iris melanomas harboring *GNAQ* mutations.[20] Select chromosomal alterations have been investigated in a few studies. Frequent (71%) partial or complete losses of chromosome 3 in 19 iris melanomas were detected by microsatellite assay (MSA).[30] Another approach identified frequent chromosome 3 (45%) as well as chromosome 9p (35%) losses in 20 iris melanomas by fluorescent in-situ hybridization (FISH).[31] A recent study failed to identify *BRAF* mutations, but reported chromosome 3 losses as well as rarer chromosomes 6p and 8q gains by MSA or multiplex ligation-dependent probe amplification (MLPA).[29] These reports are in part somewhat unexpected, as *BRAF* mutations and chromosome 9p losses are typical of conjunctival and cutaneous melanoma,[32] whereas *GNAQ* mutations and chromosome 3 losses are typical of ciliary body and choroidal melanoma.[23]

A study of gene expression signatures identified the uveal melanoma classes 1 and 2 signatures in approximately 2/3 and 1/3 of tumors, respectively.[33] Despite many tumors having the poor prognostic class 2 signature, no patient had a poor prognosis (e.g., recurrence of disease, metastasis, and so forth).

The goal of our study was to analyze iris melanomas genetically and to determine whether they harbor genetic alterations common to conjunctival or cutaneous melanoma (e.g., *BRAF* or *NRAS* mutations) or characteristic of other uveal melanomas (e.g., *GNAQ* or *GNA11* mutations). A novel amplicon-based targeted next-generation sequencing approach was used, allowing a more detailed analysis of multiple genes from minimal amounts of DNA than was technically feasible in the past.

Materials and Methods

Sample Selection

A total of 48 formalin-fixed and paraffin-embedded (FFPE) tumor samples of iris melanoma, ciliary body melanoma, ring melanoma, and iris nevi were obtained from the archives of the Institute of Pathology, University Hospital Essen ($n = 41$) and University Hospital Tübingen ($n = 7$) from patients treated in the Departments of Ophthalmology of the University Hospital Essen and Tübingen, Germany. In many cases, samples represented biopsies with minimal amounts of material for analysis. Patient data, such as sex, age, laterality of tumor, applied therapy, and occurrence of local relapse and metastatic disease, were obtained from patient medical records.

The tumor type and origin was defined primarily clinically. All patients underwent a detailed examination, including slit-lamp examination, gonioscopy, ultrasound biomicroscopy and diasphanoscopy. Based on these results, tumors could be distinguished as arising from the iris, ciliary body or 360° radial spread tumors (ring melanomas).

The study was performed with written patient informed consent in accordance with the tenets of the Declaration of Helsinki and the guidelines put forth by the ethics committee of the University of Duisburg-Essen, Germany.

DNA Isolation and Next-Generation Sequencing

In cases where sufficient material was present, FPPE tumor blocks were cut in 5 μm thick sections. Tumor material was obtained by anterior segment biopsy performing iridectomy, iridocyclectomy, or by aspiration cutter-assisted anterior chamber biopsy. For iris melanomas, the sample frequently was fully sectioned initially (5–20 sections) for routine pathologic analysis based on minimal amounts of isolated tissue and the concern that an additional attempt to section slides may prove futile. In these cases DNA was isolated from the remaining nonstained sections that had not been required for routine pathology. Sections were deparaffinized according to standard methods. For genomic DNA isolation, we applied the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction.

Three different custom designed amplicon-based sequencing panels were applied. The panels covered between 9 and 29 genes known to be mutated recurrently in cutaneous and uveal melanoma.[34,35] After initially applying two panels covering cutaneous and uveal gene mutations and only detecting recurrent mutations in genes recurrently mutated in uveal melanoma (i.e., *GNAQ* and *GNA11*), we further applied a 9-gene panel focusing on mutations in uveal melanoma (Table 1). This enabled us to focus on the obviously relevant recurrent mutations in uveal melanoma and obtain a higher coverage rate. At the same time, the frequent activating cutaneous and conjunctival melanoma mutations *BRAF*, *NRAS*, and *KIT* still were covered by the panel. Only samples sequenced with this panel were included in our study.

