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ORIGINAL ARTICLE

Frequent and Yet Unreported *GNAQ* and *GNA11* Mutations are Found in Uveal Melanomas

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Abstract
Malignant melanoma of the uvea is the most common primary malignant tumor in the eye. We aimed to analyze *GNAQ* and *GNA11* mutations in uveal melanomas using formalin-fixed, paraffin-embedded material and correlate the results with clinicopathological parameters. Tumor tissue was microdissected followed by amplification of *GNAQ* exon 4 and 5, *GNA11* exon 4 and 5, and finally analyzed by Sanger sequencing. A total of 64.4 *GNA11/GNAQ* mutations, including ten yet unreported, were found. Two cases showed multiple mutations. Overall survival was significantly shorter in the uveal melanoma cohort with *GNAQ* exon 5 mutation. In concordance with previous studies, high frequencies of mutations in *GNAQ* or *GNA11* were detected. Interestingly, in about 20% of UM, not yet reported mutations in *GNAQ* or *GNA11* were seen. Rarely, uveal melanoma may harbor double mutations in *GNAQ* and/or *GNA11*. Recent data imply, that implementation of *GNAQ/GNA11* mutation analysis in routine diagnostic procedures might be helpful for future therapeutic decisions.

Keywords Uveal melanoma · GNAQ/GNA11 mutation · Driver mutation frequency · Survival

Introduction

Malignant melanoma of the uvea involves melanomas of the iris, the ciliary body and the choroid [1]. Among these, choroidal melanoma is the most frequent primary intraocular tumor in adults [1]. The incidence in the white population is 2 to 8 cases/million/year [2]. About 50% of uveal melanomas (UM) show an aggressive behavior and develop metastases [2].

In skin melanomas, the profile of early driver mutations is quite well known and involves mutations of the RAF and RAS family, leading to an activation of the MAPK/MEK/ERK pathway [3, 4]. However, due to most studies, in primary UM *RAF* and *RAS* mutations are almost never found [4, 5]. Previous studies have identified high frequencies of activating somatic mutations in the *GNA11* and *GNAQ* genes in UM [6–14]. *GNAQ* and *GNA11* belong to a family of heterotrimeric guanine nucleotide-binding protein G which encode the subunit αq and α11, respectively [9, 15]. Mutations in these genes also lead to a constitutive activation of the MAPK/MEK/ERK pathway in UM, similarly as in cutaneous melanomas [8, 9]. Furthermore, the transcriptional coactivator YAP, part of the Hippo pathway, is activated in a GNAQ/GNA11 mutation specific manner in uveal melanomas [16]. The reported frequencies of *GNAQ* or *GNA11* mutations in UM range between 20 and 60% [6–14]. Also, the data on *BRAF* mutations are somewhat variable but mostly low frequency [4, 5, 17–22].

Therefore, we aimed to analyze *GNAQ, GNA11* in uveal melanomas, using formalin-fixed, paraffin-embedded (FFPE) samples and correlate these results with clinicopathological parameters. *BRAF* analysis was also performed to ensure that the analyzed samples derived from primary uveal melanoma and not from metastases of cutaneous melanoma.

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Material and Methods

Patients

A total of 49 patients with UM were included in this study. Among these, 35 choroidal melanomas, 4 melanomas of the iris, 2 melanomas of the ciliary body and 8 UM of the choroidea with ciliary body involvement were analyzed. FFPE samples of 49 patients were retrieved from the archives of Pathology at the University of Rostock, diagnosed between 2000 and March 2014. All patients had undergone enucleation or biopsy (iridectomy) at the Department of Ophthalmology, University Medicine Rostock, between 2000 and March 2014. The study was performed in accordance with the Helsinki declaration and German laws concerning data safety, approved by the Ethics Committee of the University of Rostock (Reference number: A2015–0171) and with informed written consent from all patients prior to surgery. All cases were reviewed and reclassified according to the American Joint Committee on Cancer (AJCC) classification [23]. Callendar cell type was defined as reviewed in [1].

Clinical Data

Clinical and follow-up data were obtained by reviewing the charts of the Department of Opthalmology, Medical University Rostock, and the Clinical Cancer Registry, University Medicine Rostock. These data were unidentified by source and included sex, age at diagnosis, grade, stage, preoperative radiotherapy (brachytherapy or Cyberknife therapy), thermotherapy, overall survival (OS) and progression-free survival (PFS).

