Pyrophosphorolysis-activated polymerization detects circulating tumor DNA in metastatic uveal melanoma.

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Abstract

Purpose: To develop a molecular tool to detect circulating tumor-derived DNA (ctDNA) in the plasma from patients with uveal melanoma (UM) as a marker of tumor burden and monitor treatment efficacy.

Experimental Design: A real-time PCR was developed based on bi-directional pyrophosphorolysis-activated polymerization (bi-PAP) for the quantification of ctDNA using 3'blocked primer pairs specific for the 3 recurrent mutually exclusive mutations of Ga subunits GNAQ and GNA11.

Results: Sensitivity and specificity of bi-PAP were assessed on serial dilutions of tumor DNA in normal DNA for the 3 recurrent mutations. Each assay could detect a single mutated molecule per reaction, while $10^4$ copies of normal DNA were not detected. CtDNA was readily detected in plasma of mice bearing UM xenografts in amounts proportional to circulating human DNA. Finally, plasma were almost always found positive (20 out of 21 tested patients) in a prospective analysis of metastatic UM patients.

Conclusions: Bi-PAP assays detect and quantify ctDNA in metastatic UM patients. A prospective study is ongoing to assess the clinical usefulness of ctDNA level in UM.

Translational relevance

Despite continuous improvement in primary tumor treatment, prognosis of metastatic uveal melanoma (MUM) remains dismal. It is likely that adjuvant therapies will benefit more to patients with low tumor burden, but imaging and biological tools are rather insensitive and positive only in metastatic patients with high tumor mass. We thus aimed to detect disseminated disease at early stage by evaluating circulating blood tumor DNA taking advantages of the highly recurrent $GNAQ/II^{Q209}$ mutations in this disease, and the pyrophosphorolysis-activated polymerization (PAP) technique, an exquisitely sensitive and specific molecular technique derived from the polymerase chain reaction (PCR). We here show...
that PAP detects a single mutated molecule among $10^4$ genome-equivalent of normal DNA and detected tumor DNA in all GNAQ/11 mutated UM xenograft models. More importantly, PAP was positive in plasmatic DNA for 20/21 MUM patients and quantification of tumor DNA correlated well with metastasis volume estimated by imaging. This new technique, which required no special equipment, may enable the selection of patients with minimal tumor mass and facilitate the early evaluation of therapeutic efficiency.
Introduction

Uveal melanoma (UM) is the most common primary intraocular malignancy in adults with an incidence of six cases per million per year. Metastatic spread occurs via the hematogenous route and almost invariably involves the liver (1). Despite improvement of diagnosis and treatment of the primary tumor, there is no effective treatment of metastatic disease and approximately half of patients will die within one year or less following metastases detection (2). Several parameters are associated with metastasis and poor survival, including (i) clinical factors such age, tumor diameter and thickness, extra-ocular extension, (ii) monosomy 3 and (iii) gene expression profiling after enucleation or fine needle aspiration biopsies of the primary tumor (3-6). An issue in high risk patients is to detect relapse at the earliest stage after primary tumor treatment. Unfortunately, clinically available methods to assess the extent of the disease such as detection by liver function tests (LFTs) or imaging are generally positive when the metastatic tumor burden is already high (7, 8). Indeed, the detection of small lesions remains a real challenge especially for the miliary form of metastases, which is frequent in UM (9). More sensitive methods are needed to assess tumor burden and to improve the monitoring of response to treatment in metastatic patients. Such tools could be also very useful to detect disease recurrence at an early stage in order to select patients who may benefit the most from adjuvant therapies.

Circulating cell-free DNA (cfcDNA) represents such a suitable marker and several studies have demonstrated its presence in human blood in various malignancies (for a review, see (10). In cancer patients, the circulating tumor DNA (ctDNA) represents a fraction of cfcDNA bearing the same genetic and epigenetic alterations than the related primary tumor. The majority of ctDNA is derived from tissue tumor cells rather than from circulating tumor cells and even primary tumors have been shown to release ctDNA in the bloodstream (11). A milestone contribution from Diehl and Coll was the demonstration that detection and quantification of
specific mutations in ctDNA fragments could be successfully used to assess tumor burden and monitor treatment efficacy in colorectal carcinomas (12).