All samples were prepared applying the GeneRead Library Prep Kit from Qiagen according to manufacturer's instruction. For each individual sample the NEBNext Ultra DNA Library Prep Mastermix Set and NEBNext Multiplex Oligos for Illumina from New England Biolabs (Ipswich, MA, USA) were used for adapter ligation and barcoding. Maximum twelve samples were sequence in parallel on an Illumina MiSeq next generation sequencer.

Sequence analysis was performed applying CLC Cancer Res. Workbench from Qiagen. A detailed description of the analysis steps performed has been described previously.[35] Mutations were reported if the overall coverage of the mutation site was $\geq$30 reads, $\geq$10 reads reported the mutated variant, and the frequency of mutated reads was $\geq$10%. The mean coverage achieved for all samples was 19,034-fold with 93% of the target area having a coverage >30 reads. Samples were excluded (14 of 48 tumor samples) if they had poor coverage (<50% of the target region having >30 reads) or too little DNA was available to allow library prep (in most of these cases the DNA concentration was not measurable). In tumor samples sequenced with two panels, mutations detected in both analyses were reported.

Table 1. Genes Covered in the Applied Sequencing Panel

No.	Gene	Chromosome	Location GRCh37	Target Exons	Selection of Mutations Covered	Mutation Type	Target Bases	Bases Covered	Primer Pairs
1	*BRAF*	7	140453065	11, 15	G463, G465,V600	Activating	275	275	4
2	*NRAS*	1	115256411	1, 2	G12, G13, Q61	Activating	345	345	5
3	*KIT*	4	55593572	11, 13, 17	L576, K642, N822	Activating	418	418	8
4	*GNAQ*	9	80409369	4, 5	R183, Q209	Activating	297	297	6
5	*GNA11*	19	3114932	4, 5	R183, Q209	Activating	297	225	3
6	*CYSLTR2*	13	49281314	1	L129	Activating	50	50	1
7	*SF3B1*	2	198267458	14	R625	Alters function	50	50	1
8	*EIF1AX*	X	20156647	1, 2	Mutations exons 1 + 2	Alters function	143	143	3
9	*BAP1*	3	52436293	All	Mutations in all exons	Inactivating	2599	2459	41

BAP1 Immunohistochemistry

Only in a few tumor samples ($n = 5$) was sufficient material available to perform immunohistochemistry (IHC). FFPE tissue was cut in 5-µm thin sections. The applied BAP1 antibody recognizes a synthetic peptide corresponding to amino acids 430 to 729 of the BAP1 molecule (clone C-4; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The primary antibody to detect BAP1 (diluted 1:50 at 36°C for 24 minutes) was used in combination with a highly sensitive and specific polymer detection system applying the chromogen permanent red, resulting in an orange-red reaction product (Ultra view universal alkaline phosphatase detection kit, Ventana). The sections were counterstained with hematoxylin for 5 min. All stainings were performed by Ventana Benchmark XT Autostainer. Tumors were scored as positive or negative according to nuclear staining of BAP1.

Statistical Analyses

For statistical analysis, SPSS Statistics software (version 22.0; SPSS, Inc., Chicago, IL, USA) was used. Associations of categorical variables (Tables 2, 3) were performed using χ^2 and Fisher exact tests, as appropriate. A P value of <0.05 was interpreted as being statistically significant.

Results

Patients and Tumor Samples

The successfully sequenced 34 tumor samples included in the study consisted of 19 iris melanomas, eight ciliary body melanomas, three ring melanomas and four iris nevi. The mean follow-up was 30 months (median 19 months; range, 0–112 months). The mean age at first diagnosis of all patients was 61.3 years (median 65.7 years; range, 26.9–90.6 years). There was no difference in age between the different tumor groups. The tumor samples were collected from 18 men and 15 women. In one ciliary body melanoma patient sex was unknown. Tumors arose in the right and left eyes in 21 and 11 cases, respectively. In two cases, the affected eye was unknown.

Histopathologic analysis reported 28 tumors having a spindle-cell morphology (18 iris melanoma, five ciliary body melanoma, and three iris nevi, and two ring melanoma). One ring melanoma and two ciliary body melanomas were reported as mixed cell-type. In three cases, histologic cell-type was not known.

