Circulating Cell-Free Tumor DNA Analysis of 50 Genes by Next-Generation Sequencing in the Prospective MOSCATO Trial

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Abstract

Purpose: Liquid biopsies based on circulating cell-free DNA (cfDNA) analysis are described as surrogate samples for molecular analysis. We evaluated the concordance between tumor DNA (tDNA) and cfDNA analysis on a large cohort of patients with advanced or metastatic solid tumor, eligible for phase I trial and with good performance status, enrolled in MOSCATO 01 trial (clinical trial NCT01566019).

Experimental Design: Blood samples were collected at inclusion and cfDNA was extracted from plasma for 334 patients. Hotspot mutations were screened using next-generation sequencing (clinical trial NCT01566019).

Results: Among the 283 patients with tDNA-cfDNA pairs, 121 had mutation in both, 99 in tDNA only, 5 in cfDNA only, and for 58 patients no mutation was detected, leading to a 55.0% estimated sensitivity [95% confidence interval (CI), 48.4%–61.6%] at the patient level. Among the 220 patients with mutations in tDNA, the sensitivity of cfDNA analysis was significantly linked to the number of metastatic sites, albumin level, tumor type, and number of lines of treatment. A sensitivity prediction score could be derived from clinical parameters. Sensitivity is 83% in patients with a high score (≥8). In addition, we analyzed cfDNA for 51 patients without available tissue sample. Mutations were detected for 22 patients, including 19 oncogenic variants and 8 actionable mutations.

Conclusions: Detection of somatic mutations in cfDNA is feasible for prescreening phase I candidates with a satisfactory specificity; overall sensitivity can be improved by a sensitivity score allowing to select patients for whom cfDNA constitutes a reliable noninvasive surrogate to screen mutations.

Introduction

Personalized medicine in oncology, which has seen a rapid development in the last decade due to advances in cancer genomic research and technology, consists of proposing a treatment tailored to the individual patient's molecular profile (1). This assumes that the identified genomic mutations are driver alterations, and that matched targeted drugs are available (2). With the use of high-throughput molecular technologies, the feasibility of such approach has been demonstrated in several pilot trials (3, 4) as well as in large scale studies (5).

To characterize the genomic alterations, tissue from primary tumor or metastasis is generally used for molecular analysis. In the absence of suitable tissue samples from surgical or endoscopic resections, targeted biopsy is required. However, biopsy techniques present ethical and logistic challenges, due to their invasiveness, morbidity, cost, variable accessibility of the tumor sites, as well as potential sampling bias due to intratumor genetic heterogeneity (6,7). In contrast, tumor-derived nucleic acids in plasma (also known as circulating cell-free tumor DNA - cfDNA), only requires a blood sample, can be collected easily and repeatedly, and may be a noninvasive alternative to tissue biopsy (8).

cfDNA is composed of small circulating DNA fragments shed into the plasma following apoptosis or necrosis of tumor cells, or...
through spontaneous release from cancer tissue, and possibly circulating tumor cells (CTC; refs. 9, 10). The detection and clinical use of cfDNA have long remained challenging due to the mixture between tumor circulating free DNA and normal circulating free DNA, the low levels of cfDNA, and the difficulty to accurately quantify the number of DNA fragments. However, recent technological advances now enable the detection, quantification, and analysis of cfDNA (11), thereby opening up possibilities for clinical use. Added to the minimal invasiveness of blood sampling, the attractiveness of liquid biopsy also consists in avoiding cost and delay linked to the tissue biopsy.

The potential applications of cfDNA are currently under extensive investigations, and include diagnosis, patient selection for targeted therapies, prognostic assessment, monitoring of treatment response and resistance, as well as screening for acquired mutations at the time of resistance (10, 12). Recent studies have shown a correlation between cfDNA levels and treatment response in breast cancer (13), the ability of cfDNA to identify mutations associated with acquired resistance (14), and its potential for diagnosis and patient selection of targeted therapy (15).