Several issues concerning sample processing, assay specificity and sensibility remain to be addressed. The main difficulty is to set up techniques enabling detection and quantification of tumor-specific single base changes in a large excess of wild-type DNA. For example, PCR-based methods using 5' hydrolysis probes fail to detect less than 1% mutant DNA diluted in normal DNA (13). To overcome this problem, digital PCR methods compartmentalize the ctDNA fragments through water-in-oil emulsion (BEAMing) or using a droplet-based microfluidic system before individual analysis and quantification (11, 14, 15). However, these strategies remain delicate and expensive to perform, requiring instrumentations and expertise beyond most diagnosis laboratory setting. A promising method developed by Sommer and Coll relies on the use of pyrophosphorolysis-activated polymerization (PAP), a highly specific PCR-derived method that can detect point mutations in the presence of a great excess of wild-type DNA (16, 17). The specificity of this method, which totally discriminates a single nucleotide change, derives from the serial coupling of activation of two opposing 3'-blocked pyrophosphorolysis activatable oligonucleotides (bi-PAP) with extension of the unblocked oligonucleotides (18) (Figure 1). These blocked oligonucleotides must perfectly anneal to their complementary target for their activation to occur, limiting the risk of false-positive and self-dimerization.

The present study describes the development and the validation of a bi-PAP real-time PCR approach for the detection of tumor specific mutations in ctDNA of UM patients. More than 80 percent of UM patients have mutually exclusive somatic mutations in GNAQ or its paralog GNA11, both genes encoding alpha subunits of heterotrimeric G proteins (19, 20). Mutations occur almost exclusively at nucleotide 626, leading to substitution of the glutamine at codon 209 into a leucine for GNAQ<sup>626A>T</sup> (Glu209Leu) and GNA11<sup>626A>T</sup> (Glu209Leu), or a proline
for \(GNAQ^{626A>C}\) (Glu209Pro), and turning \(GNAQ\) and \(GNA11\) into dominant acting oncogenes. These mutations are not clearly associated to a poor prognosis (21). These mutations are thought to occur at the early phase of oncogenesis as they are also found in benign proliferations of melanocytic origin, such as blue naevus and naevus of Ota (19, 20).

In order to detect and quantify these mutations in ctDNA, three bi-PAP assays targeting \(GNAQ^{626A>T}\), \(GNAQ^{626A>C}\) and \(GNA11^{626A>T}\) were designed. These assays were validated in terms of sensitivity and specificity in reconstruction experiments and plasma from xenografted mice. More importantly, these assays allowed a quantitative detection of ctDNA in plasma from patients with metastatic UM.
Materials and Methods

Biological samples

Uveal melanoma xenografts established at Institut Curie were described in (22). The following 11 xenografts models were used in this study: MM26, MM33, MM52, MM66, MP34, MP38, MP41, MP42, MP46, MP55 and MP80. In addition, a cell line derived for an UM xenograft (MP65) was used for set-up. Peripheral blood samples (100 to 500 µl) were drawn into EDTA tubes at different stages of engraftment. Tumor size was measured at time of the sampling. Human blood samples (15 ml) were collected in EDTA tubes from a series of 21 patients with a confirmed diagnosis of metastatic UM (MUM), at distance (more than 6 months) of any invasive exploration and systemic treatment for 16 patients, 3 weeks after chemotherapy for 3 patients, one week after liver biopsy for a patient and 2 months after liver surgery for another patient. The primary tumor has either been surgically removed or treated by brachytherapy or proton therapy, and locally controlled in all cases. Patients were prospectively enrolled in this study at any time of the metastatic disease course. The protocol was approved by Ethical and Clinical committees and all patients signed an informed consent. Blood samples from 20 healthy human individuals were used as control.

