Biology of Human Tumors

Clinical Cancer Research

Combination Approach for Detecting Different Types of Alterations in Circulating Tumor DNA in Leiomyosarcoma

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Abstract

Purpose: The clinical utility of circulating tumor DNA (ctDNA) monitoring has been shown in tumors that harbor highly recurrent mutations. Leiomyosarcoma represents a type of tumor with a wide spectrum of heterogeneous genomic abnormalities; thus, targeting hotspot mutations or a narrow genomic region for ctDNA detection may not be practical. Here, we demonstrate a combinatorial approach that integrates different sequencing protocols for the orthogonal detection of single-nucleotide variants (SNV), small indels, and copy-number alterations (CNA) in ctDNA.

Experimental Design: We employed Cancer Personalized Profiling by deep Sequencing (CAPP-Seq) for the analysis of SNVs and indels, together with a genome-wide interrogation of CNAs by Genome Representation Profiling (GRP). We profiled 28 longitudinal plasma samples and 25 tumor specimens from 7 patients with leiomyosarcoma.

Results: We detected ctDNA in 6 of 7 of these patients with >98% specificity for mutant allele fractions down to a level of 0.01%. We show that results from CAPP-Seq and GRP are highly concordant, and the combination of these methods allows for more comprehensive monitoring of ctDNA by profiling a wide spectrum of tumor-specific markers. By analyzing multiple tumor specimens in individual patients obtained from different sites and at different times during treatment, we observed clonal evolution of these tumors that was reflected by ctDNA profiles.

Conclusions: Our strategy allows for the comprehensive monitoring of a broad spectrum of tumor-specific markers in plasma. Our approach may be clinically useful not only in leiomyosarcoma but also in other tumor types that lack recurrent genomic alterations. *Clin Cancer Res; 24(11); 2688–99. ©2018 AACR.*

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Introduction

Recent improvements in next-generation sequencing platforms have paved the way for the highly sensitive detection of circulating tumor DNA (ctDNA) in plasma specimens. Current strategies for ctDNA analysis may be divided into three categories: (1) patient-specific approaches that utilize personalized assays (1–4); (2) tumor type–specific targeted sequencing that does not require patient-specific optimization (5–7); and (3) tumor type–independent genome-wide analyses (8–11). The first approach is highly sensitive but is technically challenging and expensive. The second approach involves targeted sequencing methods such as Cancer Personalized Profiling by deep Sequencing (CAPP-Seq; refs. 5, 6). It is highly sensitive, but is most practical in patients with tumor types that harbor highly recurrent aberrations that can be sequenced with a capture panel of a relatively limited size. The third approach, although broadly applicable, does not reach the sensitivity of the first two targeted approaches. In cancer types that are characterized by intermediate levels of recurrent single-nucleotide variants (SNV), small insertions and deletions (indels), or copy-number alterations (CNA), a combination of multiple assays may improve the sensitivity of ctDNA detection while still retaining the benefits of broad applicability and cost-effectiveness. Leiomyosarcoma is a suitable disease to explore the potential of such a combination approach for the orthogonal detection of multiple classes of alterations because these tumors are characterized by a wide range of DNA abnormalities spread across the whole genome. These include complex CNAs, as well as point mutations affecting multiple tumor-suppressor genes such as *TP53*, *RB1*, *ATM*, *ATR*, *ATRX*, and *PTEN* (12, 13).

Leiomyosarcoma patients, like many other cancer patients, could greatly benefit from a noninvasive monitoring of tumor burden by liquid biopsies. Currently, the decision to initiate adjuvant treatment in leiomyosarcoma patients is based on the

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Translational Relevance

The detection of circulating tumor DNA (ctDNA) has potential to improve prognostication, molecular profiling, and surveillance, especially in cancer types with highly recurrent genomic alterations. Leiomyosarcoma represents a type of tumor that harbors a wide spectrum of heterogeneous genomic abnormalities; thus, targeting a narrow genomic region for ctDNA monitoring may not be practical. In this study, we demonstrate a combination approach that integrates different sequencing protocols for the orthogonal detection of multiple types of alterations in ctDNA. This strategy substantially increases the number of molecular markers that can be tracked in plasma and improves the confidence of ctDNA detection in leiomyosarcoma patients and could be applied to a wide range of tumors characterized by genomic profiles of comparable complexity.

assessment of multiple prognostic factors related to patient performance, stage of the disease, and type of surgery, as well as the potential benefits and side effects of the treatment. Leiomyosarcoma ctDNA testing may improve the patients' clinical outcome through earlier identification of candidates for adjuvant therapy. Longitudinal monitoring of ctDNA may also complement imaging-based regimens for long-term surveillance of leiomyosarcoma patients for disease recurrence.