Most patients received brachytherapy or proton therapy after initial biopsy. In three cases (including one ciliary body and two ring melanomas) initial enucleation was necessary due to tumor size. Seven patients received no treatment at all, including all four patients with iris nevi and also three patients with iris melanomas who refused adjuvant therapy. Local relapse occurred in four cases, including one iris melanoma, two ciliary body melanomas, and one ring melanoma. During follow-up, four patients underwent enucleation due to local relapse ($n = 3$) or secondary glaucoma ($n = 1$). One patient suffered from metastatic disease during the follow-up period. Patient and tumor data are summarized in Table 2.

Gene Mutation Profiles

In total, 34 tumor samples were sequenced successfully and analyzed for the genes *GNAQ, GNA11, CYSLTR2, EIF1AX, SF3B1, BAP1, BRAF, NRAS*, and *KIT* (Table 1). An overview of the results is depicted in the Figure.

The most frequent mutations detected in the 19 iris melanomas analyzed were activating hotspot mutations in *GNAQ* in 68% of tumor samples (10 c.626A>T Q209L; two c.625_626delCAinsTT Q209L, and one c.626A>G Q209R). Activating hotspot *GNA11* mutations were somewhat less frequent, occurring in 16% of iris melanoma (three c.626A>T Q209L). Overall, 84% of iris melanoma harbored either a *GNAQ* or *GNA11* mutation. The identified mutations occurred in a mutually exclusive fashion.

EIF1AX mutations were identified in 8 of 19 (42%) iris melanomas (one c.5C>G P2R, one c.5C>T P2L, one c.7A>C K3E, one c.12T>A N4K, one c.16G>A G6S, two c.22G>A G8R, and one c.23G>A G8E mutation). IHC analysis of BAP1 protein expression was possible only in three samples, two of which were BAP1-positive and one BAP1-negative. Genetically, no mutation in the *BAP1* gene was detected. In three iris melanoma samples no mutations were detected in any of the analyzed genes. Correlations of mutation status with clinical parameters were not observed (Tables 2, 3).

In ciliary body melanomas with iris involvement, *GNAQ* and *GNA11* hotspot mutations occurred in 100% samples. This included six *GNAQ* mutations (four c.626A>T Q209L, two c.625_626delCAinsTT Q209L) and two *GNA11* mutations (all c.626A>T Q209L). *BAP1* mutations were detected in 25% (two of eight) of ciliary body melanomas (one c.485_486delTG V162fs, one c.9delC N413fs). *EIF1AX* mutations occurred in two ciliary body melanoma samples (c. 17G>A G6D; c.12T>A N4K). In the *SF3B1* gene no mutation was identified. None of the ciliary body melanomas was assessed for BAP1 expression by IHC.

GNA11 mutations were detected in all three ring melanoma (all c.626A>T Q209L). One ring melanoma demonstrated a truncating *BAP1* mutation (c.79delG V27fs) correlating to the observed loss of protein expression by IHC. One of the other ring melanomas harbored a *BAP1* mutation genetically (c.47C>G N102K) and demonstrated retained nuclear BAP1 expression by IHC.

Of the four tumors diagnosed as iris nevi that were available for analysis only one *GNAQ* mutation (c.625_626delCAinsTT

Table 2. Clinical and Pathologic Characteristics of the Tumor Cohort

	Total	Iris Nevi, $n = 4$	Iris Melanoma, $n = 19$	Ciliary Body Melanoma,* $n = 8$	Ring Melanoma, $n = 3$	*P* Value†
Median age, y	65.7	71.3	70.1	54.4	66.2	
Sex						
Female	15	4	8	2	1	0.12
Male	18	0	11	5	2	
Unknown	1	0	0	1	0	
Eye						
Right	21	3	11	6	1	0.55
Left	11	1	8	1	1	
Unknown	2	0	0	1	1	
Histotype						
Spindle cell	28	3	18	5	2	0.07
Epithelioid	0	0	0	0	0	
Mixed	3	0	0	2	1	
Unknown	3	1	1	1	0	
Therapy						
Brachytherapy	9	0	5	3	1	<0.001
Proton therapy	12	0	11	1	0	
Enucleation	3	0	0	1	2	
Excision	2	0	0	2	0	
None	7	4	3	0	0	
Unknown	1	0	0	1	0	
Relapse						
Yes	4	0	1	2	1	0.26
No	30	4	18	6	2	
Mutation status						
GNAQ only	12	1	8	3	0	0.08
GNA11 only	2	0	0	1	1	
GNAQ+BAP1	1	0	0	1	0	
GNA11+BAP1	3	0	0	1	2	
GNAQ+EIF1AX	7	0	5	2	0	
GNA11+EIF1AX	3	0	3	0	0	
None detected	6	2	3	0	0	

* For one ciliary body melanoma no data were available.
† Based on Fischer exact test or χ^2.

