Ultradeep sequencing detects GNAQ and GNA11 mutations in cell-free DNA from plasma of patients with uveal melanoma

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Keywords
Biomarker, cell-free DNA, GNAQ, ultradeep sequencing, uveal melanoma

Abstract
Elevated levels of cell-free DNA (cfDNA) are frequently observed in tumor patients. Activating mutations in exon 4 (R183) and exon 5 (Q209) of GNAQ and GNA11 are almost exclusively found in uveal melanoma, thus providing a highly specific marker for the presence of circulating tumor DNA (ctDNA). To establish a reliable, noninvasive assay that might allow early detection and monitoring of metastatic disease, we determined the proportion of GNAQ or GNA11 mutant reads in cfDNA of uveal melanoma patients by ultradeep sequencing. Cell-free DNA from 28 uveal melanoma patients with metastases or extraocular growth was isolated and quantified by real-time polymerase chain reaction (PCR) (7–1550 ng DNA/mL plasma). GNAQ and GNA11 regions of interest were amplified in 22 of 28 patients and ultradeep sequencing of amplicons was performed to detect even low proportions of mutant reads. We detected Q209 mutations (2–38% mutant reads) in either GNAQ or GNA11 in the plasma of 9 of 22 metastasized patients. No correlation between the proportion of mutant reads and the concentration of cfDNA could be detected. Among the nine ctDNA-positive patients, four had metastases in bone, whereas no metastases were detected in the 13 ctDNA-negative patients at this location (P = 0.025). Furthermore, ctDNA-positive patients tended to be younger at initial diagnosis and show larger metastases. The results show that ultradeep amplicon sequencing can be used to detect tumor DNA in plasma of metastasized uveal melanoma patients. It remains to be shown if this approach can be used for early detection of disseminated tumor disease.

Introduction
Uveal melanoma is the most common primary intraocular tumor in adults [1]. Molecular analyses have revealed that there are two distinct classes of this tumor. This knowledge is of clinical relevance because metastasizing disease, which affects 50% of patients, almost exclusively originates from tumors that belong to only one of these classes [2, 3]. The latency period of uveal melanoma metastases is highly variable, and the preferred site of metastasis is the liver [4]. In some patients, metastases have become evident more than 15 years after successful treatment of the primary tumor [5]. No effective treatment options for metastatic disease are known, and therefore, prognosis of patients with metastases has remained poor [6]. Liver function tests including ultrasound imaging of the liver and detection of lactate dehydrogenase, alkaline phosphatase, and gamma glutamyl transpeptidase have been used to detect metastatic disease [6, 7]. However, there is no study showing that these markers permit reliable preclinical detection of metastatic disease in uveal melanoma patients.

Serum and plasma of cancer patients as well as healthy individuals are known to contain cell-free DNA (cfDNA) [8]. Although already identified in the 1940s by Mandel et al., analysis of cfDNA has lagged in the following
decades due to the absence of sensitive and suitable methods of cfDNA detection [9, 10]. Until now, controversial opinions exist concerning the origin and exact mechanism of release of cfDNA in healthy individuals as well as in tumor patients. It has been reported that in healthy individuals most of the cfDNA originates from (mainly hematopoietic) apoptotic cells [11]. Necrosis does not contribute to the cfDNA in healthy individuals [12]. However, all living tumor and nontumor cells may also actively release DNA as reported by Stroun et al. in 2001 [13]. In addition to apoptosis, necrosis of cancer cells, especially those with a high cellular turnover rate, contributes to the frequently observed elevated levels of cfDNA in cancer patients [10, 14]. It has been suggested that quantification of cfDNA has to be combined with mutation analysis to provide the specificity needed to distinguish between cell-free plasma DNA originating from tumor or nontumor cells [10].

