SHORT COMMUNICATION

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Patient survival in uveal melanoma is not affected by oncogenic mutations in *GNAQ* and *GNA11*

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Background: Mutations in *GNAQ* and *GNA11*, encoding the oncogenic G-protein alpha subunit q and 11, respectively, occur frequently in the majority of uveal melanomas.

Methods: Exons 4 and 5 from *GNAQ* and *GNA11* were amplified and sequenced from 92 ciliary body and choroidal melanomas. The mutation status was correlated with disease-free survival (DFS) and other parameters.

Results: None of the tumours harboured a *GNAQ* exon 4 mutation. A *GNAQ* mutation in exon 5 codon 209 was found in 46 out of 92 (50.0%) of the tumours. Only 1 out of 92 (1.1%) melanomas showed a mutation in *GNA11* exon 4 codon 183, whereas 39 out of 92 (42.4%) harboured a mutation in exon 5 of *GNA11* codon 209. Six tumours did not show any mutations in exons 4 and 5 of these genes. Univariate analyses showed no correlation between DFS and the mutation status.

Conclusion: *GNAQ* and *GNA11* mutations are, in equal matter, not associated with patient outcome.

Previous studies identified high frequencies of activating somatic mutations in the *GNAQ* and *GNA11* genes in uveal melanoma (Onken *et al*, 2008; Bauer *et al*, 2009; Van Raamsdonk *et al*, 2009b, 2010). *GNAQ* and *GNA11* encode the heterotrimeric guanine nucleotide-binding protein G subunit alpha q and 11, respectively. Mutations in *GNAQ*, or its paralog *GNA11* (together referred to as Gα genes), occur mutually exclusively in codon 183 (exon 4) or 209 (exon 5), leading to a constitutive activation of the MAP kinase (MAPK) pathway (Van Raamsdonk *et al*, 2009a, 2010). Limited information is available on correlation of the mutations with survival. We examined to what extent oncogenic *GNAQ* and *GNA11* mutations are correlated with the patient survival.

MATERIALS AND METHODS

Uveal melanomas were collected from enucleated patients at the Erasmus University Medical Centre and the Rotterdam Eye Hospital (Rotterdam, the Netherlands). Informed consent was obtained before the operation, and the study was performed according to the tenets of the Declaration of Helsinki. Fresh tumour material was obtained within 1 h of enucleation and processed for fluorescence *in situ* hybridisation as described previously (Kilic *et al*, 2005). Part of the tumour was snap-frozen and stored in liquid nitrogen. The remainder of the eye was embedded in paraffin. All tumours were histopathologically confirmed. Only tumours located in the ciliary body and choroid were included in this study. Fluorescence *in situ* hybridisation analysis was performed on directly fixated tumour cells for chromosome 1, 3, 6 and 8 using centromeric or locus-specific probes (Kilic *et al*, 2005). High-resolution whole-genome analysis was performed on tumour-derived DNA, using the Illumina BeadChip HumanCytoSNP-12 v2 (Illumina, San Diego, CA, USA) according to the manufacturer's protocol. Filtering, normalisation and data analysis were done using version 6 of the Nexus software program (Biodiscovery, Inc., El Segundo, CA, USA). In total,

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Table 1. Mutations found in *GNAQ* and *GNA11* in detail

Gene	Mutation	No. of cases	Total (%)
GNAQ exon 4	—	0	0
GNAQ exon 5	Heterozygous Q209L	16	50.0
	Heterozygous Q209P	28	
	Homozygous Q209P	1	
	Heterozygous Q209R	1	
GNA11 exon 4	Heterozygous R183C	1	1.1
GNA11 exon 5	Heterozygous Q209L	37	42.4
	Heterozygous Q209P	1	
	Heterozygous Q209L + heterozygous R214M	1	

Abbreviations: *GNAQ* = G-protein alpha subunit q; *GNA11* = G-protein alpha subunit 11.

92 patients were selected for whom follow-up and clinical, histopathological, and cytogenetic data were available.

