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Circulating Tumor DNA as a Prognostic Factor in Patients With Resectable Hepatic Metastases of Uveal Melanoma

Pascale Mariani, MD,* François-Clément Bidard, MD, PhD,†‡§
 Aurore Rampanou, BA,† Alexandre Houy, MS,|| Vincent Servois, MD,¶
 Toulsie Ramtohol, MD, PhD,¶ Gaele Pierron, PhD,# Marion Chevrier, PhD,**
 Benjamin Renouf, PhD,†† Olivier Lantz, MD, PhD,‡‡§§||| Sophie Gardrat, MD,¶¶
 Anne Vincent-Salomon, MD, PhD,¶¶ Sergio Roman-Roman, PharmD, PhD,##
 Manuel Rodrigues, MD, PhD,‡|| Sophie Piperno-Neumann, MD,‡
 Nathalie Cassoux, MD, PhD,* Marc-Henri Stern, MD, PhD,||⊠
 and Shufang Renault, PhD†⊠

Objective: We report here the results of a prospective study of circulating tumor DNA (ctDNA) detection in patients undergoing uveal melanoma (UM) liver metastases resection (NCT02849145).

Background: In UM patients, the liver is the most common and often only site of metastases. Local treatments of liver metastases, such as surgical resection, have a likely benefit in selected patients.

Methods: Upon enrollment, metastatic UM patients eligible for curative liver surgery had plasma samples collected before and after surgery. *GNAQ/GNA11* mutations were identified in archived tumor tissue and used to quantify ctDNA by droplet digital polymerase chain reaction which was then associated with the patient's surgical outcomes.

Results: Forty-seven patients were included. Liver surgery was associated with a major increase of cell-free circulating DNA levels, with a peak 2 days after surgery (~20-fold). Among 40 evaluable patients, 14 (35%) had detectable ctDNA before surgery, with a median allelic frequency of 1.1%. These patients experienced statistically shorter relapse-free survival

(RFS) versus patients with no detectable ctDNA before surgery (median RFS: 5.5 vs 12.2 months; hazard ratio=2.23, 95% CI: 1.06-4.69, $P=0.04$), and had a numerically shorter overall survival (OS) (median OS: 27.0 vs 42.3 months). ctDNA positivity at postsurgery time points was also associated with RFS and OS.

Conclusions: This study is the first to report ctDNA detection rate and prognostic impact in UM patients eligible for surgical resection of their liver metastases. If confirmed by further studies in this setting, this noninvasive biomarker could inform treatment decisions in UM patients with liver metastases.

Keywords: circulating tumor DNA, ddPCR, liver metastases resection, prognostic value, uveal melanoma

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Uveal melanoma (UM), arising from melanocytes of the choroid (90%), the iris (4%), and the ciliary body (6%),¹ is the most common primary intraocular tumor in adults with an incidence of 2 to 8 new cases per million per year.^{2,3} Up to 50% of patients with primary UM eventually develop a metastatic disease, mainly in the liver (90%)⁴ with then a median progression-free survival and overall survival (OS) of 3.3 and 10.2 months, respectively.⁵ Recently, tebentafusp demonstrated a clinical benefit in HLA-A02:01-positive patients.⁶ However, for oligometastatic patients, surgical resection of liver metastases is still an efficient option that yields a 2- to 3-fold longer median survival in retrospective studies on selected patients.⁷⁻⁹ Despite the surgical treatment of UM liver metastases, including complete resection with no positive margins (R0), some patients relapse within the next few months, most often in the liver. Therefore, an easily accessible, sensitive, and specific method that would allow a better selection of patients for surgical treatment of hepatic metastases of UM is a medically relevant need.

Circulating tumor DNA (ctDNA), a fraction of total cell-free circulating DNA (cfcDNA), has emerged in the last decades as a promising biomarker for diagnosis, prognosis, and monitoring treatment efficacy in different cancer types.¹⁰⁻¹² UM could be considered as a convenient model for investigating the clinical validity of ctDNA detection since most UMs display mutually exclusive, clonal, and activating hotspot mutation of either *GNAQ* or *GNA11*, not detected in healthy subjects.^{13,14} Prior reports from our group and others¹⁵⁻²⁰ have shown that

From the *Department of Surgical Oncology, Institut Curie, Paris, PSL Research University, Paris, France; †Circulating Tumor Biomarkers Laboratory, Inserm CIC-BT, Department of Translational Research, Institut Curie, Paris, France; ‡Department of Medical Oncology, Institut Curie, Paris and Saint-Cloud, France; §UVSQ, Paris-Saclay University, Saint Cloud, Paris, France; ||Inserm U830, DNA Repair and Uveal Melanoma (D.R.U.M.) Team, Institut Curie, PSL Research University, Paris, France; ¶Department of Radiology, Institut Curie, PSL Research University, Paris, France; #Somatic Genetic Unit, Department of Genetics, Institut Curie, PSL Research University, Paris, France; **Biometry Unit, Institut Curie, PSL Research University, Paris and Saint-Cloud, France; ††Direction of the Clinical Research, Institut Curie, Paris, France; ‡‡INSERM U932, Institut Curie, PSL University, Paris, France; §§Clinical Immunology Laboratory, Institut Curie, Paris, France; |||Inserm CIC-BT1428, Institut Curie, Paris, France; ¶¶Department of Diagnostic and Therapeutic Medicine, Institut Curie, PSL Research University, Paris, France; and ##Department of Translational Research, Institut Curie, PSL Research University, Paris, France.