Genetic alterations of oncogenes or tumor-suppressor genes that occur during tumorigenesis provide highly specific markers that allow identification and quantification of circulating tumor DNA (ctDNA) [15]. Such tumor-specific mutations have been detected in plasma of patients suffering from various solid cancers or hematopoietic malignancies (for review see [8]). Diehl et al. determined the number of adenomatous polyposis coli (APC) gene fragments in cfDNA of advanced colorectal cancer patients and found elevated mutant proportions in patients with advanced tumor stages [16]. Later, this group used the same approach to monitor tumor dynamics of colorectal cancer patients undergoing chemotherapy or surgery [17]. Mutations of either GNAQ or GNA11 can be detected in 83% of all (primary or metastatic) uveal melanomas [18]. Both oncogenes encode for the alpha subunit of a heterotrimeric GTP-binding protein [19]. Activating somatic mutations in both genes can affect codon Q209 in exon 5 or codon R183 in exon 4. GNAQ mutations at codon Q209 were found in 45% of primary uveal melanomas, 22% of uveal melanoma metastases, and 55% of blue nevi. Mutations in GNA11 at codon Q209 were found in 32% of primary uveal melanomas, 57% of the uveal melanoma metastases, and 7% of blue nevi. Mutations at codon R183 of either GNAQ or GNA11 are rare, affecting about 6% of uveal melanomas [18]. As a consequence of these mutations, the intrinsic GTPase activity of this alpha subunit is blocked and the protein remains in its active state. The resulting MAP-kinase cascade activation leads to stimulation of cell proliferation, for example, by activation of transcriptional cell cycle genes like CDKN1 [18, 20]. Although the MAP-kinase cascade is a mutational target in several tumor entities, activation of this pathway by mutations in either GNAQ or GNA11 is specific for uveal melanomas and other nonepidermic melanocytic lesions like blue nevi [19–21]. It has been suggested that GNAQ/GNA11 mutations are present throughout the different stages of the disease and are early events in tumorigenesis [20, 22]. In this respect, these mutant alleles are suitable markers for detection of cfDNA in plasma of uveal melanoma patients.

Analysis of somatic genetic alterations in cfDNA has been used in many cancers and is a promising tool for noninvasive diagnosis of progressive cancer [15]. Several approaches have been used to detect and quantify ctDNA in tumor patients [14, 16, 17, 23]. More recently, ultra-deep sequencing technologies have been developed, which enable sequencing of tremendous amounts of DNA fragments in parallel at reasonable cost, thus providing an unbiased view on the ratio of mutant-to-normal reads in amplicons generated on cfDNA from cancer patients [24, 25]. In particular, the assay can be applied without prior knowledge about the precise nature of the expected genetic alteration.

The goal of our study was to establish an assay for the detection of mutant alleles of GNAQ and GNA11 based on ultra-deep amplicon sequencing. We analyzed a consecutive case series of patients with uveal melanoma and extraocular tumor (clinically evident metastases or extraocular growth) to assess if this assay detects ctDNA in these patients.

Materials and Methods

Patient samples

This study was approved by the Ethics Committee of the University of Duisburg-Essen (Germany). Informed consent was given by all patients and the Declaration of Helsinki protocols were followed. A consecutive series of 28 patients with a diagnosis of metastatic or extraocular uveal melanoma that had been referred to the University of Duisburg-Essen between November 2010 and September 2011 were asked to participate in this study. Most patients had not received any treatment for metastatic disease at the time of sample collection (see Table 1). Seven patients were under therapy with the multikinase inhibitor sorafenib (varying dosages) and one patient had received intrahepatic chemoembolization 1 month prior to collection of the blood sample. We also included one patient with extraocular growth of the primary tumor who donated blood 3 months prior to clinical diagnosis of metastases. In addition to blood samples, tissue from primary or metastatic tumors was available in nine cases. Plasma samples of seven noncancer individuals were included as negative controls in our study (see Table 2).
Table 1. Clinical characteristics of patients and GNAQ/GNA11 mutation status of tumors and cfDNA.