Q209L) was detected. Mutations in other genes were not observed. Due to lack of available material, analysis for BAP1 status by IHC was not possible.

SF3B1 mutations were not detected in any of the analyzed tumor samples. In none of the other genes assessed were mutations detected, in particular not in the *BRAF, NRAS*, and *KIT*, genes known to be mutated in conjunctival and cutaneous melanoma.

Discussion

In our study of a sizeable cohort of iris melanomas, we were able to demonstrate recurrent *GNAQ*, *GNA11*, and *EIF1AX*

Table 3. Associations of Clinical and Pathologic Parameters With *GNAQ*, *GNA11* or *EIF1AX* Mutation Status in Iris Melanomas

Parameter	Level	Iris Melanoma, $n = 19$	$GNAQ^{wt}$, $n = 6$	$GNAQ^{mut}$, $n = 13$	*P* Value*	$GNA11^{wt}$, $n = 16$	$GNA11^{mut}$, $n = 3$	*P* Value*	$EIF1AX^{wt}$, $n = 11$	$EIF1AX^{mut}$, $n = 8$	*P* Value*
Sex	Female	8	2	6	0.49	8	0	0.17	6	2	0.21
	Male	11	4	7		8	3		5	6	
Histologic type	Spindle cell	18	6	12	N/A	15	3	N/A	10	8	N/A
	Epithelioid	0	0	0		0	0		0	0	
	Unknown	1	0	1		1	0		1	0	
Relapse	No	18	6	12	0.68	15	3	0.84	11	7	0.42
	Yes	1	0	1		1	0		0	1	

^{wt}, wild-type; ^{mut}, mutant; N/A, not assessable.
* Based on Fisher exact test.

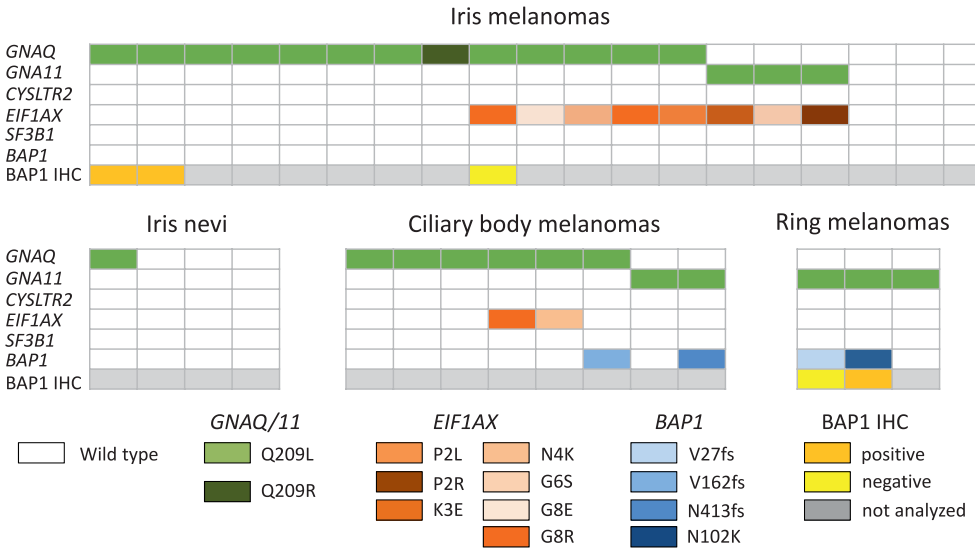

Figure. Mutations identified in iris and ciliary body melanocytic tumors. Shown are the mutations identified by targeted next generation sequencing in all tumor samples (iris nevi, iris melanoma, ciliary body melanoma, ring melanoma). The protein alterations occurring through the different mutations are signified by different *colors*, annotated at the bottom of the Figure. IHC, immunohistochemistry.

mutations, demonstrating a genetic profile highly reminiscent of other uveal (ciliary body and choroidal) melanomas and distinct from that of conjunctival melanomas. The mutational profile identified may be of value in determining clinical prognosis and therapy.