✉marc-henri.stern@curie.fr; shufang.renault@curie.fr

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ctDNA is detectable in metastatic uveal melanoma (MUM) patients by various techniques, such as bidirectional pyrophosphorolysis-activated polymerization polymerase chain reaction, droplet digital polymerase chain reaction (ddPCR) and next-generation sequencing; these reports suggested ctDNA detection is a prognostic marker in patients undergoing systemic treatment for a MUM. We report here the result of a prospective study assessing the clinical validity of ctDNA detection in the specific context of MUM patients undergoing a surgical resection for UM liver metastases.

METHODS

Patients and Blood Sampling

From August 2014 to February 2018, 318 patients with documented liver metastases were reviewed by a multidisciplinary board in our institute to assess the indication of complete surgical resection. Eligibility criteria of the study were as follows: patients aged 18 years and above with MUM eligible for curative liver surgery, treated at Institut Curie (Paris, France) with no other distant metastases. Fifty-three patients, diagnosed with MUM eligible for curative liver surgery with no other distant metastases, were initially included. Intraoperative ultrasound was performed by the surgeon to all patients to search for all the liver metastases detected preoperatively on liver magnetic resonance imaging (MRI). Patients classified as R2 resection (macroscopically incomplete resection) with more liver metastases than expected preoperatively ($n=6$) were excluded. Patients classified as R1 resection (microscopically incomplete resection) were confirmed with no macroscopically visible residual disease following resection. Finally, 47 patients were enrolled in this study. All patients signed a written informed consent to this ethically approved study, registered with clinicaltrials.gov (NCT02849145). For each patient, longitudinal blood samples were collected within 4 weeks before surgery and in the following days: day 2 (D2), day 8 (D8), and months 4, 8, 12, 16, 20, and 24 (noted below as M4, M8, M12, M16, M20, and M24, respectively) during 2 years or until relapse. For the time points at 4, 8, 12, 16, 20, and 24 months, an earlier blood sampling might be performed if relapsed. Tumor volume before surgery was obtained by summing the individual volume of each liver metastasis, measured on MRI by an expert radiologist (V. S.). After surgery, liver MRI was performed every 4 months to monitor liver relapse. Histologic type, presence of tumor-infiltrating lymphocytes (yes/no), and of tumor necrosis (yes/no) within the resected liver metastases were assessed per routine by experienced pathologists. Patient characteristics and survival data were prospectively registered.

Plasma Sample Preparation, Storage, DNA Extraction, and Quantification

Blood samples were collected in EDTA tubes and plasma was isolated within 2 hours by a 2-step centrifugation: 820 g for 10 minutes, then 16000 g for 10 minutes at 4°C. DNA was extracted using the QIAmp Circulating Nucleic Acid Kit (Qiagen) according to the manufacturer's instructions and quantified by Qubit 2.0 fluorometer (Invitrogen) using the dsDNA HS Assay (Invitrogen). Extracted DNA was stored at -20°C before use.

Mutation Detection With the Use of ddPCR Assays

For the patients with no *GNAQ* and *GNA11* mutation status available in our clinical database, ddPCR assays were

used for tracking somatic mutations in tumor DNA. Following this, identified *GNAQ* or *GNA11* mutations were used for ctDNA monitoring in plasma by ddPCR. DNA from cell lines: OMM2.3 (*GNAQ* p.Q209P c.626 A>C), MP46 (*GNAQ* p.Q209L c.626 A>T), OMM1 (*GNA11* p.Q209L c.626 A>T)²¹ or tumor tissues carrying the following mutations: *GNAQ* p.Q209R c.626 A>G, *GNAQ* p.R183Q c.548 G>A or *GNA11* p.R183C c.547 C>T was used as positive control. Genomic DNA from healthy donors purchased from Promega was used as a negative control. Primers and probes for detecting *GNAQ* p.Q209P, *GNAQ* p.Q209L, and *GNA11* p.Q209L mutations are described in Supplementary Table 1 (<http://links.lww.com/SLA/E449>). Assays for detecting *GNAQ* p.Q209R (dHsaMD-S971099482), *GNAQ* p.R183Q (dHsaMDS533896396), and *GNA11* p.R183C (dHsaMDS314447910) were purchased from Biorad Laboratories. ddPCR assays were performed using the BioRad QX100 system (Biorad Laboratories) with 900 nM of each primer, 250 nM of each probe with one probe targeting the wide-type sequence and the other one targeting the mutant sequence being labeled with different fluorophores. The amplification was under the following conditions: 95°C for 10 minutes (1 cycle); 40 cycles of 94°C for 30 seconds, and hybridization at a specific temperature for each mutation for 60 seconds, and 98°C for 1 minute (1 cycle). The hybridization temperatures used were as follows: *GNAQ* p.Q209P and *GNAQ* p.Q209L at 56°C, *GNA11* p.Q209L at 61.6°C, *GNAQ* p.Q209R, *GNAQ* p.R183Q, and *GNA11* p.R183C at 53.9°C. Cluster thresholding and quantification were performed with QuantaSoft, v.1.7.4 software. Droplets were manually assigned as wild-type (WT) or mutant (MUT) based on their fluorescence amplitude: WT: VIC⁺ or HEX⁺; MUT: FAM⁺. The mutant allelic frequencies were calculated as follows: copy numbers of MUT/(copy numbers of WT+copy numbers of MUT). The total copy number of cfcDNA in each patient was calculated as the sum of the copy numbers of WT and MUT sequences. All experiments met the minimum requirements for digital polymerase chain reaction data.^{22,23}