| ID | Age (years) | Latency PT and M (days) | cfDNA (ng/mL plasma) | Site of metastases at the time of blood sampling | Therapy | Largest diameter (mm) | GNAQ/GNA11 mutation |
|----|-------------|------------------------|----------------------|-----------------------------------------------|---------|----------------------|---------------------|
|    |             |                        |                      |                                               | PT      | M                    | Tumor tissue        |
|    |             |                        |                      |                                               | M       |                      | Plasma              |
|    |             |                        |                      |                                               | Type    |                      | Total reads         |
|    |             |                        |                      |                                               | Mutant reads (%) |
| P1 | 58          | 773 69                 | L                    | E                                              | NT      | 16.2                 | 82                  | NA                  |
|    |             |                        |                      |                                               | GNA11 Q209L | 2089                | 3.9                 |
| P2 | 68          | 803 31                 | L, LN                | B                                              | S       | 11.2                 | 36                  | NA                  |
|    |             |                        |                      |                                               | GNA11 Q209L | 861                 | 4.2                 |
| P3 | 41          | 3285 33                | L                    | B                                              | NT      | 20                   | 111                 | GNA11 Q209L1         |
|    |             |                        |                      |                                               | GNA11 Q209L | 281                 | 4.3                 |
| P4 | 31          | 869 1550               | L, K, LN             | B                                              | NT      | 16.9                 | 54                  | NA                  |
|    |             |                        |                      |                                               | GNA11 Q209L | 3961                | 38.4                |
| P5 | 29          | 4526 82                | L, B                 | Pr                                             | NT      | 8                    | 101                 | NA                  |
|    |             |                        |                      |                                               | GNA11 Q209L | 1986                | 21.7                |
| P6 | 59          | 448 20                 | L, B, LN             | ER                                             | NT      | 17.7                 | 30                  | GNA11 Q209L1         |
|    |             |                        |                      |                                               | GNA11 Q209L | 1275                | 2                   |
| P7 | 49          | 3150 510               | L, B, Mu, LN         | B                                              | S       | 13.7                 | 64                  | NA                  |
|    |             |                        |                      |                                               | GNA11 Q209L | 3588                | 24.2                |
| P8 | 75          | 2774 63                | L, B, S              | E                                              | S       | 17.4                 | 77                  | GNAQ Q209L1          |
|    |             |                        |                      |                                               | GNAQ Q209L | 94                  | 6.7                 |
| P9 | 56          | 346 436               | L                    | B                                              | Ch      | 12.2                | NA                  |
|    |             |                        |                      |                                               | GNAQ Q209P | 2951                | 2.7                 |
| P10| 49          | 1249 80               | L                    | B                                              | NT      | 9.4                 | 48                  | NA                  |
|    |             |                        |                      |                                               | GNA11 Q209 | 2523                | 0                   |
|    |             |                        |                      |                                               | GNAQ Q209 | 2701                | 0                   |
| P11| 70          | 2604 18               | L                    | B                                              | NT      | 13                  | 80                  | NA                  |
|    |             |                        |                      |                                               | GNA11 Q209L | 1212                | 0.49                |
| P12| 65          | 2564 242              | L                    | B                                              | S       | 20                   | 35                  | NA                  |
|    |             |                        |                      |                                               | GNA11 Q209L | 3071                | 0                   |
| P13| 52          | 457 20                | L                    | E                                              | S       | 9                    | 39                  | NA                  |
|    |             |                        |                      |                                               | GNA11 Q209P | 1973                | 0.05                |
| P14| 60          | 2054 132             | L, AC                | B                                              | S       | 15.7                 | 15                  | NA                  |
|    |             |                        |                      |                                               | GNA11 Q209L | 2559                | 0.04                |
| P15| 52          | 623 41                | L                    | B                                              | NT      | 12.9                 | 32                  | NA                  |
|    |             |                        |                      |                                               | GNA11 Q209P | 1646                | 0                   |
| P16| 56          | 956 36                | L                    | E                                              | NT      | 13.8                 | 24                  | NA                  |
|    |             |                        |                      |                                               | GNA11 Q209L | 3150                | 0.26                |
| P17| 59          | 2841 53               | L, Pe                | B                                              | NT      | 11                  | 59                  | NA                  |
|    |             |                        |                      |                                               | GNA11 Q209L | 3281                | 0.09                |
| P18| 70          | 1222 61               | L, SG, ST, Sc, Lu    | E                                              | S       | 15.7                 | 39                  | NA                  |
|    |             |                        |                      |                                               | GNA11 Q209P | 4267                | 0                   |
| P19| 75          | 524 39                | –                    | E                                              | NT      | 20                   | NA                  |
|    |             |                        |                      |                                               | GNA11 Q209L | 477                 | 0                   |
| P20| 75          | 13 7                 | L                    | NT                                             | NT      | 20                  | 19                  | GNA11 Q209L          |
|    |             |                        |                      |                                               | GNA11 Q209L | 1442                | 0.07                |
| P21| 55          | 556 25                | L                    | P                                              | NT      | 11.7                 | 19                  | NA                  |
|    |             |                        |                      |                                               | GNA11 Q209L | 1549                | 0.06                |
| P22| 64          | 1684 26               | L, LN                | B                                              | NT      | 10.8                 | 29.5                | NA                  |
|    |             |                        |                      |                                               | GNA11 Q209L | 2146                | 0.09                |
| P23| 61          | 4251 59               | L                    | B                                              | NT      | 6.4                  | 34                  | No mutation2         |
|    |             |                        |                      |                                               | GNAQ Q209 | 4703                | 0.02                |
| P24| 69          | 6 14                 | L, Pa                | E                                              | NT      | 9.8                  | 18.4                | GNAQ Q2091          |
|    |             |                        |                      |                                               | GNAQ Q209 | 3346                | 0                   |
| P25| 67          | 524 98                | L, Pa                | E                                              | NT      | 16.4                 | 60                  | No mutation1         |
|    |             |                        |                      |                                               | GNAQ Q209 | 2146                | 0.09                |
| P26| 37          | 1449 17               | L                    | Pr                                             | NT      | 17.3                 | 42                  | NA                  |
|    |             |                        |                      |                                               | GNAQ Q209 | 2146                | 0.09                |
| P27| 76          | 16 37                | L, B                 | E                                              | NT      | 19                  | 14                  | GNAQ Q2091          |
|    |             |                        |                      |                                               | GNAQ Q209 | 2146                | 0.09                |
| P28| 76          | 1411 22              | L, Sp, ST, B         | B                                              | NT      | 8.2                  | 35                  | NA                  |
|    |             |                        |                      |                                               | GNAQ Q209 | 2146                | 0.09                |