DNA isolation. To examine tumour content, H&E staining was conducted on a 5-μm section of snap-frozen tumour. Depending on the size of the tumour, 10–15 sections of 20 μm were used for DNA isolation using QIAmp DNA-mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA concentration was measured with the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

***GNAQ* and *GNA11* mutation analysis.** In a previous study, our group performed mutation analysis of *GNAQ* exon 5 in 75 samples (Bauer *et al*, 2009). In the present study, we amplified *GNAQ* exon 5 in 17 other tumour samples with PCR using the primers 5′-ACCATTTTGCTTGGCACAGATAAGG-3′ and 5′-GTAAGTT CACTCCATTCCCCACACC-3′. *GNAQ* exon 4 and *GNA11* exon 4 and 5 were amplified using the primers: 5′-TCTTTTTCTCC CACCCCTTGC-3′ and 5′-TTGTTTTGAAGCCTACACAT GATTCC-3′ to examine *GNAQ* exon 4; 5′-GTGCTGTGTCC CTGTCCTG-3′ and 5′-GGCAAATGAGCCTCTCAGTG-3′ to examine *GNA11* exon 4; and 5′-GATTGCAGATTGGGCC TTGG-3′ and 5′-TCTCCTCCATCCGGTTCTGG-3′ to examine *GNA11* exon 5. PCR products were purified using ExoSAP-IT (USB, Staufen, Germany) and sequenced using BigDye Terminator chemistry v3.1 on an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequences were aligned and compared with reference sequence hg19 from the Ensemble genome database (ENST00000286548 and ENST00000078429) using SeqScape software version 2.6 (Applied Biosystems).

Statistical analysis. The primary end point for disease-free survival (DFS) was defined as the time to the development of metastatic disease, whereby death due to other causes was treated as censored. The influence of single prognostic factors on DFS was assessed using the Kaplan–Meier method (for categorical variables) or the Cox proportional hazard analysis (for continuous variables). To identify the independent value of the prognostic factors on DFS, we used a multivariate Cox proportional hazard analysis with a forward stepwise method based on likelihood ratios. An effect was considered significant if the *P*-value was $\leqslant 0.05$. The statistical analyses were performed with the SPSS software version 20.0 (SPSS Inc., Chicago, IL, USA).

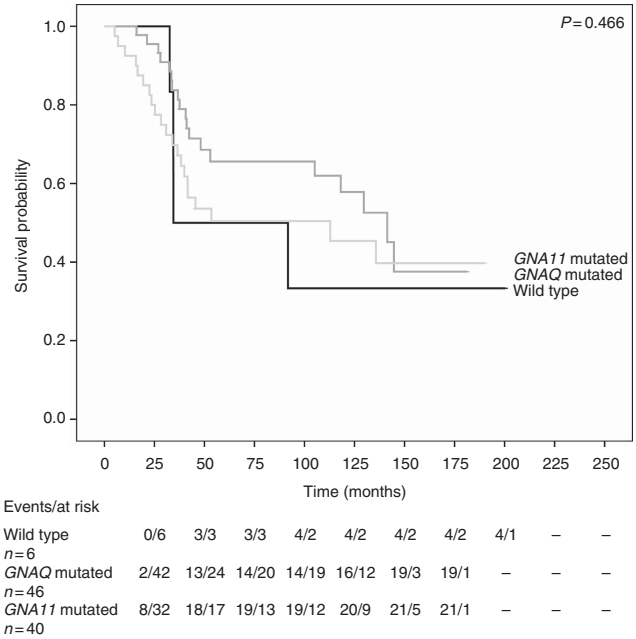

Figure 1. **Kaplan–Meier estimate of DFS in patients with tumours harbouring either a *GNAQ* or *GNA11* mutation compared with tumours harbouring no mutation (wild type).** The table shows the number of events and cases at risk over time at the respective time point. Log-rank test was used to compare survival distributions across subgroups.

RESULTS

A total of 92 patients were included in the study. Forty-eight of the patients were male and 44 were female. The median age was 62 years (range 21–86); the mean largest tumour diameter was 13.3 mm (range 7.0–19.0) and the mean tumour thickness was 8.3 mm (range 1.5–22.0). On the basis of cell type, 15 tumours were classified as epithelioid, 38 as mixed, and 39 as spindle-cell tumours. Most tumours were localised in the choroid; only six were localised in the ciliary body. The mean follow-up was 74.9 months (range 5.2–200.5), and 44 patients developed metastases, from which 39 died. Sixteen patients died due to another cause, and 32 patients were still alive at the end of the study.