ddPCR Data Analysis

The false-positive rate of each assay was estimated as previously reported^{24,25} using ≥ 19 replicates of WT DNA. The limit of blank, defined as the upper 95% confidence limit of the mean false-positive measurements, was estimated at 0.009% for *GNAQ* p.Q209L, 0.008% *GNA11* for p.Q209L, 0.008% for *GNAQ* p.R183Q, 0.005% for *GNAQ* p.Q209R assays, 0.012% for *GNAQ* p.Q209P, and 0.018% for *GNA11* p.R183C.

Samples were considered as positive when mutant allelic frequency was higher than limit of blank with >2 positive droplets for *GNA11* p.R183C or 1 positive droplet for *GNAQ* p.Q209P, p.R183Q, p.Q209L, p.Q209R, and *GNA11* p.Q209L detected per analysis. When <300 amplifiable genomes were detected by ddPCR, the sample was not considered for further ctDNA analysis.

Statistical Analyses

This is the first study investigating ctDNA detection and prognostic impact in the specific clinical setting of UM liver metastases resection. Due to its exploratory nature, the study had no prespecified power. Categorical variables were compared using the Pearson's χ^2 test or Fisher's exact test. When considered as continuous variables, Mann-Whitney and Wilcoxon rank-sum tests were used for continuous data. The correlation was assessed using the Pearson coefficient. Survival analysis was performed using Kaplan-Meier plots with significance tested

using likelihood ratio test. Relapse-free survival (RFS), defined as the time from surgery in the study to progression of disease, was collected prospectively. Patients with no disease progression were censored at the last follow-up visit. OS was defined as the time from surgery in the study to the date of death from any cause.

Cox proportional hazards modeling was used for univariate and multivariate analyses to evaluate the prognostic significance of different predictors collected before surgery. The variables tested in model 1 were ctDNA detection as a quantitative continuous variable and metastasis-free interval (months) (calculated from the time of primary UM diagnosis to the time of first liver metastasis diagnosis). Other clinical and pathologic characteristics were excluded from multivariate analysis due to their P value >0.1 in univariate analysis (Supplementary Table 2, Supplemental Digital Content 2, <http://links.lww.com/SLA/E450>), the lack of data in all the patients (eg, the histology data of the primary UM were collected only in the patients with enucleation) or their correlation with other independent variables (eg, number of liver metastases). Eventually, metastasis-free interval was the only clinicopathologic factor feasible for multivariate analysis. In model 2, metastasis-free interval (months) was included as independent variable and in model 3, only ctDNA detection as a quantitative continuous variable was tested. The Akaike Information Criterion score was used to estimate the best model.

Statistical analyses were performed with GraphPad Prism (version 8.0) or R software (version 4.1.1). P values ≤ 0.05 were considered statistically significant.

RESULTS

Clinical Characteristics of the Patients

Forty-seven patients, diagnosed with MUM eligible for curative liver surgery with no other distant metastases, were included in this study (Fig. 1). According to the histologic residual tumor (R) classification by the International Union Against Cancer (UICC),^{26,27} 5 patients were classified as R1 (microscopically incomplete resection) after surgery and 42 patients were classified as R0 (microscopically complete

resection). Patient characteristics are shown in Supplementary Table 3 (Supplemental Digital Content 3, <http://links.lww.com/SLA/E451>).

Identification of Trackable Somatic Mutations

All patients ($N=47$) had an available archived tumor tissue, either from the primary tumor or the resected liver metastases. Finally, 43 patients were detected with one somatic mutation in tumor tissue DNA (Fig. 1). These patients were then assessed for cfcDNA and ctDNA levels before and after surgery by targeting the mutation identified in the tumor.

cfcDNA Kinetics

cfcDNA quantification was performed with the use of ddPCR. As shown in Figure 2A, a median of 20.0-fold higher cfcDNA levels was observed at day 2 (D2) after surgery compared to those before surgery (mean = 50.6, 95% CI: 20.6–96.4, $P < 0.0001$) (Supplementary Fig. 1A, Supplemental Digital Content 4, <http://links.lww.com/SLA/E452>). This post-operative increase of cfcDNA at D2 was independent of the patients' age ($P = 0.30$, $r = 0.17$) (Supplementary Fig. 1B, Supplemental Digital Content 4, <http://links.lww.com/SLA/E452>) or tumor volume before surgery ($P = 0.54$, $r = 0.10$) (Supplementary Fig. 1C, Supplemental Digital Content 4, <http://links.lww.com/SLA/E452>). A follow-up of the cfcDNA level was performed at day 8 (D8), 4 months (M4), and every 4 months for 2 years or until relapse. A decrease of cfcDNA was detected at D8 ($P < 0.0001$), before returning to preoperative levels at M4 (Fig. 2A), illustrating the impact of the surgical trauma and wounding process kinetics.