Plasma preparation and DNA extraction

Plasma samples were prepared as described by Diehl and Coll. (12). Briefly, blood was centrifuged at 820g for 10 min. The supernatant was transferred to sterile tubes, centrifuged at 16,000g for 10 min at room temperature, and the supernatant was stored at -80°C. The overall process from blood collection to plasma storage did not exceed 3 hours. DNA was extracted from plasma of patients and xenografts using the QIAamp circulating nucleic acid and MinElute virus vacuum kits (Qiagen, Courtaboeuf, France), respectively. Extraction was performed according to the manufacturer instructions except that only 1µg RNA carrier was used. Nucleic acids were stored at -20°C.
Mutation screening of \textit{GNAQ} and \textit{GNA11} in tumors and xenografts.

For tumors, one microgram of RNA was reverse-transcribed using SuperScript II reverse transcriptase (Life Technologies, Saint Aubin, France). A 300 bp region was amplified from tumor cDNA or xenograft DNA using the following primer set: GNAQ\textsubscript{Fwd}, 5’-GATGTGCTTAGAGTTCGAGTCC-3’, GNAQ\textsubscript{Rev}, 5’-TTCTCATTGTCTGACTCCACGA-3’, GNA11\textsubscript{Fwd} 5’-TACCAGCTCTCGACTCTGC-3’ and GNA11\textsubscript{Rev}, 5’-TTGGTCGTATTGCCGTGAGG-3’. Purified PCR products were sequenced with dideoxynucleotides (BigDye Terminator v1.1, Life Technologies), 3.2 pmol of specific primer in 20 µl, purified on a Sephadex G50 column, and analyzed with a capillary sequencing machine (3500xl Genetic Analyzer, Life Technologies).

Real-time PCR assays

In bi-PAP real-time PCR assays, primers blocked at their 3’ termini by a dideoxy-nucleotide (dd) and specific for \textit{GNAQ}\textsubscript{626A>T} Fwd, 5’-ACCTTGCAGAATGGTCGATGTAGGGGGCC-ddT-3’, \textit{GNAQ}\textsubscript{626A>T} Rev, 5’-AGTGTATCCATTTCTTCTCTCTGACCTT-ddA-3’, \textit{GNAQ}\textsubscript{626A>C} Fwd, 5’-ACCTTGCAGAATGGTCGATGTAGGGGGCC-ddC-3’, \textit{GNAQ}\textsubscript{626A>C} Rev, 5’-AGTGTATCCATTTCTTCTCTCTGACCTT-ddG-3’ and \textit{GNA11}\textsubscript{626A>T} Fwd, 5’-TCCTTTCAGGATGGTGGATGTGGGGGGCC-ddT-3’ and \textit{GNA11}\textsubscript{626A>T} Rev, 5’ – AGTGGATCCACTTTCCGCTCGAGGACCACG-ddA- 3’ were obtained from Eurogentec (Liege, Belgium) and Biosearch Technologies (Novato, CA, USA). The PCR reaction was done in a total volume of 25 µl including DNA sample, 1 U KlenTaq-S (Scientech Corp, MO, USA), 1X KlenTaq-S buffer, 8% (v/v) dimethyl sulfoxide (DMSO), 25 µM of each dNTP, 1 mM dithiothreitol (DTT), 0.1X ROX calibration dye, 0.2X SYBR Green I dye (Life Technologies) and optimized concentrations of primers and Na\textsubscript{4}PPI as follow: the PCR assay targeting \textit{GNA11}\textsubscript{626A>T} and \textit{GNAQ}\textsubscript{626A>T} contained 5 pmol of each primer, and 30 or 40 µM Na\textsubscript{4}PPI, respectively. The PCR assay targeting \textit{GNAQ}\textsubscript{626A>C} contained 2.5 pmol of each primer and 30
µM Na₄PPi. The cycling conditions consisted of an initial denaturation step at 94°C for 2 min followed by 40 to 45 cycles of 94°C for 20 s, 60°C for 30 s, 64°C for 30 s, 72°C for 30 s. The final step involved the generation of a melting curve analysis.