Molecular genetic analysis. All uveal melanomas were analysed for *GNAQ* and *GNA11* mutations and for chromosomal aberrations in chromosome 1, 3, 6, and 8. No mutations were found in *GNAQ* exon 4. Forty-six tumours (50.0%) harboured a mutation in *GNAQ* exon 5 codon 209; details are shown in Table 1. Although only one mutated case was found in *GNA11* exon 4, 39 tumours (42.4%) harboured a mutation in *GNA11* exon 5. Six out of 92 tumours contained no mutations in exons 4 and 5 of both genes. One tumour (EOM-0179) showed two mutations in *GNA11* exon 5 (resulting in p.Q209L and p.R214M). Tumour sample EOM-0179 was therefore subjected to deep sequencing with a custom-designed HaloPlex Target Enrichment kit for Illumina (Agilent Technologies, Santa Clara, CA, USA), and both variants were located within the same read (Koopmans *et al*, manuscript in preparation). No DNA from blood of this patient was available to determine whether variant R214M is a germline variant. Therefore, we isolated DNA from formalin-fixed paraffin-embedded retina tissue, and Sanger sequencing of *GNA11* exon 5 revealed a wild-type status.

Statistical analysis. Univariate analyses showed that the DFS was significantly shorter in patients with tumours with loss of chromosome 3, loss of chromosome 8p and gain of chromosome 8q.

Table 2. Correlations between GNAQ and GNA11 mutations and clinical, histopathological and chromosomal data

	***GNAQ* mutation status**			***GNA11* mutation status**		
	Mutated	**Wild type**		**Mutated**	**Wild type**	
Variable	***n* = 46**	***n* = 46**	***P*-value**	***n* = 40**	***n* = 52**	***P*-value**
Mean age (years)	58.9 ± 13.2	65.0 ± 13.5	**0.017**[a]	66.5 ± 12.4	58.4 ± 13.6	**0.004**[a]
Mean largest tumour diameter (mm)	13.6 ± 3.0	13.0 ± 2.9	0.350[a]	13.3 ± 2.6	13.3 ± 3.1	0.915[a]
Mean tumour thickness (mm)	8.5 ± 4.0	8.0 ± 3.2	0.885[a]	8.1 ± 3.3	8.4 ± 3.8	0.968[a]
Gender *n* (%)						
Male	25 (27.2%)	23 (25.0%)	0.676[b]	21 (22.8%)	27 (29.3%)	0.956[b]
Female	21 (22.8%)	23 (25.0%)		19 (20.7%)	25 (27.2%)	
Cell type *n* (%)						
Spindle	17 (18.5%)	14 (15.2%)	0.508[b]	11 (12.0%)	20 (21.7%)	0.270[b]
Mixed/epithelioid	29 (31.5%)	32 (34.8%)		29 (31.5%)	32 (34.8%)	
Involvement of the ciliary body *n* (%)						
Yes	7 (7.6%)	10 (10.9%)	0.420[b]	8 (8.7%)	9 (9.8%)	0.742[b]
No	39 (42.4%)	36 (39.1%)		32 (34.8%)	43 (46.7%)	
Chromosome 1p loss *n* (%)						
Yes	16 (17.4%)	15 (16.3%)	1.000[b]	13 (14.1%)	18 (19.6%)	1.000[b]
No	30 (32.6%)	31 (33.7%)		27 (29.3%)	34 (37.0%)	
Chromosome 3 loss *n* (%)						
Yes	25 (27.2%)	33 (35.9%)	0.130[b]	29 (31.5%)	29 (31.5%)	0.128[b]
No	21 (22.8%)	13 (14.1%)		11 (12.0%)	23 (25.0%)	
Chromosome 6p gain *n* (%)						
Yes	19 (20.9%)	16 (17.6%)	0.668[b]	14 (15.4%)	21 (23.1%)	0.828[b]
No	27 (29.7%)	29 (31.9%)		25 (27.5%)	31 (34.1%)	
Chromosome 6q loss *n* (%)						
Yes	12 (13.2%)	19 (20.9%)	0.125[b]	18 (19.8%)	13 (14.3%)	**0.045**[b]
No	34 (37.3%)	26 (28.6%)		21 (23.1%)	39 (42.8%)	
Chromosome 6q gain *n* (%)						
Yes	3 (3.3%)	4 (4.4%)	0.714[b]	4 (4.4%)	3 (3.3%)	0.456[b]
No	43 (47.3%)	41 (45.0%)		35 (38.5%)	49 (53.8%)	
Chromosome 8p loss *n* (%)						
Yes	9 (9.8%)	10 (10.9%)	1.000[b]	10 (10.9%)	9 (9.8%)	0.440[b]
No	37 (40.2%)	36 (39.1%)		30 (32.6%)	43 (46.7%)	
Chromosome 8p gain *n* (%)						
Yes	9 (9.8%)	11 (12.0%)	0.801[b]	9 (9.8%)	11 (12.0%)	1.000[b]
No	37 (40.2%)	35 (38.0%)		31 (33.7%)	41 (44.5%)	
Chromosome 8q gain *n* (%)						
Yes	26 (28.3%)	34 (37.0%)	0.125[b]	30 (32.6%)	30 (32.6%)	0.122[b]
No	20 (21.7%)	12 (13.0%)		10 (10.9%)	22 (23.9%)	