Iris melanomas are very rare and the available samples usually have extremely limited tumor material, which is likely the reason why they have not yet been investigated in greater detail. Our analysis method enabled a panel of genes to be sequenced from very small amounts (5 ng and less) of DNA isolated from FFPE material. This allowed us to detect *GNAQ* and *GNA11* mutations in 84% of iris melanomas. *GNAQ* mutations were identified in 13 (68%) and *GNA11* mutations in 3 (16%) iris melanomas. No activating mutation was detected in three tumor samples. While *GNAQ* mutations were reported previously,[20] to our knowledge our study is the first to report *GNA11* mutations in iris melanoma. The frequency of 84% of iris melanoma harboring *GNAQ* or *GNA11* mutations in a mutually exclusive fashion is highly similar to published results for ciliary body and choroidal melanoma, in which approximately 90% of tumors harbored mutations, also in a largely mutually exclusive fashion.[14,15,36]

GNAQ and *GNA11* mutations are expected to be early or initiating oncogenic events in uveal melanoma.[20] This is supported by our detecting a *GNAQ* mutation in an iris nevus. Our cohort of iris melanomas demonstrated considerably more *GNAQ* than *GNA11* mutations. This could be coincidence; however, a predominance of *GNAQ* mutations has been found in other tumors frequently exhibiting benign behavior, including blue nevi and melanocytomas (benign primary leptomeningeal melanocytic tumors).[34,35] Future studies will need to validate if *GNAQ* mutations are really considerably more frequent than *GNA11* mutations in iris melanoma and if this may have prognostic relevance.

A recent study proposed that A>T mutations in *GNAQ* and *GNA11* may be associated with light exposure.[37] These alterations were identified primarily in tumors arising in the anterior part of the uvea, as well as in patients with lighter colored eyes, whereas A>C mutations were detected primarily in choroidal melanomas of the posterior uvea and patients with darker eye colors. The iris receives more ultraviolet (UV) exposure than other parts of the uvea and our finding of primarily A>T mutations in iris melanomas is in keeping with this proposal. However, additional studies will be necessary to validate this concept.

The high frequency of *GNAQ* and *GNA11* mutations in iris melanomas, along with a complete lack of *BRAF*, *NRAS*, and *KIT* mutations, is similar to findings in other uveal (ciliary body and choroidal) melanomas. In addition to activating mutations in *GNAQ* and *GNA11* (rarely also *CYSLTR2* and *PLCB4*), these tumors frequently harbor additional mutations in *EIF1AX*, *SF3B1*, or *BAP1*. The panel we applied covered all known mutation hotspots for these genes. The only gene recurrently mutated in iris melanomas was *EIF1AX* in 42% of tumor samples. *EIF1AX* mutations occur at a rate of approximately 13% in ciliary body and choroidal melanoma[23,36,38,39] and are associated with a favorable prognosis. *EIF1AX* mutations being considerably more frequent in iris melanomas (42%) than other uveal (ciliary body or choroidal) melanomas (13%) would appear to fit well with iris melanomas generally having a more favorable prognosis. In our cohort, only 1 of 8 iris melanomas (sample #23) with an *EIF1AX* gene mutation demonstrated a local relapse. While our tumor cohort and the available follow-up data are too small to allow conclusive statements, we believe a potential association of *EIF1AX* mutations with prognosis should be assessed in larger cohorts.

SF3B1 mutations occur in approximately 18% of ciliary body and choroidal melanomas.[36] As *SF3B1* mutations occur in a mutually exclusive fashion with *EIF1AX* mutations in ciliary body and choroidal melanomas with favorable prognosis, it is surprising that no *SF3B1* mutations were identified in our cohort of iris melanomas. This finding is unlikely to be due to technical reasons, as the panel used has repeatedly and reliably detected *SF3B1* R625 mutations[40] (and nonpublished data). Potentially it is a bias of our tumor cohort. Additional studies will need to further assess the presence of *SF3B1* mutations in larger cohorts of iris melanoma.