Total human circulating cell-free DNA (cfcDNA) was quantified using the LINE1 real-time PCR assay (LINE1 PCR) as described in Rago et al. (23). A serial dilution of normal human peripheral blood mononuclear cells (PBMC) DNA was incorporated in each plate as standard. All real-time PCR assays were carried out in a 7500 Fast real-time PCR system (Life Technologies). The baseline and the cycle threshold value (Ct) were set using the Life Technologies software.

**Efficiency, sensitivity and specificity of the bi-PAP real-time PCR assays**

DNA extracted from UM xenografts or cell line (MM33, MP38, MP65) and from PBMC were used as positive and negative controls, respectively. DNA concentration was determined by spectrophotometry using a Nanodrop 2000c apparatus. The diploid genome-equivalent (GE) was calculated assuming a DNA content of 6.6 pg per cell. Specificity and sensitivity were determined on reconstruction experiments with serial dilutions of tumor DNA (1 to $10^4$ GE) in $10^4$ GE of normal human PBMC DNA.

**Quantitative detection of ctDNA in human samples**

DNA was extracted from 5 ml plasma and eluted in a final volume of 36 µl. Bi-PAP PCR assays were performed on 10 µl DNA in 3 independent experiments. The sensitivity and the specificity of the assays were controlled on each plate by using serial dilutions of tumor DNA in normal DNA including 10 replicates of mixtures containing 1 GE of the targeted genes and 6 replicates of $10^3$ GE of normal DNA, respectively.

**Estimation of liver metastasis volume by imaging**

The volume of the metastatic lesions was estimated on liver MRIs containing morphological T1 and T2 sequences, T1 dynamic sequences after injection of a Gadolinium chelate as well as a
diffusion-weighted sequence with respiratory synchronization, and performed near the time of blood sampling. For each patient (n=16) with nodular lesions, all the round and well limited lesions were manually measured. Their volume was calculated according to the formula of the volume of a sphere. For the patients (n=5) presenting lesions with irregular shape, ill-defined borders or too numerous, the volume was calculated on the sequence of imaging offering the best contrast with the healthy liver on a dedicated imaging workstation by means of a thresholding method.

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism version 5.0 (San Diego, CA, USA). Correlation between the log transformed variables were estimated using linear regression models (Pearson) and confirmed using a non-parametric Spearman test. Mann-Witney U test was used to compare cfcDNA concentration between MUM patient and control group. *P* values lower than 0.05 were considered statistically significant.
Results