Detection of ctDNA Before and After Surgery and Association With R0/R1 Resection

ctDNA levels before and after surgery were analyzed using the above-mentioned ddPCR assays and different patterns of ctDNA positivity in the patients were observed (Fig. 2B, C). Three patients with <300 amplifiable genomes detected by ddPCR before surgery were removed from the analysis, leaving 40 evaluable patients. Among these patients, $N = 14/40$ (35.0%) patients showed detectable ctDNA before surgery with a median allelic frequency of 1.07% (interquartile range = 0.3%–3.8%) (Supplementary Table 4, Supplemental Digital Content 5, <http://links.lww.com/>

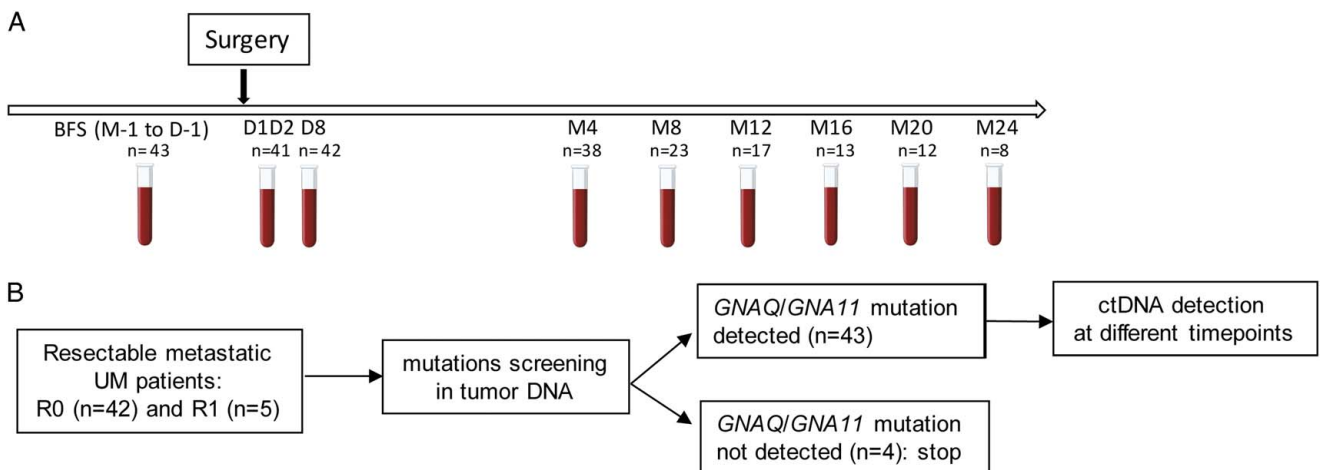


FIGURE 1. Blood sampling (A) and workflow of the study (B). BFS indicates before surgery; R0, microscopically complete resection; R1, microscopically incomplete resection.