GNAQ and GNA11 and GNA11 Q209L mutations in cell-free plasma DNA of metastasized uveal melanoma patients and one patient with extraocular growth of primary tumor.

Age, Age at diagnosis of primary tumor; P, patient; NP, healthy individual; PT, primary tumor; M, metastasis; L, liver; LN, lymph node; K, kidney; B, bone; Mu, muscle; Pa, pancreas; Sp, spleen; AC, adrenal cortex; Pe, peritomeum; SG, suprarenal gland; ST, soft tissue; Sc, subcutis; Lu, lung; E, enucleation; B, brachytherapy; Pr, proton beam irradiation; ER, transretinal endoresection; S, Sorafenib; Ch, chemoembolization; NA, not available; NT, no treatment.

1Mutation found in primary tumor.

2Mutation found in metastasis.

3Patient with extraocular tumor growth, but no metastases at the time of blood sampling, patient developed metastases later on (GNA11 Q209 positive).
To quantify cfDNA [16, 23]. We used an MGB (minor groove binder) TaqMan assay that targets a genomic 239-bp fragment located in the AS-SRO region on chromosome 15 as described elsewhere [27]. All samples were analyzed in duplicate.

### PCR reaction and ultradeep sequencing

Amplification of the GNAQ Q209 (298 bp), GNAQ R183 (212 bp), GNA11 Q209 (150 bp), and GNA11 R183 (249 bp) regions was performed using the universal tailed amplicon sequencing strategy as described in the GS Junior System Guidelines for Amplicon Experimental Design (Roche, Mannheim, Germany). Briefly, the region of interest was amplified in a first PCR using template-specific primers (for sequences, see [18, 19]) with a universal sequence at the 5′ end that serves as target for the primers of the second PCR. Different universal tags were used for forward and reverse primers.