The ongoing MOSCATO 01 (Molecular Screening for Cancer Treatment Optimization) trial (clinical trial NCT01566019) is a prospective molecular screening program of patients eligible for phase 1 trials, which uses high-throughput technologies [next-generation sequencing (NGS) and comparative genome hybridization (CGH)] to match patients’ tumor molecular profile with targeted therapy and evaluates the clinical benefit of such approach (16, 17).

In the current study, we evaluated whether detection of mutations in cfDNA is a reliable alternative to tissue biopsies for molecular screening, by determining the performance of cfDNA analysis to detect mutations as compared to tumor tissue (tDNA).

**Patients and Methods**

**Patients and sample collection**

Patients referred to the Drug Development Department (DITEP) at the Gustave Roussy Cancer Center (Villejuif, France) were eligible for inclusion in the MOSCATO 01 trial if they had an advanced or metastatic solid tumor progressive after at least one line of standard therapy, with a good performance status (ECOG = 0 or 1) and a primary tumor or metastasis accessible to biopsy. The study was approved by the local Institutional review board and all patients provided written informed consent for genetic analysis of their tumor and plasma samples prior to participation in this study. CT scan or ultrasound-guided biopsies were performed in metastatic or primary tumor sites to carry out a comprehensive molecular characterization. The percentage of tumor cells out of all nucleated cells was assessed on a 3-μm section after a hematoxylin and eosin staining by a senior pathologist.

Samples with at least 10% of tumor cells were considered for further cfDNA analysis through NGS; at least 30% of tumor cells within the sample was required for CGH analysis. Patients with less than 10% of tumor cells on the tissue sample underwent only cfDNA analysis. A weekly molecular tumor board reviewed all the NGS and CGH results and determined the most relevant targeted therapy available through early clinical trials according to the molecular profile. Treatment efficacy was evaluated by Response Evaluation Criteria in solid tumors (RECIST) version 1.1 (18).

Peripheral blood samples were collected just before the tissue biopsy.

**Extraction and quantification of cfDNA**

Blood samples (3–10 mL) were collected in EDTA tubes (BD Vacutainer, Beckton Dickinson and Company) and centrifuged for 10 minutes at 1,000 × g within 4 hours of the blood draw. The supernatant containing the plasma was further centrifuged at 14,000 × g for 10 minutes at room temperature and stored at −80°C until analysis. Initially, the plasma were selected on the basis of their availability, and then, consecutively.

DNA was extracted from 500 μL of plasma using the QIAamp DSP virus kit (Qiagen), according to the manufacturer’s instructions, and resuspended in 40 μL of elution buffer. A real-time quantitative PCR TaqMan assay targeting GAPDH was used to measure plasma DNA concentration.

tDNA was extracted from the fresh frozen biopsy sample using the AllPrep DNA/RNA Mini Kit (Qiagen) and quantified with Qubit 2.0 (Life technologies).

**Identification of somatic genomic mutations by NGS**

Targeted sequencing libraries were generated using the Ion AmpliSeq Library kit 2.0 according to the manufacturer’s instructions (Life Technologies). Tumor and plasma samples were analyzed independently with Cancer Hotspot Panel v2(CHP2) targeting 50 cancer genes covered by 207 amplicons (Life Technologies). For this study, any other genomic alterations found with other biopsy analysis (additional NGS panel, CGH, FISH, etc.) performed in the MOSCATO trial were not considered.