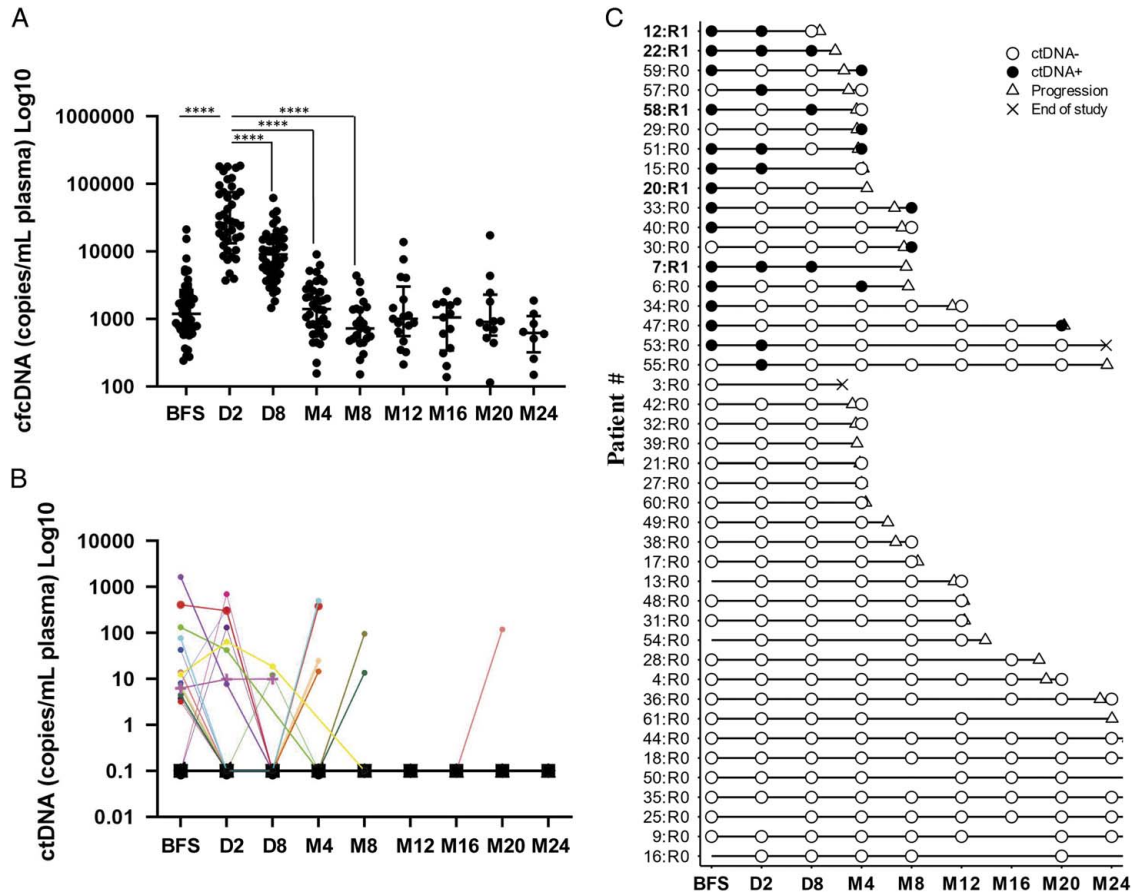


FIGURE 2. cfcDNA and ctDNA detection before and after surgery. cfcDNA (A) and ctDNA (B) levels before (BFS) and after surgery quantified by ddPCR. Each dot represents the level of cfcDNA or ctDNA of the patient measured at that time point. The Mann-Whitney test was used to compare cfcDNA levels at different time points. **** $P < 0.0001$. C, Swimmer plot showing the liver metastasis resection status (R0 vs R1), dynamic changes of ctDNA levels, timing of clinical relapse, and “end of study” (after 2 years on study with no relapse), if applicable.

SLA/E453) which was correlated with tumor volume ($P = 0.03$; $r = 0.35$) (Supplementary Fig. 2, Supplemental Digital Content 6, <http://links.lww.com/SLA/E454>). Preoperative ctDNA detection was also significantly associated with necrosis patterns within liver metastases, as revealed by blinded histopathologic examination of surgical specimens ($P = 0.04$, Supplementary Table 3, Supplemental Digital Content 3, <http://links.lww.com/SLA/E451>).

Two days after surgery, 41 patients were evaluable, 8 of whom were ctDNA-positive. Among the 14 patients who were ctDNA-positive before surgery, residual ctDNA was detected in only 6 (Fig. 2C). Two other patients (P55, P57) who were ctDNA-negative before surgery also emerged as ctDNA-positive at D2. At D8, among 42 patients, further ctDNA clearance was observed, with only 3 patients (P7, P22, P58) tested positive. Two of them (P7, P22) had ctDNA detected before surgery and at D2, while the third patient (P58) had ctDNA detected before surgery but not at D2 (Fig. 2C).

ctDNA monitoring was also performed at M4 and every 4 months until relapse. Among the patients who had available ctDNA analysis at M4 ($N = 36$), 4 patients (P6, P29, P51, P59) were detected with ctDNA (Fig. 2C). Three of them (P6, P51, P59) were ctDNA-positive before surgery, with one maintaining ctDNA detection at D2 (P51), none at D8, while 1 patient (P29) showed no

ctDNA detection before and after surgery until M4 (Fig. 2C). A limited number of patients underwent blood sampling at later follow-up time points, with $N = 21$, 16, 11, 11, and 6 patients evaluable for ctDNA analysis at 8, 12, 16, 20, and 24 months, respectively. Among them, 2 (P30, P33) and one (P47) were found ctDNA-positive at 8 and 20 months, respectively. The timing of the clinical relapse of the different patients is shown in Figure 2C.

As previously explained, patients with complete and curative tumor resection in the liver were assigned to the R0 group ($N = 38$), and patients with microscopic residual tumor after surgery to the R1 group ($N = 5$). All the 5 R1 patients (P7, P12, P20, P22, P58) were positive for ctDNA before surgery and 3 of them (P7, P22, P58) maintained ctDNA detection at D8 (Fig. 2C). 74.3% of R0 patients showed no ctDNA detected before surgery (Fig. 2C) and an association between lower ctDNA levels (mean) before surgery and eventual R0 resection was observed ($P = 0.001$) (Supplementary Fig. 3, Supplemental Digital Content 7, <http://links.lww.com/SLA/E455>). A maintained ctDNA detection at D8 was associated with R1 resection (Supplementary Fig. 3, Supplemental Digital Content 7, <http://links.lww.com/SLA/E455>). At M4, except for P58 with no ctDNA detection, no blood sampling was available for the other 4 R1 patients, noting that P12, P22 experienced a relapse before M4.