In the first PCR, 6 ng of cell-free plasma DNA was used in a final reaction volume of 20 μL containing 0.25 mmol/L of each dNTP, 3.1 mmol/L MgCl2 (Applied Biosystems, Darmstadt, Germany), 2.0 μL tenfold Buffer II (Applied Biosystems), 0.5 μmol/L of each primer, and 2U AmpliTaq Gold (Applied Biosystems). After initial denaturation at 95°C for 5 min, the PCR reactions were subjected to 10 cycles of 95°C (20 sec), 63°C (1 min), and 72°C (1 min), followed by 30 cycles of 95°C (20 sec), 56°C (1 min), and 72°C (1 min) and a final extension at 72°C for 7 min. The Univ-A and Univ-B sequences, which are required for the sequencing process on the Roche GS Junior device, were joined to each amplicon in a second round of PCR. Different Univ-A and Univ-B primers, each tagged with a patient-specific multiplex identifier (MID) sequence, were used to provide the ability to sequence multiple samples in a single run. The second PCR was performed in a reaction volume of 50 μL containing 0.15 mmol/L of each dNTP, 3.1 mmol/L MgCl2, 5 μL tenfold Buffer II, 0.2 μmol/L of each of the primers Univ-A-MID and Univ-B-MID, and 2U AmpliTaq Gold polymerase. After initial denaturation at 95°C for 10 min, PCR was performed for 30 cycles at 95°C (20 sec) and at 72°C (45 sec), followed by elongation at 72°C (7 min). PCR products were separated on a 2% agarose gel and visualized by ethidium bromide staining under UV light. The desired PCR products were extracted from the gel using the QIAamp MinElute gel extraction kit (Qiagen) according to manufacturer’s recommendations to get rid of unincorporated nucleotides and primers. Ultradeep amplicon sequencing was performed using a Roche GS Junior sequencing device following the manufacturer’s protocol. The GS Amplicon Variant Analyzer software (Roche) was employed to analyze the read data.

Mutation analysis of GNAQ/GNA11 exons 4 and 5 was performed on DNA from primary or metastatic tumors.

### Plasma preparation and cfDNA isolation/quantification

Blood samples were obtained by venipuncture (for clinical characteristics of participating patients see Table 1) using 7.5-mL-sized K-EDTA blood collection tubes (ID 01.1605.001, Sarstedt, Nümbrecht, Germany). The blood was centrifuged at 1500 g for 10 min within 30 min after sampling. The supernatant (plasma) was collected after sampling. The supernatant (plasma) was collected at −20°C for later use in DNA preparation. Samples with visual signs of hemolysis (red-colored supernatant) were discarded. Cell-free DNA was isolated from 1 mL of plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) following the manufacturer’s protocol. DNA was eluted in 40 μL AVE Buffer and stored at 5°C. Genomic DNA from primary and metastatic tumor tissue was isolated as described previously [26].

Real-time polymerase chain reaction (PCR) was used to quantify cfDNA [16, 23]. We used an MGB (minor groove binder) TaqMan assay that targets a genomic 239-bp fragment located in the AS-SRO region on chromosome 15 as described elsewhere [27]. All samples were analyzed in duplicate.

### Table 2. GNAQ and GNA11 ultradeep sequencing in cell-free plasma DNA of age-matched healthy individuals.

For legend see Table 1.