Meta-Analysis of *GNAQ*, *GNA11* and *BRAF* Mutation in Uveal Melanomas.

Data from full text papers found in the PubMed database (URL: https://www.ncbi.nlm.nih.gov/pubmed) were extracted. For each eligible study, the following items were extracted: authors, publication year, numbers of cases, frequencies of mutations in *GNAQ*, *GNA11* and/or *BRAF*.

GNAQ, *GNA11* and *BRAF* Mutation Analysis.

DNA was extracted from FFPE sections after microdissection of tumor tissue, followed by deparaffinization, proteinase K digestion and subsequent clean-up using Wizard DNA Clean-up System (Promega, Mannheim, Germany) according to standard protocols. The following genomic regions of interest were amplified by PCR: *GNAQ* exon 4 (forward primer: GCTTTGGT GTGATGGTGTCA, reverse primer: TCATGGACTCAGTT ACTACCTGA), *GNAQ* exon 5 (forward primer: TTTCCCTA AGTTTGTAAGTAGTGCT, reverse primer: CCATTCCC CACACCCTACTT), *GNA11* exons 4 (forward primer: TGCTGTGTCCCTGTCCTG, reverse primer: CACACCGG GCAAATGAGC), *GNA11* exons 5 (forward primer: GATTGCAGATTGGGCCTTGG, reverse primer: CTTGGCAGGTGGGGAAGG) and *BRAF* exon 15 (forward primer: TCATAATGCTTGCTCTGATAGGA, reverse primer: CTTTCTAGTAACTCAGCAGC). Twenty-five microliter of reaction mixtures contained 0.2 μl MyTaq polymerase with 5 μl 5× PCR buffer (Bioline, Luckenwalde, Germany), 1 μM of each primer set and 75 ng of template DNA. PCR reaction was carried out as follows: reactions were started at 95 °C for 1 min. This was followed by 35 cycles at 95 °C for 15 s, 58 °C (*GNAQ/GNA11*) or 60 °C (*BRAF*) for 15 s and 72 °C for 10s. As control, 10 μl of each PCR product were visualized on an agarose gel. PCR products were purified with alkaline phosphatase (Thermo Scientific, Dreieich, Germany) and exonuclease I (Thermo Scientific). Subsequently, sequencing reaction was performed using BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany) with each pair of forward and reverse primers, followed by analysis on a 3500 genetic analyzer (Applied Biosystems). The sequence data were compared with reference sequences (*GNAQ*: ENSG00000156052, *GNA11*: ENSG00000088256, *BRAF*: ENSG00000157764) using SeqScape Software v2.7 (Applied Biosystems).

Statistical Analysis

Statistical analysis was performed using the Statistical Package of Social Sciences (SPSS 22.0, IBM, Ehnigen, Germany). Descriptive statistics were computed for continuous and categorical variables. The parameters include mean and standard deviation of continuous variables, frequencies and relative frequencies of categorical variables. Progression-free survival (PFS) and overall survival (OS) were analyzed by the Kaplan-Maier method and compared by the logrank test. PFS was defined as the time elapsed between histopathological diagnosis and tumor progression. OS was defined as the time elapsed between histopathological diagnosis and patient death. Patients who were progression-free and/or still alive at their last visit were then censored for PFS and/or OS. The Pearson χ^2 test was applied to analyze the correlation between mutation status and different clinocopathological parameters. All p values were obtained using two-sided statistical tests and values of $p < 0.05$ were considered to be statistically significant.

Results

Tumor and Patient Characteristics

The demographic and clinicopathological data of 49 patients with UMs are given in Table 1. The mean age at diagnosis was

Table 1 Patient and tumor characteristics

Number of patients	49	
Age and gender	29 males, range 34–88 yrs., mean 61.76 yrs.	
	20 females, range 50–92 yrs., mean 68.75 yrs	
Neoadjuvant radiotherapy (%)	11/49 (22.4)	
Largest basal diameter	mean 12.49 mm, range 3–26 mm	
Tumor prominence	mean 7.64 mm, range 2–18 mm	
T-stage (%)	1	9 (18.4)
	2	14 (28.6)
	3	19 (38.8)
	4	7 (14.3)
M-stage (%)	0	47 (95.9)
	1	2 (4.1)
R-stage (%)	0	41 (91.1)
	1	4 (8.9)
	Total	45 (100)
Scleral invasion (%)	None	14 (31.8)
	scleral invasion	30 (68.2)
	total	44 (100)
Callender cell type (%)	epitheloid cell type	12 (24.5)
	spindle cell type	16 (32.7)
	(A + B)	21 (42.9)
	mixed cell type	
Location	choroidea	35 (71.4)
	iris with or without ciliary body involvement	4 (8.2)
	ciliary body	2 (4.1)
	choroidal melanoma with ciliary body involvement	8 (16.3)