Prognostic Value of ctDNA Detection Before and After Surgery

The prognostic impact of ctDNA detection before and after surgery was then assessed. With a median follow-up of 45.3 months (range: 8.6–67.6 months), disease relapses were observed in 37 patients (86.0%), and deaths were observed in 22 patients (51.2%). Patients with no ctDNA detected before surgery were found with a significantly longer RFS [median: 12.2 vs 5.5 months; hazard ratio (HR)=2.23, 95% CI: 1.06–4.69, $P=0.04$] and a numerically although not statistically longer OS (median: 42.3 vs 27.0 months, HR=1.88, 95% CI: 0.77–4.57; $P=0.18$) (Fig. 3A). At D2, detectable ctDNA (N=8) had no prognostic impact on RFS ($P=0.21$) and OS ($P=0.63$) (Fig. 3B). At D8, a trend was observed suggesting a shorter RFS in ctDNA-positive patients (HR=3.83, 95% CI: 1.12–13.17, $P=0.07$; Fig. 3C).

Among the patients who had ctDNA detection during follow-up, relapse was detected by MRI in 9 patients after

4 months of surgery (Fig. 2C). The clinical validity of molecular relapse detection with ctDNA at M4 and M8 was then assessed. ctDNA detection at M4 preceded metastatic relapse, diagnosed by liver MRI surveillance in 1 patient (P6), with a lead time of ~4 months. However, in 3 other patients (P29, P51, P59), ctDNA positivity at M4 was synchronous or posterior to metastatic relapse diagnosis by MRI (Fig. 2C). Postsurgical RFS and OS, by ctDNA status observed 4 months after surgery, are shown in Figure 4A and a highly prognostic value in ctDNA-negative versus positive patients was observed (Fig. 4A). The same trend was observed at M8, but the limited number of patients (only 2 positive patients out of 21) precluded statistical analyses (Fig. 4B).

Finally, to evaluate the prognostic value of clinicopathologic factors collected before surgery and ctDNA detection as a quantitative continuous variable, 3 different univariate, and multivariate proportional hazards models were set up. As shown in Table 1 and Supplementary Table 2 (Supplemental Digital