| ID | Age at blood sampling (years) | cfDNA (ng/mL plasma) | Gene | Total reads | Mutation type | Mutant reads (%) |
|----|-----------------------------|----------------------|------|-------------|---------------|-----------------|
| NP1 | 33 | 6 | GNA11 | 3700 | Q209P | 0 |
| | | | | | Q209L | 0 |
| | | | GNAQ | 2073 | Q209P | 0.05 |
| | | | | | Q209L | 0 |
| NP2 | 31 | 4 | GNA11 | 3170 | Q209P | 0.03 |
| | | | | | Q209L | 0 |
| | | | GNAQ | 2460 | Q209P | 0 |
| | | | | | Q209L | 0 |
| NP3 | 75 | 117 | GNA11 | 2301 | Q209P | 0 |
| | | | | | Q209L | 0 |
| | | | GNAQ | 1077 | Q209P | 0 |
| | | | | | Q209L | 0 |
| NP4 | 62 | 8 | GNA11 | 2969 | Q209P | 0.1 |
| | | | | | Q209L | 0 |
| | | | GNAQ | 1532 | Q209P | 0 |
| | | | | | Q209L | 0 |
| NP5 | 51 | 6 | GNA11 | 3841 | Q209P | 0.05 |
| | | | | | Q209L | 0.03 |
| | | | GNAQ | 1645 | Q209P | 0 |
| | | | | | Q209L | 0 |
| NP6 | 61 | 13 | GNA11 | 2764 | Q209P | 0.04 |
| | | | | | Q209L | 0.03 |
| | | | GNAQ | 3875 | Q209P | 0 |
| | | | | | Q209L | 0.06 |
| NP7 | 55 | 3 | GNA11 | 5421 | Q209P | 0 |
| | | | | | Q209L | 0 |
| | | | GNAQ | 3743 | Q209P | 0 |
| | | | | | Q209L | 0 |
Results

cfDNA in plasma

We extracted cfDNA from plasma samples obtained from a series of 28 consecutive patients diagnosed with primary uveal melanoma and either metastatic disease or extraocular extension of the primary tumor (for details see Table 1). Quantification of cfDNA in plasma was performed by real-time PCR targeting the AS-SRO2 region [27]. The AS-SRO2 amplicon (239 bp) is a suitable reference, as it is similar sized to the GNAQ and GNA11 target regions (150–298 bp). In the metastatic uveal melanoma patient cohort, the results showed a wide range of cfDNA concentrations ranging from 7 to 1550 ng/mL of plasma (median = 40 ng/mL plasma; quartiles: lower quartile = 24 ng/mL plasma; upper quartile = 80 ng/mL plasma). The results in the noncancer cohort showed an overall lower cfDNA concentrations ranging from 4 to 117 ng/mL plasma (Table 2).

Identification of mutations in exons 4 and 5 of GNAQ or GNA11 in primary or metastatic tumors by Sanger sequencing

Primary tumor or metastatic tissue was available from 9 of 28 patients. We sequenced exons 4 and 5 of GNAQ and GNA11 in DNA from these samples. In seven of nine patients, a somatic mutation was identified (Table 1), each in heterozygous state. The plasma samples from the two patients without mutations in the primary tumor (P23, P25) were excluded from further ultradeep amplicon sequencing.

Ultradeep sequencing of GNAQ/GNA11 amplicons from cfDNA

We performed PCR using 6 ng cfDNA, the equivalent of the genome of about 1000 cells. For 3 of the 26 remaining patients (P24, P26, P28), we did not have a sufficient amount of cfDNA needed for deep amplicon sequencing on each of the four amplicons. PCR on cfDNA of one patient (P27) repeatedly failed to produce sufficient PCR product for further analysis. Thus, amplicon sequencing was performed on cfDNA from 22 patients.

Sanger sequencing of tumor DNA had previously shown GNAQ/GNA11 mutant alleles in 5 of the 22 patients. In three of these five samples, the tumor-specific mutation was detected in cfDNA, albeit with low absolute mutant read numbers (P3, P6, and P8 with 12, 26, and 6 mutant reads, respectively). The tumor-specific mutation was not detected in amplicons of cfDNA of the remaining two patients (P19 and P20), although the number of sequence reads would have permitted detection if it was present at a proportion of over 1%.