64.61 years (range 34 to 92). Survival data (OS and PFS) were available in 27 cases (mean OS 39.3 months, range 3–108) and 27 cases (mean PFS 36.3 months, range 3–108), respectively. The following tumor subgroups were included in this study: 35 (71.4%) melanoma of the choroidea, 4 (8.2%) melanoma of the iris with or without involvement of the ciliary body, 2 (4.1%) melanoma of the ciliary body and 8 (16.3%) choroidal melanomas with involvement of the ciliary body. In 11/49 (22.4%) cases, enucleation followed primarily administered radiotherapy and/or thermotherapy.

Molecular Analysis.

The results of the meta-analysis of reported frequencies of mutations in *GNAQ*, *GNA11* and/or *BRAF* are listed in Table 2.

In 45 UM cases which were suitable for *GNAQ/GNA11* mutation analysis, 4 *GNAQ* exon 4 mutations, 6 *GNAQ* exon 5 mutations, 2 *GNA11* exon 4 mutations, and 18 *GNA11* exon 5 mutations were found (Table 3, and Fig. 1a). In sum, we detected 10 *GNAQ* and 20 *GNA11* mutations. PCR amplification failed in 0/45 (0%), 17/45 (37.8%), 15/45 (33.3%), and 7/45 (15.5%) UM which were analyzed for mutations in exon 4 *GNAQ*, exon 5 *GNAQ*, exon 4 *GNA11*, and exon 5 *GNA11*, respectively. Reasons were poor DNA quality of old archived specimens and excessive melanin pigmentation especially in the irradiated cases. In 6/10 irradiated cases, that were sequenced, all target sequences could be evaluated.

In 4/45 (8.9%), 2/28 (7.1%), 1/30 (3.3%) and 2/38 (5.3%) UM yet unreported mutations in exon 4 *GNAQ* (P170S, I189T, Q176R, P193L), exon 5 *GNAQ* (F228 L, M203 V), exon 4 *GNA11* (E191G), and exon 5 *GNA11* (*E234K, E221D*) were detected, respectively (Table 3). In one non-irradiated choroidal melanoma simultaneous mutations in exon 5 *GNAQ* (Q209P) and exon 5 *GNA11* (Q209L) were found. Another irradiated case of choroideal melanoma harbored simultaneous mutations in exon 5 *GNAQ* (Q209P) and exon 4 *GNAQ* (P193L).

Taking all cases, also the partially failed and those with double hits, into account, GNAQ mutations were detected in 20% and GNA11 mutations were detected in 44% (Fig. 1b).

Although the reported frequency is very low, apart from iris melanoma, BRAF mutation analysis was performed, first to characterize the included iris melanomas and second to emphasize the primary character of the uveal tumors. This analysis was successful for 34 cases. In one choroidal melanoma a rare *BRAF* V600A mutation was found (Table 3). Two cases showed an unreported S614F mutation, in one of them

Table 2 Reported frequencies of *GNAQ, GNA11* and *BRAF* mutations in uveal melanomas

Year of publication, reference	*GNAQ* mutation n (%)	*GNA11* mutation n (%)	*GNAQ* and *GNA11* mutation (%)	*BRAF* mutation n (%)	Case numbers
2003 [17]	Not done	Not done	–	0	29
2003 [18]	Not done	Not done	–	0	62
2003 [19]	Not done	Not done	–	0	48
2003 [20]	Not done	Not done	–	0	40
2003 [5]	Not done	Not done	–	0	42
2004 [21]	Not done	Not done	–	1 (2.3)	44
2005 [4]	Not done	Not done	–	1 (3.3)	30
2007 [22]	Not done	Not done	–	9 (47.4)	19
2008 [6]	33 (49)	Not done	–	Not done	67
2009 [7], 2013 [12]	46 (50)	40 (43.5)	93.5	Not done	92
2009 [8], 2010 [9]	73 (44.8)	52 (32)	76.8	Not reported	163
2011 [10]	8 (36.4)	Not done	–	Not done	22
2012 [11]	39/83 (47)	40/91 (44)	91	Not reported	123
2014 [13]	6 (20)	18 (60)	80	Not done	30
2014 [14]	19/45 (42.2)	15/46 (32.6)	74.8	Not done	50
present study	9 (20)	20 (44.4)	64.4	3/34 (8.8)	45