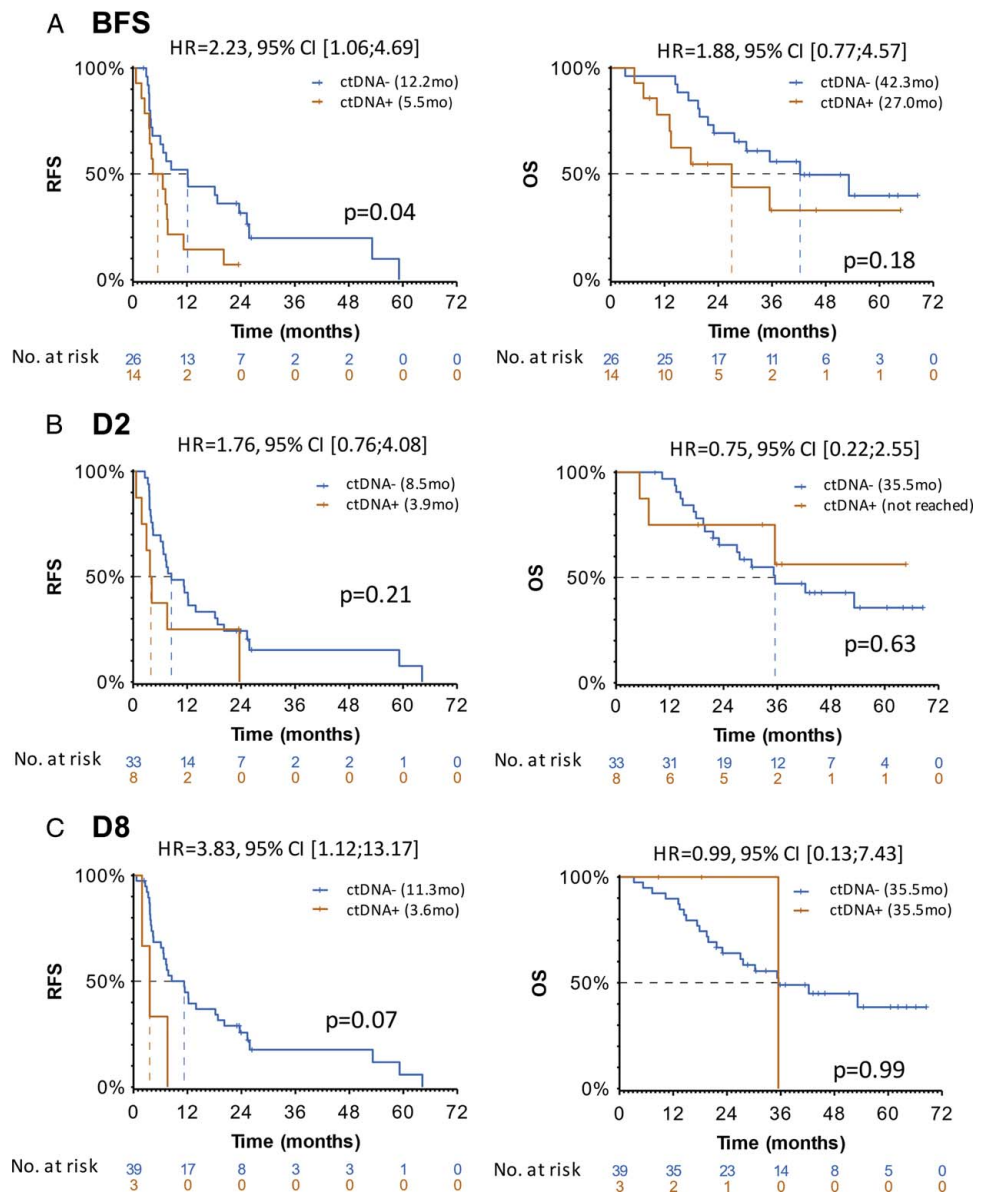


FIGURE 3. Prognostic value of ctDNA detection before (BFS) and after surgery (D2 and D8). RFS and OS curves before surgery (A: BFS) and at days 2 and 8 after surgery (B: D2 and C: D8).

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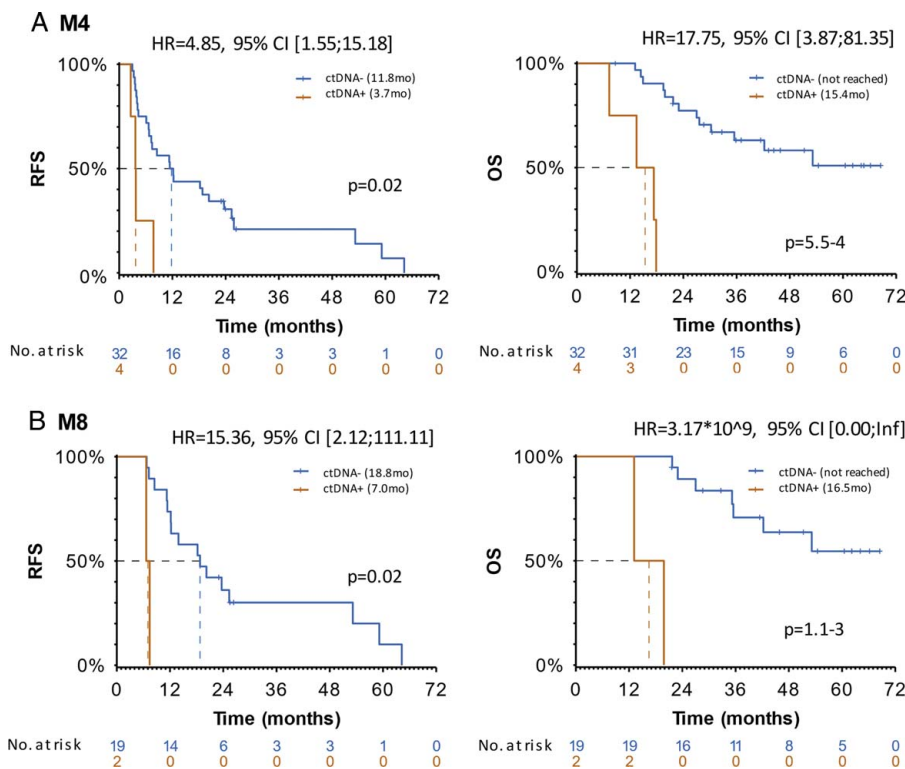


FIGURE 4. Prognostic value of ctDNA detection at M4 and M8. RFS and OS curves at M4 (A: M4) and M8 (B: M8).

Content 2, <http://links.lww.com/SLA/E450>), ctDNA detection (model 3) and metastasis-free interval (model 2) were significantly associated with RFS and OS in univariate analysis with the latter presenting a better prognostic value. When metastasis-free interval, the only clinicopathologic factor feasible for multivariate analysis, was analyzed with ctDNA detection in multivariate analysis (model 1), both factors were confirmed as independent prognostic factors for RFS and the lowest Akaike Information Criterion score was obtained indicating the best model for RFS prediction. However, different from RFS, ctDNA detection was no more significantly associated with OS in multivariate analysis. The best model for predicting OS was metastasis-free interval as an independent variable (Table 1).

DISCUSSION

This study is the first to assess and report the prognostic impact of ctDNA detection in a prospective homogeneous study on MUM patients undergoing surgical resection of their liver

metastases. In the present study, cfcDNA and ctDNA levels were quantified prospectively by ddPCR before and after complete resection.

In MUM, ctDNA was detected in ~41% to 100% of patients with the use of different detection technologies (ultra-deep sequencing or ddPCR).²⁸⁻³² In the present study, 35.0% of MUM patients showed detectable ctDNA in plasma before surgery by tracing the driver mutation detected in tumor DNA, suggesting a limited release of ctDNA in patients with resectable liver UM metastases. This is likely attributable to limited tumor burden, less tumor necrosis, and the absence of other metastatic sites in our patient population, as compared to the previously reported studies in unselected MUM patients.¹⁷⁻¹⁹ A similar lower yield of ctDNA in patients with localized liver metastases (vs in the general population of metastatic patients) was previously observed by our group in colorectal cancer.³³ Despite the limited number of patients with ctDNA detected before surgery, a negative prognostic impact was clearly demonstrated, with a 2-fold longer median RFS in patients with no ctDNA detected.