Here, we describe a proof-of-principle study to determine the feasibility of ctDNA analysis in patients diagnosed with tumors of moderate genomic complexity, through a simultaneous application of two separate methods, CAPP-Seq and Genome Representation Profiling (GRP) in leiomyosarcoma. The former is a deep, targeted sequencing approach optimized for ctDNA detection, which is ideal for the ultrasensitive quantitative analysis of SNVs, indels, and fusion breakpoints. The clinical utility of monitoring ctDNA by CAPP-Seq has been previously demonstrated in patients with lung cancer and diffuse large B-cell lymphoma (5, 6, 14–16). The second approach, GRP, is based on shallow whole-genome sequencing for the assessment of genome-wide CNAs and has been shown to detect ctDNA in patients with ovarian carcinoma, Hodgkin lymphoma, and follicular lymphoma (9). Successful monitoring of CNAs in plasma has been also described previously in prostate cancer patients (11). In the present study, we demonstrate that the combination of these two techniques enables the reliable monitoring of a wide spectrum of molecular markers in ctDNA, and this approach has a significant translational potential in leiomyosarcoma and other cancer types characterized with a comparable genomic complexity.

Materials and Methods

Leiomyosarcoma patient cohort

Nine leiomyosarcoma patients treated at the Stanford Cancer Institute provided informed consent to participate in the study and donated serial blood samples throughout the course of their treatment. The study was approved by the Stanford University Institutional Review Board (approvals IRB-31067 and IRB-31596). Clinical features of the patients included in this study are summarized in Supplementary Table S1. Data from two patients have been excluded from the analysis due to failed quality control (QC) or the absence of SNV/indels in tumor within the genomic region covered by CAPP-Seq panel. The data from the remaining 7 leiomyosarcoma patients have been used for the final analysis comparing CAPP-Seq and GRP. All leiomyosarcoma patients in the ctDNA monitoring analysis had either a primary tumor or metastatic disease confirmed by imaging at all blood collection time points.

Healthy donors

Blood specimens from 24 healthy donors used for CAPP-Seq analysis were collected into ethylenediaminetetraacetic acid (EDTA) tubes (Beckton Dickinson). Plasma specimens from 428 volunteers (214 females and 214 males) used for GRP analysis were collected into cell-free DNA BCT tubes (Streck). Collection of plasma from these asymptomatic donors was approved by the local Institutional Review Boards.

Leiomyosarcoma-specific CAPP-Seq selector design

Whole-exome sequencing data from 77 matched tumor-normal specimens from leiomyosarcoma patients from The Cancer Genome Atlas (TCGA) were used to design a leiomyosarcoma-specific CAPP-Seq capture panel. The analyses presented in the current publication are based on the use of study data downloaded from the dbGaP web site, under phs000178.v8.p7 (17). Paired-end sequencing reads were aligned to the human reference genome (GRCh37/hg19) using BWA-MEM (version 0.7.13) with the default settings (18). SAMtools (version 1.3) was used for converting SAM to BAM format, sorting, and indexing the alignments (19). Picard (version 1.96) was used for the removal of duplicate reads (20). The GATK framework (version 3.3-0) was used for the local realignment and base call recalibration (20). SNVs and indels were identified using VarScan2 (version 2.3.7; ref. 21). Variants identified by Varscan2 were annotated with ANNOVAR (22) and filtered for exonic or splice site nonsynonymous SNVs, frameshift indels, stopgain, and stoploss variants, requiring 0% variant allele fraction (VAF) in the matched germline DNA. All variants were required to be present on at least one forward and one reverse sequencing read. Variants reported at >0.01% frequency in 1000Genomes and ExAC databases were excluded from the analysis. In addition, genes that are likely to produce false-positive variant calls in next-generation sequencing data were filtered out (including *MUC*, *GOLG*, *NBP*, *ZNF*, *OR*, and *WDR* family genes). Somatic variants obtained after this prefiltering were used to identify all exons mutated in at least 2 leiomyosarcoma patients. This output was further filtered based on the effective size of each exon, i.e., based on the recurrence index (5) defined as the number of patients with the mutation divided by the exon length in kb. Only exons with recurrence index > 0.5 were retained. Exons with poor mappability according to the Uniqueness of 35 bp Windows from ENCODE/OpenChrom (Duke) track in UCSC Genome Browser GRCh37/hg19 were excluded (23). This analysis yielded a panel of 281 exons from 82 genes that were recurrently mutated in TCGA leiomyosarcoma cases. This panel covered 98.7% (76/77 cases) of the TCGA cohort with a median of 3 SNVs/indels per patient. This panel was extended with 25 exons from 7 genes (*MED12*, *KRAS*, *CDKN2A*, *CDH1*, *KIT*, *HRAS*, and *KDM6A*) that carried mutations reported in 114 leiomyosarcoma patients in the COSMIC database v74 (accessed on October 23, 2015). The final leiomyosarcoma-specific selector for CAPP-Seq covered the region of 184 kb and included 306 exons from 89 genes (Supplementary Table S2). A custom SeqCap EZ Choice