The primers used for amplification were partially digested by FuPa enzyme. The digested product was then ligated with adapters and barcodes, then amplified and purified using Agencourt beads. The quantity of the libraries was assessed using the Qubit 2.0 Fluorometer. An equal amount of each library was pooled and amplified with the Ion OneTouch 2 system by the emulsion PCR with the Ion PGM Template OT2 200 Kit (Life Technologies). The enrichment was then performed with the Ion One Touch ES (Enrichment System). The enriched Ion Spheres were loaded into a 316v.2 Ion Sequencing Chip. Sequencing was made using Ion Personal Genome Machine (PGM, Life Technologies) using real-time measurement of the hydrogen ions produced during DNA replication. The sequencing data were analyzed with the Torrent Suite Variant Caller 4.2 software and reported somatic variants.
were compared with the reference genome hg19. The variants were called if \( \geq 5 \) reads supported the variant and/or total base depth \( >50 \) and/or variant allele frequency \( >1\% \) was observed. All the variants identified were visually controlled on .bam files using Alamut v2.4.2 software (Interactive Biosoftware). All the germline variants found in 1000 Genomes Project or ESP (Exome Sequencing Project database) with frequency \( >0.1\% \) were removed. All somatic mutations were annotated, sorted, and interpreted by an expert molecular biologist according to available databases (COSMIC, TCGA) and medical literature. Analyses of tDNA and cfDNA were performed and interpreted independently.

For validation of our analysis method, we used a Quantitative DNA Reference Standard control (Horizon Diagnostics) harboring 16 mutations in 9 different genes. Ten nanograms of DNA Reference Standard were spiked in 1 mL of control plasma (pool of healthy patients). Two independent analyses were performed with CH2P panel using the DNA analysis protocol. All the mutations detected in the plasma were analyzed down to variant with 1% allele frequency. Nevertheless, the 1% allele frequency threshold could not be considered for all the samples in our analysis, as the assay sensitivity depends on the coverage depth which is variable between samples and genomic positions.

MiSeq resequencing analysis (Illumina), using CH2P library amplicons, was performed.

Statistical analysis

All patients with available tDNA and cfDNA results were included in the main analysis. We estimated sensitivity and specificity of cfDNA analysis compared with tDNA considered as the reference test. The analysis was performed both at the gene level, with possibly multiple mutated genes per patient, and at the patient level. Kappa agreement coefficient was estimated at the gene level in the subset of patients who were consecutively included. Among patients with mutations found in tumor, the cfDNA result was classified as fully concordant if all mutations identified in tumor were also found in cfDNA, and partially concordant if only part of the mutations found in tumor were identified in cfDNA. At the patient level, the sensitivity of cfDNA was computed as the probability of a positive concordant cfDNA result (fully or partially concordant) in patients for whom mutations were found in tumor.

We then modeled this probability of a positive concordant cfDNA result in patients whose mutations were found in tumor tissue, and built a sensitivity prediction score using a logistic regression. All variables associated with a \( P < 0.15 \) in univariable model were then evaluated in multivariable analysis using a backward procedure. We first considered only patient characteristics at study entry (age, gender, ECOG performance status, primary tumor site, number of metastatic sites, LDH level, albumin level, and number of prior lines of treatment), to build a clinical score, available before tumor biopsy and DNA extraction. We also evaluated the diagnostic value of the RMH score, which is a score developed by the team from Royal Marsden Hospital for prognosis purpose in the setting of oncology phase I trial score (19). We then evaluated the additional value of cfDNA concentration adjusted on the clinical score. Continuous variables (age, LDH and albumin levels, number of metastatic sites and prior lines of treatment, RMH score, cfDNA concentration) were entered in the model assuming a log linear relationship, after checking the shape of the relation in the model. Models were compared using Akaike Information Criterion (AIC; ref. 20).

Calibration and discriminative ability of the final model were evaluated using Hosmer–Lemeshow test (21) and AIC (22). We set the \( P \) value threshold at 0.05 in the multivariable analysis. The final model was validated using the 10-fold cross-validation approach. All estimates are given with their 95% confidence intervals (95% CI) and tests are two-sided.

All analyses were performed with SAS v9.3 (SAS Institute).