Features of the bi-PAP real-time PCR assays

We developed 3 bi-PAP assays to detect the three most recurrent \textit{GNAQ} and \textit{GNA11} point mutations in uveal melanoma. Primer pairs were designed to target a short DNA sequence (less than 60 bp) as detection of short amplicon (<100pb) has been shown to be more suitable for tumor DNA detection in plasma (ctDNA) (24). Efficiency, linearity, sensitivity and specificity of the bi-PAP assays were assayed in reconstruction experiments in which tumor DNAs carrying the targeted mutations were serially diluted in a background of $10^4$ genome-equivalent (GE) of normal DNA. Linearity ranged over a 3-log from $10^4$ to 10 copies of targets per reaction, allowing quantitative detection. Efficiency was 74, 90 and 100\%, for \textit{GNAQ}^{626A>T}, \textit{GNAQ}^{626A>C} and \textit{GNA11}^{626A>T}, respectively (Figure 2). The sub-optimal efficiency observed in one assay might be related (i) to the opposite direction of the pyrophosphorolysis and polymerization reactions and the necessary addition of PPi, which inhibit the polymerization; (ii) to the specific sequence encompassing the detected mutation; (iii) to sub-optimal quality of the primers as 3’-blocked primers are difficult to synthesize. To further determine the limit of detection and specificity of the bi-PAP assays, 10 replicates containing 5, 2.5, 1 and 0 copies of the target genes in $10^4$ genome equivalent of normal DNA were assessed. The results are summarized in Table 1. Bi-PAP achieved a high sensitivity in all 3 assays. Five copies of the targets were consistently detected for \textit{GNAQ}^{626A>T}, \textit{GNAQ}^{626A>C}, and in 8 out of 10 assays for \textit{GNA11}^{626A>T}, while 2.5 copies were detected in 8, 10 and 7 out of 10 assays for \textit{GNAQ}^{626A>T}, \textit{GNAQ}^{626A>C} and \textit{GNA11}^{626A>T}, respectively. A single copy of the target was detected in 6 out of 10 replicates with the \textit{GNAQ}^{626A>T} and \textit{GNA11}^{626A>T} bi-PAP assays and 4 out of 10 replicates with the \textit{GNAQ}^{626A>C} bi-PAP assay. These results are compatible with a Poisson distribution, and indicate a sensitivity of 1 copy per reaction. No positive detection occurred when cross-testing tumor DNAs and the 3 bi-PAP assays specific for the irrelevant
$GNAQ/GNA11$ mutations (data not shown). Finally, no positive detection was observed for the 3 assays in 10 replicates containing only $10^4$ GE of normal DNA. Some signal was observed at the last PCR cycles for the $GNA11^{626A>T}$ assay, but with a high Ct and a different melting temperature.

**Validation of the bi-PAP real-time PCR assays on plasma of mice bearing xenografts**

We then assessed the ctDNA detection assays in experimental models of UM. A total of 11 xenografts were tested for their $GNA11/GNAQ$ mutations. The $GNA11^{626A>T}$ mutation was found in 6 xenografts models, while $GNAQ^{626A>C}$ and $GNAQ^{626A>T}$ mutations were found in 2 models respectively. No mutation was found in the MM52 model. At time of sacrifice, the tumor was measured and plasma collected for DNA extraction. Plasma DNAs were then assayed with the LINE1 PCR assay for human cfcDNA detection and the 3 bi-PAP PCR assays for the detection of $GNAQ/GNA11$ activating mutations. Circulating cell-free human DNA concentration ranged from 1 to 152 GE per 20 µl of plasma in xenografted mice. In 6 ungrafted mice, cfcDNA concentration ranged from 0.056 to 0.136 GE per 20 µl of plasma, representing either some non-specific amplification of murine DNA or an unavoidable very low level of human DNA contamination (23). Mutations targeted by the 3 bi-PAP assays were detected in plasma DNAs extracted from all the xenografts accordingly to their mutational status. No positive detection was observed with the 3 bi-PAP assay on plasma from MM52 and ungrafted mice. As expected, cfcDNA and ctDNA concentration measured in plasma were positively correlated (Figure 3) further validating the bi-PAP assays.

**Quantitative detection of GNAQ/GNA11 mutations in metastatic UM patients**

We then explored whether bi-PAP assays detect ctDNA in human samples. A series of 21 MUM patients were prospectively assessed for ctDNA. Activating $GNAQ/GNA11$ mutations were determined by bi-PAP on tumor biopsies, and the relevant bi-PAP assay was performed on the corresponding plasma samples. In one case for which no tumor sample was available,
the bi-PAP assays were directly performed on plasma DNA. The cfcDNA was measured by LINE-1 PCR allowing the calculation of the proportion of ctDNA in plasma cfcDNA. The activating mutations were detected in all but one (20/21) plasma samples from metastatic UM patients whereas no positive detection was observed in plasma of 20 healthy volunteers. High variations of ctDNA amount were found among patients (Figure 4) ranging from 1.3 to 2125 copies per ml of plasma and representing 0.08 to 100% of the total cfcDNA. In the six advanced MUM patients (#8, 9, 10, 13, 18 and 19), the ctDNA fraction reached 19.2 to 100% of the total cfcDNA. The weak correlation found between ctDNA and cfcDNA levels (Supplementary Figure S1 and Supplementary Table S1) was mainly due to these advanced cases, and cfcDNA distribution largely overlapped between MUM patients and controls. Thus cfcDNA is of no apparent clinical value in UM. In some cases, very low amount of ctDNA was measured, highlighting the need to perform ctDNA quantification on large volume of plasma. Even using these large amounts of cfcDNA, ctDNA were found in some instance below 5 copies, leading to Poisson distribution with the risk of false negative as exemplified in cases #4 and 7. Variability displayed among the replicates observed in patients #1, 11, 17 and 21 might be explained by technical issues or potential presence of inhibitors in samples, which represents a caveat of assessing large volume of biological material.