TABLE 1. Different Univariate and Multivariate Models for RFS and OS

	N	Model 1		Model 2		Model 3	
		HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P
RFS							
ctDNA at BFS: MAF (%)	40	1.12 (1.02-1.23)	0.014			1.18 (1.07-1.3)	0.002
Metastasis-free interval (mo)*	40	0.97 (0.96-0.99)	<0.001	0.97 (0.95-0.99)	<0.001		
AIC		170.1		172.8		187.5	
OS							
ctDNA at BFS: MAF (%)	40	1.06 (0.96-1.17)	0.219			1.11 (1.03-1.21)	0.031
Metastasis-free interval (mo)	40	0.98 (0.96-1)	0.017	0.98 (0.96-0.99)	<0.001		
AIC		125.3		124.3		132.0	

*Metastasis-free interval (mo) was calculated from the time of primary UM diagnosis to the time of first liver metastasis diagnosis. AIC indicates Akaike Information Criterion; BFS, before surgery; MAF, mutant allelic frequency.

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Besides, ctDNA detection as a quantitative continuous variable, total number of liver metastases, and metastasis-free interval were found significantly associated with RFS and OS in univariate analysis with the latter being the strongest prognostic factor. When ctDNA detection before surgery and metastasis-free interval were included in multivariate analysis, the model was proved to be the best one to predict RFS, although not for OS. This further demonstrated ctDNA detection as a useful independent prognostic factor in UM patients eligible for surgical resection of their liver metastases. Currently, the surgical treatment of hepatic metastases from UM remains controversial and it is proposed only to a small number of oligometastatic patients. Our study showed that, combined with other clinicopathologic predictors, ctDNA detection before surgery could be a useful prognostic biomarker to help clinicians advising patients with resectable liver metastases on their surgical treatment option. This is in line with the previous study in resectable metastatic colorectal cancer.³³ Further prospective studies need to be performed to confirm these results.

After surgery, as previously observed by Henrisken et al³⁴ and Konishi and colleagues,^{35,36} an immediate increase of total cfcDNA was observed. This increase of cfcDNA was transient, with a clear decrease detected at D8 after surgery and total cfcDNA levels returning to normal after 4 months. This implied that cfcDNA increase is a common readout in different cancer types after surgical trauma. However, as ctDNA constitutes only a minor fraction of cell-free DNA circulating in cancer patients, a release of large quantities of total cfcDNA caused by surgery trauma might decrease the sensitivity of ctDNA detection. In this study, some patients showed detection of up to 180,000 cfcDNA copies/mL of plasma 2 days after surgery. We, therefore, posit that the detection of ctDNA at day 2 might have been hampered by the dramatic trauma-induced cfcDNA increase. In contrast, we also found that the majority of patients positive for ctDNA detection at D2 after surgery became negative at D8, and no prognostic impact of ctDNA status at D2 was found on RFS and OS. These results are surprising as the half-life of circulating DNA is reported of ~2 hours,³⁷ therefore with an almost complete ctDNA clearance anticipated at D2 after R0 surgery. One hypothesis is that ctDNA may have a slower clearance after surgery due to the large excess of total cfcDNA that may delay the elimination of circulating DNA. A limited value of early sampling after liver surgery (D2) is concluded here, with concerns over limited sensitivity (cfcDNA impairing ctDNA detection) and, possibly, specificity (slower circulating DNA clearance). This limitation might be circumvented by assessing blood samples more distant from surgery. In this study, ctDNA positivity before and after surgery in each patient were longitudinally monitored. Due to the different patterns of ctDNA positivity observed and ctDNA detected only in a limited number of patients at D8 and later follow-up time points, the role of the evolution of ctDNA on RFS and OS was not investigated longitudinally in the same patient.

Various studies in early-stage breast cancer³⁸ and stage III melanoma³⁹ have shown the clinical validity of using ctDNA detection to predict cancer recurrence after surgery earlier than clinical surveillance. In our study, the lead time between ctDNA positivity and relapse was apparently limited, probably because of the intense postoperative clinical surveillance undertaken in the included patients, who were all considered at high risk of further relapse and underwent liver MRI at each visit. This observation is in line with a recently reported trial in triple-negative breast cancer: patients who tested positive for ctDNA

during follow-up after breast cancer surgery often had overt metastases detected at imaging workup.⁴⁰

Of note, patients with R1 status in our study showed around 3-fold shorter RFS and OS compared to patients with complete R0 liver metastases resection, although no statistical significance was retrieved for OS. Due to the limited number of patients in the R1 subgroup, no multivariate analysis on RFS and OS was undertaken; a larger cohort would be needed to clarify this point.

In conclusion, with the use of ddPCR assays, we demonstrated that ctDNA detection before surgery is associated with more R1 resection and shorter time to relapse and death. If confirmed by further studies, and combined with other clinicopathologic predictors, the noninvasive assessment of ctDNA might become a useful tool to further select patients eligible for this treatment.

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