Results

Patient characteristics

From November 2011 to October 2014, 669 heavily pretreated patients with metastatic or advanced solid tumors were enrolled in the MOSCATO 01 trial (Fig. 1). Among the 600 patients for whom a biopsy was performed, a total of 334 (56%) patients had a cfDNA analysis. All plasma samples have been processed within 4 hours after the blood sampling, ensuring a good homogeneity of all the preanalytic and analytic steps in our cohort. Evaluable NGS results were obtained by both tDNA and cfDNA analysis in 283 patients (85%, main analysis set). The first 178 patients were selected on the basis of the plasma availability and knowing mutational status in tumor, whereas from June 2014, all consecutive patients enrolled in MOSCATO 01 trial (\( N = 105 \)) had a cfDNA analysis. For 51 additional patients without tissue samples available for molecular testing (due to the low content in tumor cells in biopsy samples), only cfDNA analysis was performed. Those patients were not included in statistical analysis.

Patient and disease baseline characteristics (\( N = 283 \)) are summarized in Table 1. Patients had a median age at biopsy of 58 years (range: 18–79). Thoracic, gastrointestinal, breast, head and neck, gynecologic and urologic cancers were the most frequent tumor locations, with 19%, 18%, 15%, 14%, 9%, and 7%, respectively. 95% of the patients were metastatic and were heavily pretreated with a median number of 3 previous lines of treatment (range: 0–11). The median cfDNA concentration found in plasma was 18.1 ng/mL (range: \( \leq 0.1–727.0 \) ng/mL). As illustrated in Fig. 2, cfDNA concentration was significantly higher in patients with a gastrointestinal tumor than in patients with other tumor sites (median: 30.4 vs. 16.2, \( P = 0.02 \)).

Mutation analysis

Overall, 283 patients had tumor and plasma samples collected at the same timepoint which were analyzed independently using the same CH2P panel. Mean depth for the cfDNA NGS analysis was \( x492 \). A total of 347 mutations were identified in tDNA and 195 mutations were identified in cfDNA. In tDNA, at least 1 mutation was detected in 220 patients (78%) (1 mutation in 129 patients, 2–4 in 91 patients). Mutations are detailed in Supplementary Table S1.

The most frequently mutated genes in tumor (\( N > 3 \)) were TP53 (47.3% of patients), \( K R A S \) (17.3%), \( P I K 3 C A \) (13.1%), \( E G F R \) (5.3%), \( C D K N 2 A \) (4.2%), \( A P C \) (3.9%), \( P T E N \) (3.9%), \( S M A D 4 \) (3.5%), \( A K T 1 \) (2.5%), \( S T K 1 1 \) (2.5%), \( C T N N B 1 \) (2.1%), \( F B X W 7 \) (2.1%), \( K I T \) (1.8%), \( E R R B 2 \) (1.4%), \( R B 1 \) (1.4%), and \( B R A F \) (1.4%). In cfDNA, at least 1 mutation was detected in 134 patients (47%; median = 1 mutation per patient, range 1–4 mutations). The most frequent mutations (\( n > 3 \)) found in cfDNA were in \( T P 5 3 \) (23.3%), \( K R A S \) (11.0%), \( P I K 3 C A \) (6.0%), \( A P C \) (3.2%), \( P T E N \) (3.2%), \( E G F R \) (2.8%), \( E R R B 2 \) (2.5%), \( S M A D 4 \) (2.1%), \( C T N N B 1 \) (2.1%).
CDKN2A (1.8%), CDKN2A (1.4%), AKT1 (1.4%), KIT (1.4%), and RB1 (1.4%) genes. No correlation was observed between allele frequency observed in tumor tissue and in plasma.

The cfDNA analysis of the 51 patients without tDNA analysis revealed at least one mutation in 22 patients (43.1%). Among the 29 mutations found, 19 were pathogenic variants and included 8 variants of therapeutic interest. Several of these cfDNA analyses have been performed in parallel to tDNA analysis of patients recently enrolled in MOSCATO 01 trial and the results were discussed in dedicated molecular tumor board. Also, cfDNA analysis could be performed in the same conditions (turn-around time and cost) as tDNA analysis.