additional to the *GNAQ* double mutation. In 3/4 iris melanomas no *BRAF* mutation was detected. In the fourth case, no FFPE material was available.

Pearson χ^2-test demonstrated significant differences between *GNAQ* mutations and tumor prominence ($p = 0.019$). No statistical significant correlations were found between *GNAQ* or *GNA11* mutations and the clinicopathological parameters pT- and M-category, R-status, largest basal tumor diameter, Callender cell type, tumor progression or survival ($p > 0.05$). Interestingly, in none of the GNAQ wild type cases metastasis occurred.

Survival Analysis

Univariate Cox regression analysis showed a significant prolonged overall survival (OS) in UM with *GNAQ* exon 5 wildtype versus UM carrying mutations in *GNAQ* exon 5 ($p = 0.018$; 95% confidence interval 1.0–120.86, HR 10.72) (Fig. 2). This result could not be confirmed by multivariate analysis. For the UM group carrying an exon 5 GNA11 mutation, a trend toward longer overall survival was seen ($p = 0.099$; 95% confidence interval 0.02–1.66, HR 1.2).

No statistical significances were seen in univariate Cox regression analysis of PFS in UM harboring exon 5 *GNAQ* or exon 5 *GNA11* mutations in comparison to the non-mutated subgroup ($p > 0.05$). Cox regression analysis failed in UM harboring exon 4 GNAQ or exon 4 GNA11 mutations in comparison to the non-mutated subgroup due to a lack of occurrences.

Discussion

Aberrant expression and activity of G proteins and G-protein-coupled receptors are frequently associated with tumorigenesis [24]. Deep sequencing studies have shown that about 4%

Table 3 Frequencies of *GNAQ, GNA11* and *BRAF* mutations in uveal melanomas

GNAQ exon 4	unreported mutations: P170S, I189T, Q176R, P193L	4/45 (8.9%)
GNAQ exon 5	Q209P or Q209L	4/28 (14.3%)
	unreported mutations: F228 L, M203 V	2/28 (7.1%)
GNA11 exon 4	R183C	1/30 (3.3%)
	unreported mutation: E191G	1/30 (3.3%)
GNA11 exon 5	Q209L	16/38 (42.1%)
	unreported mutations: E234K, E221D	2/38 (5.3%)
BRAF exon 15	V600A	1/34 (2.9%)
	unreported mutation: S614F	2/34 (5.9%)

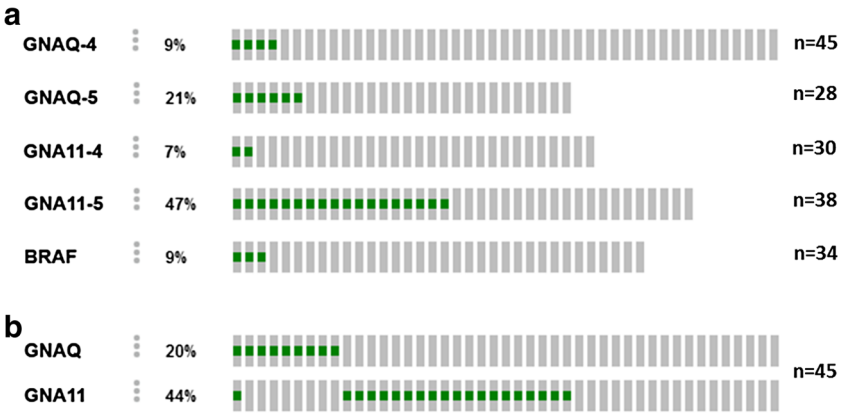

Fig. 1 Summary of mutation analysis. a OncoPrinter (URL: http://www.cbioportal.org/oncoprinter.jsp) representation of mutation analysis results for each exon, only considering the successfully analyzed samples. (**b** OncoPrinter representation of *GNAQ* and *GNA11* mutation analysis by gene, considering all cases analyzed