Abbreviations: *GNAQ* = G-protein alpha subunit Q; *GNA11* = G-protein subunit 11. The significant correlations ($P \leq 0.05$) are shown in bold.
[a]The *P*-value for the comparison of continuous variables among different subgroups was calculated with the Mann-Whitney test.
[b]The *P*-value for the comparison of categorical variables among different subgroups was calculated with the Fisher's exact test.

The DFS in patients with tumours harbouring *GNAQ* or *GNA11* mutations was not significantly less than that in the wild-type tumours (Figure 1). Correlations between the clinical and histopathological parameters, chromosomal parameters, and *GNAQ* and *GNA11* mutations using the Fisher's exact test and the Mann–Whitney test showed a weak association between age and both *GNAQ* and *GNA11* mutation status ($P = 0.017$ and 0.004, respectively; Table 2). *GNA11* mutation status was also correlated with loss of chromosome 6q ($P = 0.045$). We examined the possibility that *GNAQ* and *GNA11* mutations may affect the prognosis of patients with monosomy 3 by constructing Kaplan–Meier curves for changes in chromosome 3, stratified for *GNAQ* and *GNA11* mutations. Log-rank

tests showed that there was no significant effect on the DFS in tumours with loss of chromosome 3 and the presence of *GNAQ* or *GNA11* mutation ($P = 0.745$). Multivariate models were constructed for *GNAQ* and *GNA11* separately with positive variables from the univariate analysis. The presence of epithelioid cells, largest tumour diameter, involvement of the ciliary body, chromosome 3 loss, chromosome 8p loss, and mutations in *GNAQ* ($P = 0.587$) or *GNA11* ($P = 0.796$) were rejected. Only the variable chromosome 8q gain (HR 6.562, $P = 0.000$ for both *GNAQ* and *GNA11* mutation status) and chromosome 6p gain (HR 0.419, $P = 0.014$ for both *GNAQ* and *GNA11* mutation status) were independent predictors of DFS.

DISCUSSION

In this study, we investigated whether *GNAQ* and *GNA11* mutations in uveal melanoma are associated with patient survival. We found that these mutations occur mutually exclusive in the majority of uveal melanomas, up to 93.4%, which is in the same range as reported previously (Onken *et al*, 2008; Bauer *et al*, 2009; Van Raamsdonk *et al*, 2009b, 2010). Van Raamsdonk *et al* (2010) suggested that *GNA11* mutations might have more potent effect on melanocytes than mutations in *GNAQ*. Because the mutations occur in 93.4% of the tumours, it seems to be an early event in the development of a melanoma, and our study demonstrates that mutations in *GNAQ* and *GNA11* do not contribute to the patients' prognosis. Moreover, we conclude that *GNA11* mutations are not more harmful than *GNAQ* mutations in uveal melanoma patients.

All mutations were localised either in codon 209 (exon 5) for both *GNAQ* and *GNA11* or codon 183 (exon 4) for *GNA11* only. Surprisingly, one tumour harboured a double mutation in *GNA11* codons 209 and 214. The reported heterozygous non-synonymous variant in codon 214 results in arginine to methionine transition. A germline variant was excluded by sequencing normal retinal tissue. Using the *in silico* tool PolyPhen-2, both these transitions seem to be damaging on the structure and function of the protein. The tumour with the double mutation had no chromosomal alterations, and this patient has not developed any metastases at a follow-up time of 154.1 months. To our knowledge, this is the first reported double mutation in *GNA11* exon 5 in uveal melanoma.

Recently, the Gα genes have been investigated in metastatic lesions, showing no difference in mutation frequency between rapidly progressive and slowly progressive lesions (Abdel-Rahman *et al*, 2012). This is in line with our findings that patient outcome is not influenced by the presence of mutations in *GNAQ* or *GNA11*.