To confirm mutations identified in cfDNA with the PGM-Ion Torrent analysis, MiSeq resequencing analysis (Illumina) was performed using the CHP2 library amplicons on a set of 83 patients. The mean depth for the cfDNA analysis with MiSeq was x748 and 91 mutations were identified. MiSeq analysis allowed the detection of 16 additional mutations which were not identified in cfDNA analysis by PGM-Ion Torrent, with 11 of them (69%) present in the tDNA analysis. Conversely, PGM-Ion Torrent analysis identified 6 mutations in cfDNA, which were also found in the tDNA, but which could not be detected with MiSeq sequencing of cfDNA. Overall, MiSeq and PGM sequencing provided concordant results for the cfDNA analysis.

Concordance between tDNA and cfDNA
Among the 347 mutations identified in tDNA from 283 patients, 173 mutations were identified in both tDNA and cfDNA, and 174 mutations in tDNA, but not in cfDNA. Results for the main genes are illustrated in Fig. 3A. Moreover, 22 mutations were found in cfDNA, but not in tDNA, providing additional information. The sensitivity of using NGS for 50 targeted hotspot genes analysis in cfDNA compared with tDNA was 49.9% (95% CI, 44.6%–55.1%) and the specificity was 99.8% (95% CI, 99.8%–99.9%). Sensitivity in the five most frequent mutated genes (TP53, KRAS, PIK3CA, EGFR, and APC, representing 75.9% of all cfDNA mutations, was 47%, 59%, 43%, 53%, and 46%, respectively. The Kappa coefficient calculated on the 105 consecutive patients was 0.60 (95% CI, 0.50–0.69).

At the patient level, NGS analysis allowed us to observe: (i) 58 patients without any mutation found in tumor or in cfDNA; (ii) 5 patients with mutations found in cfDNA but not in tumor; (iii) 220 patients with mutations found in tumor and plasma. Among these latter, the mutations found in plasma were fully concordant for 87 patients (39.5%) and partially concordant for 34 patients (15.5%), whereas the mutations found in tumor were not found in the cfDNA for the 99 other patients (45%), leading to an estimated sensitivity of 55.0% (95% CI, 48.4%–61.6%) at the patient level (Fig. 3B).
Table 1. Patient and tumor characteristics

<table>
<thead>
<tr>
<th></th>
<th>Frequency (%)</th>
<th>Total N = 283</th>
</tr>
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<tbody>
<tr>
<td>Age at study entry</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (range)</td>
<td>58 (18–79)</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>149 (52.7%)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>134 (47.3%)</td>
<td></td>
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<tr>
<td>Primary tumor site</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breast</td>
<td>41 (14.5%)</td>
<td></td>
</tr>
<tr>
<td>Gastrointestinal</td>
<td>50 (17.7%)</td>
<td></td>
</tr>
<tr>
<td>Gynecologic</td>
<td>26 (9.2%)</td>
<td></td>
</tr>
<tr>
<td>Head and Neck</td>
<td>39 (13.8%)</td>
<td></td>
</tr>
<tr>
<td>Thoracic</td>
<td>52 (18.4%)</td>
<td></td>
</tr>
<tr>
<td>Urologic</td>
<td>21 (7.4%)</td>
<td></td>
</tr>
<tr>
<td>Othera</td>
<td>52 (18.4%)</td>
<td></td>
</tr>
<tr>
<td>Number of previous lines of therapy Median (Range)</td>
<td>3 (0–11)</td>
<td></td>
</tr>
<tr>
<td>Number of metastatic sites</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>15 (5.3%)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>101 (35.7%)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>87 (30.7%)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>51 (18%)</td>
<td></td>
</tr>
<tr>
<td>&gt;3</td>
<td>29 (10.2%)</td>
<td></td>
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<tr>
<td>LDH level (U/L; 7 MD)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (range)</td>
<td>220 (4–6,980)</td>
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</tr>
<tr>
<td>RMH score (10 MD)b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>102 (37.4%)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>106 (38.8%)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>53 (19.4%)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>12 (4.4%)</td>
<td></td>
</tr>
<tr>
<td>cfDNA Concentration (ng/mL; 2 MD) Median (range)</td>
<td>18.1 (c–727)</td>
<td></td>
</tr>
<tr>
<td>Number of mutated genes/patient</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>63 (22.3%)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>129 (45.6%)</td>
<td></td>
</tr>
<tr>
<td>&gt;1</td>
<td>91 (32.2%)</td>
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Abbreviation: MD, missing data.