BAP1 mutations occur in approximately 35% of ciliary body and choroid melanomas and are associated with chromosome 3 monosomy and poor prognosis.[25,36,41] While we did detect loss-of-function mutations in ciliary body and ring melanomas (see Figure), no mutations were identified in iris melanomas. Immunohistochemistry could be performed only in select cases with available tissue; however, of 3 iris melanoma

samples analyzed, one demonstrated BAP1 loss. This iris melanoma sample (#9) had demonstrated *GNAQ* and *EIF1AX* but no *BAP1* mutations by sequencing (Supplementary Table S1). Another interesting case is the tumor harboring an *EIF1AX* mutation, which recurred and required enucleation (#23, Supplementary Table S1). Although not part of our genetic analysis, a clinical cytogenetics report in the patient's file reported the tumor as having monosomy 3. This would suggest BAP1 loss; however, our sequencing identified no *BAP1* mutations and, unfortunately, BAP1 IHC could not be performed. These cases where no *BAP1* mutation was identified, but chromosome 3 loss or BAP1 IHC loss was demonstrated, could be a result of the difficulties involved in reliably detecting *BAP1* gene alterations. Our sequence analysis covers 95% of the *BAP1* gene (Table 1) and did reliably detect *BAP1* mutations in ciliary body and ring melanomas. However, 5% of the gene was not covered and potentially mutations in these regions could have been missed. More likely, some mutations (e.g., large-scale deletions of the entire gene or whole exons) may not be detected by panel and other sequencing methods. The remaining DNA from surrounding wild-type tissue may be amplified and sequenced, or the alteration is not identified by the bioinformatics analysis. Promoter methylation, resulting in gene expression silencing and loss of BAP1 protein expression, is another mechanism of BAP1 loss not detected by our sequencing panel. Due to these various possible mechanisms of inactivating BAP1 that are not reliably detected by sequencing, we generally perform IHC BAP1 staining in tumors in which sufficient material is available.[41,42] Due to the extremely limited amount of available tissue, this frequently was not possible in our sample cohort.

Our study has some limitations. Due to very limited quantities of DNA and tissue, we were required to focus analysis on a very select group of genes and generally could not perform IHC analysis of BAP1 status. Where we are convinced the mutations identified are real, the poor DNA quality (a result of its isolation from archival FFPE tissue) may well have resulted in our missing other mutations. This could particularly apply to *BAP1* (as discussed above). Additionally, chromosomal copy number alterations could not be detected by our panel, which would have been valuable, in particular for determining chromosome 3 status. Despite these limitations, the sequencing approach we applied enabled the most detailed analysis of iris melanomas presented to date allowing us to gain more information about the pathogenetic gene mutations involved than any previous study.

Our findings clearly support iris melanoma being part of the nonepidermal derived melanocytic tumor group, consisting of melanocytic tumors of the uvea (ciliary body and choroid), cutaneous dermis (i.e., blue nevi)[34] and the central nervous system (primary leptomeningeal melanocytic tumors).[35,40,43,44] These tumors frequently (70% to 90%) harbor activating *GNAQ* or *GNA11* mutations. Additionally, *EIF1AX*, *SF3B1*, or *BAP1* mutations occur in malignant tumors (melanomas) with *BAP1* mutations associated with a particularly poor prognosis. Although in addition to highly recurrent *GNAQ* and *GNA11* mutations our study only detected highly recurrent *EIF1AX* mutations, we do find it likely *SF3B1* and *BAP1* alterations also may occur. Samples obtained from iris melanomas are very small; however, performing a concise NGS genetic analysis, similar to our presented panel, screening for mutations in *GNAQ, GNA11, EIF1AX, SF3B1*, and *BAP1* as well as performing BAP1 IHC or determining chromosome 3 status should be possible and could provide information allowing prognostic assessment. This could prove highly beneficial for planning treatment and follow-up regimens (e.g., patients with *BAP1*-mutant tumors might benefit from additional adjuvant treatment and/or more frequent follow-up).

In summary, our study identified recurrent mutations in *GNAQ*, *GNA11*, and *EIF1AX* in iris melanoma. This formally supported iris melanoma being genetically related to ciliary body and choroidal melanoma and being distinct from cutaneous and conjunctival melanoma. Detecting the presence of these mutations, particularly *EIF1AX*, could prove to be of prognostic relevance. However, this will need to be assessed in future studies with larger cohorts of iris melanoma patients with detailed follow-up information.

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