**Correlation between ctDNA and tumor burden as assessed by imaging**

CtDNA levels are probably related to both tumor burden and tumor cell death rate. We therefore looked for a possible correlation between ctDNA as measured by bi-PAP and tumor burden assessed by liver MRI. Figure 5 show a linear relationship between the 2 variables ($R^2=0.5827$; $p$-value < 0.001), indicating that the main determining factor of ctDNA is tumor mass. Noteworthy, the only patient with no detectable ctDNA had a low tumor burden, with a liver metastasis volume estimated at 0.46 cm$^3$. Therefore ctDNA represents a potential biomarker for tumor burden assessment.
Discussion

This study demonstrates that pyrophosphorolysis-activated polymerization developed over years by Sommer and Coll is a reliable, exquisitely sensitive and specific technique able to detect circulating tumor DNA identified by a single nucleotide variation (16, 17). An important advantage of PAP over other proposed techniques is its simplicity, allowing its implementation in most clinical molecular pathology laboratories. Once the assay has been set-up, the per-sample cost is low, enabling longitudinal studies of large cohorts. All recurrent (mostly activating) oncogenic mutations could potentially be assessed by PAP, UM representing a remarkable model situation in which 3 mutations encompass 85% of patients. Other biomarkers of dissemination have been proposed in UM, mainly based on the detection of circulating tumor cell (CTC) either by RT-quantitative PCR (25, 26), or by magnetic immunopurification followed by immunochemistry (IHC) (27, 28). However illegitimate transcription could cause false positive results using RT-PCR, and CTC immunopurification/IHC is costly, labor intensive and require a trained pathologist. Comparisons of ctDNA and CTC assessments in large UM cohorts will be necessary to determine the respective significance and clinical usefulness of both strategies.

PAP assay was shown to be consistently positive in metastatic UM and is an easy and reliable measure of tumor burden. In this disease, local eradication is achieved in the vast majority of cases, and subsequent presence of ctDNA is likely to reflect metastatic disease. However, one cannot exclude that the irradiated ocular tumor tissues could release ctDNA for a long period of time. A prospective longitudinal study analyzing patients undergoing brachy- or proton-therapies is required to address this possibility. Therefore, at the present time, the presence of ctDNA cannot be used to ascertain a disseminated disease.

In 2 patients with low tumor burden, we observed negative replicates reflecting the stochastic nature of the bi-PAP assay. This highlights the need to process large quantity of biological
material to lower the limit of detection. However, this may lead to the presence of polymerase inhibitors, which should be minimized by optimizing DNA extraction and purification protocol. In addition, false negative replicates were observed especially during the implementation of the technique in patients with high copy level (patients #11, 17 & 21), and were probably due to technical issues. These issues are now fully controlled by performing replicates in independent experiments, and by verifying the sensitivity of the bi-PAP in every experiment. This includes low and high positive controls, 10 replicates at 1 copy per reaction and several negative controls.