*aOther tumor sites: prostate (N = 18), cancer of unknown primary (N = 9), testis (N = 5), thyroid (N = 4), bone (N = 4), skin (N = 3), penis (N = 2), peritoneum (N = 2), connective tissue and soft tissue (N = 5), lip (N = 1), scalp and neck (N = 1).

bRMH Score: Prognostic score developed by the team from Royal Marsden Hospital in the setting of oncology phase I trials (ref Arkenau JCO 2009), based on LDH level, albumin level and number of sites of metastases, weighted equally: LDH normal (0) versus LDH > upper limit of normal (1), albumin ≥ 35 g/L (2) versus albumin < 35 g/L (1), and sites of metastases ≤ 2 (0) versus sites of metastases > 2 (1). The prognostic score for each individual was computed as the sum of the three components.

Table 2 details the modeling of cfDNA positivity among the 220 patients with mutations found in the tumor, including 121 patients with fully or partially concordant cfDNA result. The sensitivity of cfDNA test significantly increased with the number of metastatic sites (Fig. 4A, P = 0.0006 in multivariable model), a decreased albumin level (Fig. 4B, P = 0.00007), and the number of prior treatment lines (Fig. 4C, P = 0.047); it was also significantly associated with the primary tumor site (Fig. 4D, P = 0.002). The sensitivity was higher in breast and gastrointestinal cancers and lower in patients with head and neck or urologic cancer. The final multivariable analysis clinical model, based on these four clinical characteristics, had a good predictive power as indicated by the AUC equal to 0.78 (Supplementary Fig.S1), as well as a good goodness of fit (Hosmer and Lemeshow test, P = 0.34). Using the 10-fold cross validation, the estimated prediction error was 0.205.

The relationship between the derived sensitivity prediction score and the probability of a positive cfDNA result is illustrated in Fig. 4E. The estimated sensitivity of cfDNA was 26% when focusing on the patients with a low score (first third of the distribution), which mainly corresponded to patients with less advanced disease and/or patients with head and neck or urologic cancer. It is noticeable that cfDNA was positive in only 1 of the 22 patients with the lowest score values (the first decile which includes 21 patients with head and neck cancer). In contrast, sensitivity of cfDNA is 83% in patients with a high score value (last third of the distribution), reaching 94% (17/18) in the highest decile that is mainly represented by patients with advanced breast or gastrointestinal cancer.

The strong relationship between LDH level and cfDNA positivity observed in univariable analysis vanished in multivariable analysis (OR = 1.33, 95% CI, 0.98–1.81, P = 0.07), because LDH level was significantly correlated with the other variables included in the model (Spearman correlation coefficient between LDH and clinical score = 0.36, P < 0.0001).

The RMH score was significantly associated with cfDNA positivity. However, the performance of the multivariable model described herein above was better than that of the model based only on RMH score (AIC, 254.2 for the final clinical multivariable model vs. 264.5 for the model based only on RMH score, when
to 0.79. Clinical model was marginal: the AUC increased from 0.78 in univariable analysis and 0.045 in multivariable analysis. However, the added value of cfDNA concentration to the multivariable model was marginal: the AUC increased from 0.78 to 0.79. Good surrogate of tissue biopsy and the one for whom the cfDNA NGS analysis will not be contributive.