Oncogenic GNAQ/GNA11 mutations were identified in cfDNA of 9 of 22 patients. Mutations were restricted to codon Q209 and affected GNA11 more often than GNAQ (six and four samples, respectively). In most cases, the identified substitution was c.626A>T. A double-nucleotide substitution in GNA11 (c.626A>T [j] 627G>A) was detected in cfDNA and the primary tumor of one patient (P3). A rare mutation (c.626A>C) that has previously reported in one uveal melanoma only [18] was identified in the cfDNA of patient nine (P9). One patient, P4, showed mutations in both GNA11 and in GNAQ with a proportion of 38.4% and 2.6%, respectively, relative to normal sequence reads. The proportion of mutant GNA11/GNAQ reads obtained from cfDNA varied between patients. In 5 of 9 patients there were <5% mutant reads. Real-time PCR showed no correlation between the proportion of mutant reads and the concentration of cfDNA: the patients with the highest concentrations of cfDNA (P4 and P7 with 1550 and 510 ng/mL, respectively) also showed the highest proportion of mutant reads (38.4% and 24.2%, respectively). However, the patient with third highest cfDNA value (P9, 436 ng/mL) showed the second lowest (2.7%) proportion of mutant reads.

Clinical findings and mutational status

We compared clinical findings of patients with and without GNA11 or GNAQ mutant alleles in cfDNA as detected by deep sequencing. We found no association to characteristics of the primary tumor, like tumor height or largest basal diameter. However, we observed some relations between the cfDNA mutation status and clinical findings with respect to metastatic disease. First, four of the nine cfDNA-positive patients had metastases in bone, whereas none of the 13 cfDNA-negative patients had metastases at this location (P = 0.025, Fisher’s exact test). Second, we observed that cfDNA-positive patients were younger at initial diagnosis (mean age 52 years vs. 63 years) and had larger metastases (Fig. 1). Neither the type of treatment of the primary tumor (enucleation, brachytherapy, proton beam irradiation), nor systemic therapy of metastatic disease with multikinase inhibitor
sorafenib at the time of blood collection had any discernible effect on the cfDNA mutation status.

**Discussion**

We show here that ultradeep amplicon sequencing can be used to detect ctDNA in patients with uveal melanoma and extraocular manifestation. Mutant alleles of the GNA11 or GNAQ genes, which are highly specific for uveal melanoma, were identified in cfDNA of 9 of 22 (41%) patients. Considering that about 80% of primary tumors or uveal melanoma metastases show mutant GNA11 or GNAQ genes [18], it is plausible that only a few of the remaining 13 patients had tumors without one of these mutations, and therefore, were uninformative for our study. In fact, we could not detect any mutant reads in cfDNA (i.e., they were ctDNA negative) in two of five patients with known GNAQ/GNA11 mutations in the primary tumors. This raises the question why ultradeep sequencing did not detect these mutant alleles in plasma DNA from these patients. One factor known to limit the sensitivity of our assay is the error rate inherent to the next-generation sequencing method that we used [28]. Even in healthy individuals, we detected “mutant” reads at a frequency of up to 0.1%. Therefore we took a conservative approach by setting the cut off for detection to 1%, and setting the condition that a mutation has to be detected both in forward and reverse reads. Sequencing errors, however, are very much dependent on sequence context and type of mutation. In fact, the very mutations under study here showed few misreads, and therefore, we could have safely set a site-specific cut off for detection to levels lower than 1% for these mutations. However, applying less stringent cutoff levels to the data obtained from the two ctDNA-negative patients who had GNA11 mutations confirmed that no mutant reads were evident in their tumor cells (0% and 0.07%). A more general reason that may underlie the failure to detect rare variant DNA is of stochastic nature: presence of only a few mutant DNA molecules in cfDNA increases stochastic bias during early cycles of PCR (PCR drift) [29]. This problem was also encountered by Madic et al. (2012), who used a very elegant and highly mutation-specific PCR method to detect ctDNA in 20 of 21 patients with metastatic uveal melanoma and known GNA11 or GNAQ mutations [30]. An obvious solution for this problem is to obtain larger sample volumes as this will increase the number of mutant DNA molecules available for study. In addition, the study by Madic et al. suggests that the size spectrum of ctDNA in patients with metastatic uveal melanoma is dominated by smaller sized fragments, analogous to the finding in other tumors [31]. Consequently, another possibility to increase the number of amplifiable DNA molecules is to target the PCR primers to smaller regions. This should further increase the sensitivity to detect ctDNA by deep sequencing.