of human tumors contain activating mutations in *GNAS* (encoding Gαs), and that oncogenic activating mutations in genes encoding Gαq family members (*GNAQ* or *GNA11*) are mutually exclusive present in approximately 5.6% of tumors and in about 66% and 6% of melanomas arising in the eye and skin, respectively, where they can act as driver oncogenes [24–26]. Hotspot mutations in Gαq and Gα11 (R183 and Q209) disrupt the GTPase activity, thereby leading to constitutive activity and persistent signaling in the MAPK/MEK/ERK pathway [3, 4, 25]. The frequency of either *GNAQ* or *GNA11* mutations in about 80% of UM cases is quite high, so that they are now considered to represent the driver oncogenes in UM [8, 9]. In the literature, frequencies of *GNAQ* mutations range between 20 and 53.3%, and the rate of mutations in *GNA11* ranges between 32.6–60% [6–14].

The present analysis of mutations in *GNA11* or *GNAQ* are in line with reported frequencies [6–14], but as in the study of Griewank et al. the absolute number of *GNA11* mutations is higher than alterations in *GNAQ* [13]. Seven studies reported a frequency of *GNAQ* and *GNA11* mutations between 74.8% and 93.5% (median 85.5%) [7–14]. The present analysis revealed *GNAQ* and *GNA11* mutations in 64.4% of cases, considering that for some samples the analyses partially failed, the frequency could be a bit higher. In accordance with recent studies, we also found exclusive mutations of either *GNAQ* or *GNA11* in the majority of cases. However, two cases harbored two simultaneous mutations of *GNAQ* and/or *GNA11*, exons 4 and 5. One case of a choroidal melanoma showed two hotspot mutations in exon 5 *GNAQ* (Q209P) and exon 5 *GNA11* (Q209L). In another irradiated case of choroideal melanoma, simultaneous mutations in exon 4 *GNAQ* (P193L) and exon 5 *GNAQ* (Q209P) were discovered. These results show that double mutations seem to be rare events in UM. Concordantly, Koopmans et al. reported the first double mutation in *GNA11* codons 209 and 214 in 1 of 92 UM analyzed [12].

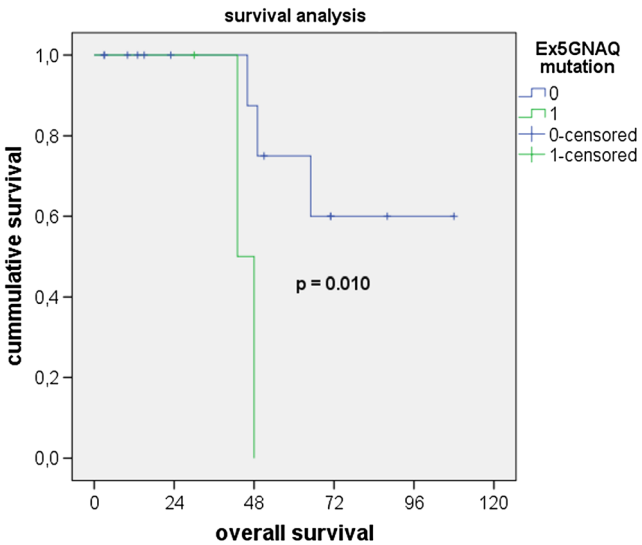

Fig. 2 Kaplan-Meier analysis: overall survival is significantly related to presence of *GNAQ* exon 5 mutation in uveal melanoma ($p = 0.018$)

Also, 9 yet unreported mutations in *GNA11* or *GNAQ* were detected. Most of these mutations are neither annotated in the COSMIC database (URL: http://cancer.sanger.ac.uk/cosmic) nor traceable in PubMed searches, only the *GNA11* E234K mutation was found in a few cases of colorectal carcinoma, but not considered as significant nor discussed as oncogenic [26]. According to the online functional prediction tool PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/index.shtml) some of these mutations might have oncogenic potential (Table 4).