Overall, the specificity was above 95%, but the sensitivity of cfDNA analysis was estimated at only 55% (121/220) in the patients with at least one mutation found in tDNA. The observed discordance could be due to several reasons: poor mutation detection in cfDNA due to low cfDNA concentrations in the bloodstream (23); presence of the mutation in allele frequencies below the detection limit of our method; true disease heterogeneity (24); and poor DNA quality (25), true genetic differences between cfDNA (released mainly from necrotic cells) and tDNA. Ongoing technological advances are likely to improve the cfDNA analysis method and further increase the concordance between tDNA and cfDNA. For low cfDNA levels, on first hand, we chose to use the limited quantity of plasma available through the prospective collection set up. Carrying out cfDNA from larger plasma volume could increase the levels of cfDNA for molecular analysis for some cases. For several samples, even using four times the volume of plasma may lead to better results; indeed, we have tested 45 additional cases using 2 mL of plasma and observed 49% of concordance (data not shown). On the other hand, using alternative sequencing method as MiSeq with better coverage did not increase the number of mutations detected. Nevertheless, our results should be also considered in a context of novel and more sensitive techniques. For example, methodologies based on digital PCR are making cfDNA analyses possible even in cases where only low levels of cfDNA are present, with a threshold of detection of 0.01% or lower (11). In addition, important progress has been made using sequence-specific assays that target predefined mutations and detect extremely rare alleles (26). False negative results (mutation found in tDNA but not in cfDNA) may be further reduced by a reestimation of the background frequency noise and the minimum allele frequency threshold. Another approach could be to prioritize screening of limited numbers of mutations with highly sensitive techniques instead of large tumor type–agnostic gene panel. However, whatever the threshold of detection, a sufficient DNA concentration or DNA copy number is needed, but was not always observed in our cohort, with DNA concentration ranging from <0.1 ng/mL to 727 ng/mL.

Even if our cohort of phase I patients could be considered as biased by low representation of some tumor types (e.g., melanoma), our results could be compared with several prior studies investigating the concordance of cfDNA and tDNA in different tumor types with varying results. Rothe and colleagues found a sensitivity of cfDNA estimated at 82% in 11 patients with mutated metastatic breast cancer (27). Another study found a sensitivity rate of 58% in 50 mutated metastatic lung cancer patients (28). The estimated sensitivity of cfDNA in patients with breast or lung cancer in our series (74% and 55%, respectively) is consistent with the published results. In a recent study based on 39 patients with different types of tumor harboring mutations, Lebofsky and colleagues estimated sensitivity of cfDNA at 59% (23/39), similar to our overall result (55%; ref. 29).

Discussion

In this study, we show that use of NGS for cfDNA analysis is feasible in routine clinical settings for molecular characterization in patients with locally advanced or metastatic solid tumors, eligible for phase I trials and displaying a good performance status. To our knowledge, this study reports the largest patient cohort used to evaluate the feasibility of NGS gene panel screening using plasma samples matched with analysis of tumor samples issued for various tumor types obtained from 283 patients. This is also the first study reporting a sensitivity prediction score enabling better selection of patients for whom liquid biopsy could be a good surrogate of tissue biopsy and the one for whom the cfDNA NGS analysis will not be contributive.
the play of chance in this small sample size series: patient characteristics were different, with, in their series compared to ours, more breast and GI cancers, less head and neck or urologic cancer, more patients with a number of metastatic sites greater than 2. In addition, most plasma samples (31/34) were collected after biopsy, often several months after the initial biopsy, when the disease could be more advanced.

Part of the disparity in sensitivity rates of cfDNA reported by different studies could also be explained by the different procedures that are used in laboratories at both preanalytic (blood collection, processing, storage, baseline of patients) and analytic phases (DNA extraction, quantification, and analysis; refs. 11, 24, 30, 31). To introduce the liquid biopsy in the clinical management of cancer patients, it is important that these parameters be standardized for consensus analysis and reporting (32).