Large prospective studies measuring ctDNA in UM patients are required to assess the clinical usefulness of this biomarker at different stages of the disease: (i) at primary diagnosis and after treatment of the primary tumor to assess the prevalence of ctDNA at early stage and its prognostic significance; (ii) during follow-up in high risk patients, to assess the respective sensitivity of ctDNA detection versus imaging for early detection of metastatic dissemination; (iii) during treatment of the metastatic disease, to assess tumor lysis potentially induced by chemotherapy and tumor burden evolution during treatment; (iv) before liver surgery, to select patients candidates to R0 (microscopically complete) resection of liver metastases.

New targeted therapies are being developed in UM. Once signs of clinical efficacy will have been demonstrated in metastatic patients, ctDNA could be used to select patients with micrometastatic disease to be included into future clinical trials in an adjuvant setting. Noteworthy, the bi-PAP technique is applicable to any recurrent oncogenic mutations such as in \textit{PI3K}, \textit{KRAS} and \textit{BRAF}. Once developed, this inexpensive assay could be used in a wide variety of frequent cancers, such as breast, colorectal and pancreatic carcinomas or cutaneous melanoma.
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References


Figure legends

**Figure 1. Principle of pyrophosphorolysis-activated polymerization (PAP) detection technique.** A. From top to bottom. A primer blocked at its 3’ extremity by a dideoxynucleotide anneals specifically to its complementary target. In presence of pyrophosphate, the dideoxynucleotide is removed by the pyrophosphorolytic activity of the polymerase. The unblocked primer can then be extended by polymerization. B. Bidirectional PAP (bi-PAP) amplification. Two opposing blocked primers overlapping by one nucleotide at their 3’- termini cannot be directly activated and extended by DNA polymerase if mismatched. In presence of the mutated target matching the primers, the blockers are removed by pyrophosphorolysis, enabling specific PCR amplification.

**Figure 2. Efficiency of bi-PAP real-time PCR assays.** The 3 bi-PAP assays were evaluated in reconstruction experiments. Left panels, representative amplification plots obtained for admixture of tumor DNAs harboring $GNAQ^{626A>C}$, $GNAQ^{626A>T}$ and $GNA11^{626A>T}$ at $10^4$, $10^3$, $10^2$, $10^1$ and 0 (WT DNA) copies in $10^4$ genome-equivalents of normal DNA. Right panels: corresponding standard curves generated by plotting the log of genome equivalents per assay (x-axis) versus the Ct value (y-axis).

**Figure 3. Correlation between cfcDNA and ctDNA in xenografted mice.** cfcDNA and ctDNA levels in plasma of xenografted mice for 11 independent models of uveal melanoma. Human cfcDNA and $GNAQ/11$ mutated ctDNA were quantified by LINE-1 PCR assay and bi-PAP PCR assays, respectively.

**Figure 4. CtDNA and cfcDNA level in metastatic UM patients.** A, ctDNA concentrations expressed as genome-equivalent per reaction (GE/PCR) in plasma samples of 21 metastatic UM (MUM) patients measured by bi-PAP assay in three independent experiments. Positive replicates are represented as plain circles. Dashed line represents the limit of sensitivity of the bi-PAP assay and empty circle below the dashed line, negative replicates.
B, cfDNA concentration measured by LINE1 PCR assay in plasma samples of 21 metastatic UM patients and in 20 healthy volunteers. Geometric mean is represented by a bar, and the P value of the Mann-Witney U test is indicated.

**Figure 5. Correlation between ctDNA and tumor burden assessed by MRI**

ctDNA levels in genome-equivalent per ml (GE.ml⁻¹) in plasma of 21 metastatic uveal melanoma patients were correlated with the volumes of liver metastases in cm³ as assessed by magnetic resonance imaging (MRI). Dashed line represents the limit of sensitivity of the bi-PAP assay, and empty circle below the dashed line, the negative bi-PAP assay.
Figure 2. Madic et al.
Figure 3. Madic et al.
Figure 4. Madic et al.
Figure 5. Madic et al.
Table 1. Sensitivity and specificity of the 3 bi-PAP assays.

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*: in genome equivalents.
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