GNAQ and *GNA11* are involved in the MAPK pathway, and mutations in these genes lead to downstream oncogenic signalling (Van Raamsdonk *et al*, 2009a, 2010). Currently, new therapeutic strategies that inhibit the downstream signalling molecules are being investigated. MEK is a potential target in the MAPK pathway, and the effects of several MEK inhibitors on uveal melanoma cell lines with Gα mutations have been described (Mitsiades *et al*, 2011; von Euw *et al*, 2012). In a preclinical study, Gα-mutant uveal melanoma cells were mildly sensitive to the MEK inhibitor AZD6244, and either moderately or highly sensitive to the MEK inhibitor TAK733. Dual-pathway inhibition of the MAPK and the PI3K/AKT pathway with MEK inhibitor GSK1120212 and PI3K inhibitor GSK2126458 resulted in induction of apoptosis in Gα-mutant uveal melanoma cells (Khalili *et al*, 2012).

In conclusion, we confirm that mutations in *GNAQ* and *GNA11* are, in equal matter, not associated with patient outcome. Also the newly found variant with a double mutation does not affect patient survival. Because the mutations occur in the majority of the tumours, and slowly growing as well as fast growing metastases, targeting of the downstream pathway seems promising. Even though there is no relation with development of metastatic disease, the new therapeutic options would be ideal in stabilising the disease process. At this moment, clinical studies are ongoing and the results have not yet been evaluated.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Abdel-Rahman MH, Cebulla CM, Verma V, Christopher BN, Carson 3rd WE, Olencki T, Davidorf FH (2012) Monosomy 3 status of uveal melanoma metastases is associated with rapidly progressive tumors and short survival. *Exp Eye Res* **100**: 26–31.

Bauer J, Kilic E, Vaarwater J, Bastian BC, Garbe C, de Klein A (2009) Oncogenic GNAQ mutations are not correlated with disease-free survival in uveal melanoma. *Br J Cancer* **101**: 813–815.

Khalili JS, Yu X, Wang J, Hayes BC, Davies MA, Lizee G, Esmaeli B, Woodman SE (2012) Combination small molecule MEK and PI3K inhibition enhances uveal melanoma cell death in a mutant GNAQ- and GNA11-dependent manner. *Clin Cancer Res* **18**: 4345–4355.

Kilic E, Naus NC, van Gils W, Klaver CC, van Til ME, Verbiest MM, Stijnen T, Mooy CM, Paridaens D, Beverloo HB, Luyten GP, de Klein A (2005) Concurrent loss of chromosome arm 1p and chromosome 3 predicts a decreased disease-free survival in uveal melanoma patients. *Invest Ophthalmol Vis Sci* **46**: 2253–2257.

Mitsiades N, Chew SA, He B, Riechardt AI, Karadedou T, Kotoula V, Poulaki V (2011) Genotype-dependent sensitivity of uveal melanoma cell lines to inhibition of B-Raf, MEK, and Akt kinases: rationale for personalized therapy. *Invest Ophthalmol Vis Sci* **52**: 7248–7255.

Onken MD, Worley LA, Long MD, Duan S, Council ML, Bowcock AM, Harbour JW (2008) Oncogenic mutations in GNAQ occur early in uveal melanoma. *Invest Ophthalmol Vis Sci* **49**: 5230–5234.

Van Raamsdonk CD, Barsh GS, Wakamatsu K, Ito S (2009a) Independent regulation of hair and skin color by two G protein-coupled pathways. *Pigment Cell Melanoma Res* **22**: 819–826.

Van Raamsdonk CD, Bezrookove V, Green G, Bauer J, Gaugler L, O'Brien JM, Simpson EM, Barsh GS, Bastian BC (2009b) Frequent somatic mutations of GNAQ in uveal melanoma and blue naevi. *Nature* **457**: 599–602.

Van Raamsdonk CD, Griewank KG, Crosby MB, Garrido MC, Vemula S, Wiesner T, Obenauf AC, Wackernagel W, Green G, Bouvier N, Sozen MM, Baimukanova G, Roy R, Heguy A, Dolgalev I, Khanin R, Busam K, Speicher MR, O'Brien J, Bastian BC (2010) Mutations in GNA11 in uveal melanoma. *N Engl J Med* **363**: 2191–2199.

von Euw E, Atefi M, Attar N, Chu C, Zachariah S, Burgess BL, Mok S, Ng C, Wong DJ, Chmielowski B, Lichter DI, Koya RC, McCannel TA, Izmailova E, Ribas A (2012) Antitumor effects of the investigational selective MEK inhibitor TAK733 against cutaneous and uveal melanoma cell lines. *Mol Cancer* **11**: 22.