Most of the published studies had a lower number of patients tested and the method of patient selection is not described in detail. Moreover, the performance status and the disease’s characteristics are generally poorly reported in most of the published studies. Those points limit the possibility of comparison with our cohort of patients having mainly performance status and a low RMH score at the time of sample collection that appear to be crucial for paired cfDNA and tDNA molecular status concordance.

In the current study, we identified mutations only in the cfDNA analysis and not in tDNA in few patients. This point was consistent with previous data (26–29). This suggests that cfDNA analysis using NGS could be also used as a complementary analysis to the tDNA to better evaluate the tumor heterogeneity (7). In addition, we analyzed the cfDNA of 51 patients without tDNA because of low tumor cellularity (less than 10% of tumor cell) and we found one or more mutations in 22 patients (43%), including 8 oncogenic variants of therapeutic interest. This data highlights the feasibility and the added value of cfDNA analysis for patients for whom tissue biopsy is not available. Nevertheless, due to relatively high rate of false negative result, it is important to identify patients for whom biopsy contribution is essential.

We showed that the sensitivity of cfDNA analysis was significantly associated with the primary tumor site and increased with the number of metastatic sites, the number of prior lines of treatment and a decrease of albumin level. For a practical use in selecting patients for whom cfDNA could be used as a surrogate molecular screening maker, we have built, for the first time, a sensitivity prediction score. Patients with a high score are more likely to have a contributive result from cfDNA analysis. Also, our findings suggest that for those patients, cfDNA is a valuable surrogate material for molecular screening by NGS analysis, whereas tumor biopsy analysis will be recommended in patients with a low sensitivity prediction score.

Therefore, molecular characterization based on cfDNA analysis could be performed in the same conditions as tDNA analysis (turn-around time and cost). cfDNA-based analysis could be used for diagnosis and treatment decisions, even for patients for whom tumor biopsy is not feasible, which represent a significant proportion of patients with metastatic cancers, or when the quality of the histologic sample is too poor for molecular analysis. But further studies on prospective validation cohorts are required to determine whether an optimized version of the assay may be applicable in routine clinical practice as a liquid biopsy.

Nevertheless, there are still improvements possible to our study, beside preanalytic points discussed previously. We evaluated only the cfDNA at baseline compared with the tDNA
regardless of the tumor types and progression rate before the inclusion in the MOSCATO trial. Further cfDNA kinetics analysis (i.e., analysis at multiple time-point samples) and correlation of the cfDNA results with the clinical outcome is warranted.

Despite its inconveniences, tumor biopsy remains the most efficient diagnostic tool at present. However, several major advantages of cfDNA justify further investigation and development as a tool to detect tumor-specific genomic alterations in the circulation, beyond what is currently possible with tumor biopsy. First, the possibility of cfDNA analysis even with low levels of cfDNA may lead to early detection of cancer (11). Second, even when biopsy is technically feasible, the minimally invasive, more convenient cfDNA quantification would be more acceptable to patients than biopsy. Third, cfDNA analysis facilitates monitoring of molecular alterations associated with tumor response or acquired resistance, while sparing the patient from repeated biopsies and their potential harms.

In conclusion, our results on a consistent cohort of patients suggest that targeted NGS for the detection of somatic mutations in cfDNA could be applicable in clinical for patient eligible for phase I clinical trials and bearing advanced or metastatic solid tumors. We have established a score that allows us to select patients for whom the analysis of cfDNA using NGS offers an attractive noninvasive option to screen mutations and to selected patients for whom it will likely be recommended to have analysis performed on tumor tissue.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Figure 4.
cfDNA result in patients with mutations found in the tumor, according to albumin level (A), number of metastatic site (B), number of previous lines of therapy (C), primary tumor site (D), and sensitivity prediction score (E). D, H&N, head and neck; Urol., urologic; Thor., thoracic; G.I., gastrointestinal; Gyne., Gynecologic.
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