Interestingly, our findings and those of Madic et al. in 2012 [30] confirm and extend the observation by Raamsdonk et al. (2010) [18] that GNA11 codon Q209 mutations were more frequent than GNAQ codon Q209 mutations in metastatic uveal melanoma than in primary uveal melanoma – of the 12 mutations that we identified in either cfDNA or DNA from metastatic tissue, eight (66%) affected codon Q209 of GNA11. The ratios observed by the groups of Madic and Raamsdonk are almost identical with 14/21 (67%) and 13/18 (72%), respectively. In the study by Raamsdonk, metastatic uveal melanoma also showed mutations affecting codon R183 of GNAQ and GNA11 (2/17, 12%). Although none of our patients showed mutations at these positions, it is important to include these positions in the analysis as mutations at these sites are more frequent in metastatic than in primary uveal melanoma.

In one patient (P4), we identified mutations in GNA11 and GNAQ with mutation rates of 38.4% and 2.6%, respectively. Obviously, the cells that contribute to ctDNA in this patient have a heterogeneous GNA11/GNAQ mutation status. One possible explanation for this is that the GNAQ mutation occurred in a cell that already had a
GNA11 mutation. This would result in a situation where all cells with mutant GNAQ are also mutant for GNA11, but only some cells with the GNA11 mutation also show a GNAQ mutation, leading to the higher rates of GNA11 mutation. However, this explanation does not support the observation that GNA11 mutations tend to be more frequent in advanced tumors [18]. Taking this into account calls for another explanation, namely, that the two mutations arose in distinct lineages of tumor cells. Under this assumption, the higher proportion of GNA11 in ctDNA from this patient indicates a greater contribution of GNA11 mutant cells to ctDNA in plasma. This would be in line with the greater role of GNA11 in advanced stages of uveal melanoma.

We found no association between the detection of ctDNA and the size of the primary tumor or treatment modality. This is to be expected, because in all but one patient, the primary tumor had been destroyed months prior to plasma sampling, and thus, it is to be expected that vital tumor cells are present outside of the eye only. We observed that patients with younger age at diagnosis tended to show larger metastases and to be ctDNA positive. Future studies should test if the relation between age at diagnosis and presence of ctDNA is valid. The relation to size was already studied by Madic et al. [30], who found that the amount of ctDNA in plasma correlates well with metastasis volume, which the authors painstakingly determined based on imaging data. We also observed an influence of the site of metastases on ctDNA status. Specifically, all patients with metastases in the bone marrow were ctDNA positive, whereas none of the ctDNA-negative patients had clinically evident metastases at this site. In view of the intimate connection between bone marrow and peripheral blood, such a correlation does not come unexpected, but has not been reported so far to our knowledge.

Detection of ctDNA by ultradep sequencing has the potential for further development. As the results are counts of specific sequence reads, the relative frequencies of these reads naturally provides quantitative information that can be used to determine ctDNA levels in blood [28]. Compared with methods based on allele-specific PCR, ultradep sequencing covers a broader spectrum of mutations per assay. This is relevant as exemplified by patient P3 in our series, who showed a rare oncogenic GNA11 mutation (c.626A>T [j] 627G>A) that may not have been detected using other methods. On the downside, the sensitivity of ultradep sequencing must be improved. However, with recent developments, it has become feasible to distinguish true mutant reads from background noise by paired-end sequencing, and thus, raise sensitivity to one variant in 5000 molecules [28].

Certainly, simply taking blood samples and performing ultradep amplicon sequencing to detect ctDNA would be a very elegant and simple noninvasive method to detect minimal residual tumor disease.

Acknowledgments

The research work was funded by the Dr. Werner Jackstädt-Stiftung. We thank Dr. Nicholas Wagner for critical reading of the manuscript.

Conflict of Interest

None declared.

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