Table 4 PolyPhen2 analysis of undescribed mutations. The calculated score predicts the probability of a mutation being potentially damaging

Mutation	Score
GNAQ P170S	0.000
GNAQ P176R	0.216
GNAQ I189T	1.000
GNAQ P193L	0.026
GNAQ M203 V	0.777
GNAQ F228 L	1.000
GNA11 E191G	1.000
GNA11 E221D	0.389
GNA11 E234K	1.000

In comparison to the described hotspot mutations [24] the functional significance of these new mutations is yet unclear. Most of normally exclusive *GNA11* or *GNAQ* mutations affect Q209 and R183, which are protein loci required for GTPase activity (reviewed in [24]). Both mutations impair GTP hydrolysis. Therefore, most *GNA11* and *GNAQ* mutations render the proteins GTPase defective and constitutively active, leading to prolonged signaling in the MAPK/MEK/ERK pathway [3, 4, 24, 25].

Another downstream effect of GNA11 and GNAQ mutation is the activation of YAP, leading to further promotion of tumorigenesis in uveal melanoma, rendering YAP as another potential therapeutical target [16, 27].

Sanger sequencing was not successful in every case. In particular, poor DNA quality of old archived material before the year 2005 or in irradiated tumors, especially in combination with strong melanin pigmentation, hampered the sequencing analysis. PCR inhibition by melanin is a well-known problem in melanoma research [28]. Nevertheless, the rate of *GNAQ* and *GNA11* mutations rank in the upper range of frequencies reported, and in >50% of irradiated UM, which were sequenced, all target sequences could be evaluated.

Most previous studies had shown none or only single *BRAF* mutations in UM [4, 17–21], but a more recent analysis of Henriquez et al. found a frequency of 47% exon 15 *BRAF* V600E mutations in 19 iris melanomas [22]. In contrast to the latter study, we found no *BRAF* mutation in any of the iris melanomas analyzed. Only one choroidal melanoma carried a rare *BRAF* (V600A) mutation. Clinical examination and follow-up data excluded the metastasis of a skin melanoma in that specific case. The *BRAF* S614F mutation found in two other samples is, like the undescribed *GNAQ/GNA11* mutations, of unknown significance, but predicted as probably damaging (0.991) by PolyPhen-2 software.

Survival analysis demonstrated a significantly worse OS in cases harboring exon 5 *GNAQ* mutations, but this result could not be confirmed in multivariate analysis. On the other hand, statistical analysis revealed a trend toward longer OS in UM carrying an exon 5 *GNA11* mutation. The case numbers and events are too low for further conclusions. Furthermore, it needs to be mentioned that we only analyzed overall survival, not disease specific survival. It is not unlikely that some death events are unrelated to the UM, particular given the fact that only two cases were recorded with metastases during follow-up. Concerning disease-free survival in UM, three studies have shown that *GNAQ* mutation status did not correlate with disease-free survival [7, 10, 12]. This is in line with the present study, as no statistical significances were seen in univariate Cox regression analysis of PFS in UM carrying exon 5 *GNAQ* or exon 5 *GNA11* mutations in comparison to the non-mutated subgroup. As similar frequencies of *GNAQ* mutations were found in all clinical stages of UM, and the mutations are not linked with chromosomal aberrations, mutations of the Gαq family members (*GNAQ* or *GNA11*) are considered to be an initiating event in UM development, which lead to cell proliferation [3, 4, 6, 7]. Additional cytogenetic changes such as monosomy 3 and / or gain of 8q are further important steps towards metastasis [29, 30]. But in the view of the functions of the mutated G-proteins leading to prolonged signaling in the MAPK/MEK/ERK pathway, as well as YAP activation, there might be opportunities for targeted therapy in UM [16, 24]. Targeting of MEK pathway seems to be challenging [31, 32], whereas YAP inhibition looks promising in GNAQ/GNA11 mutated tumors [16, 27, 33].

Conclusions

In concordance with previous studies, high frequencies of mutations in *GNAQ* and *GNA11* mutations were detected. Although more cases showed known *GNAQ* and *GNA11* mutations, about a fourth of cases demonstrated not yet reported mutations. Rarely, UM may possess double mutations in *GNAQ* and/or *GNA11*.

Because recent studies have shown that mutations in *GNAQ* and *GNA11* are sensitive to MAP kinase, protein kinase C, AKT and YAP inhibitors, there might be a therapeutic option for metastasized tumors. Hence, it is there not unlikely that the analysis of *GNAQ* and *GNA11* mutations will become a routine diagnostic procedure of UM, especially in respect to of YAP inhibitor treatment.

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