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Library (Roche) capture panel was designed using NimbleDesign software version 3.0 (Roche), with the maximum mismatches set to 1. The estimated coverage of the input genomic region of 184,871 bp was 98.1%.

Blood sample collection and processing

Peripheral blood was collected into EDTA tubes (Beckton Dickinson), and plasma was separated by centrifugation at 2,500 × *g* for 20 minutes at room temperature, and stored at −80°C. The cell pellet containing white blood cells was frozen and banked for the germline DNA extraction. cfDNA was extracted from a median of 5 mL plasma (range, 3–5 mL) using a QIAamp Circulating Nucleic Acid kit (Qiagen). Germline DNA was extracted from 200 μL of PBLs using a DNeasy Blood & Tissue kit (Qiagen). cfDNA and germline DNA were quantified using Quant-iT dsDNA High Sensitivity Assay (ThermoFisher Scientific).

Tumor specimens

Twenty-eight formalin-fixed paraffin-embedded (FFPE) tumor specimens (a median of 3 specimens per patient) from 9 leiomyosarcoma patients, collected between 2013 and 2015, were used for extraction of genomic DNA (Supplementary Table S3). The analyzed specimens included 8 primary tumors and 20 metastatic/recurrent tumors. Diagnosis was confirmed by a surgical pathologist (M. van de Rijn). Tumor specimens selected for CAPP-Seq ($n = 24$) and SNP array ($n = 26$) analysis contained a median of 95% tumor cells (range, 60%–100%). Genomic DNA was extracted using the AllPrep DNA/RNA FFPE Kit (Qiagen). Twenty and 22 samples from 7 leiomyosarcoma patients passed the QC by CAPP-Seq and SNP array analysis, respectively (Supplementary Table S3).

RNA sequencing of leiomyosarcoma tumors used for validation of CAPP-Seq selector

RNA-seq was performed on tumor specimens collected from 20 leiomyosarcoma patients treated at Stanford University Medical Center (19 FFPE and 1 frozen tumor specimen). These specimens included 8 uterine, 5 extremity, and 7 thoracic/abdominal/retroperitoneal leiomyosarcoma from 11 female and 9 male patients. Libraries were prepared using TruSeq RNA Sample Prep Kit V2 (Illumina) and sequenced on a HiSeq2000 instrument (Illumina) in 2 × 101 bp mode.

Paired-end reads were mapped to the human reference genome (GRCh37/hg19) using STAR (version 2.3.0.1; ref. 24). Duplicate reads were removed using Picard (version 1.96; ref. 20). The GATK framework (version 3.3-0) was used for Split'N'Trim, indel realignment, and base recalibration (20). SNPs and indels were identified using HaplotypeCaller in the RNA-seq mode. Variants were annotated using ANNOVAR (22) and filtered to identify exonic, nonsynonymous SNPs, and indels.

Within the 184 kb region of the leiomyosarcoma-specific CAPP-Seq panel, we identified a median of 7 SNVs/indels per patient in 18 of 20 patients from our validation cohort. The higher number of variants identified in the RNA-seq data compared with exome sequencing data from TCGA is most likely due to calling both germline and somatic mutations, as no germline control was analyzed by RNA-seq.

CAPP-Seq library construction

CAPP-Seq libraries were prepared from 32 ng of cfDNA (if less than 32 ng was available, all the cfDNA obtained was input into library preparation, input range: 18.3–32 ng; Supplementary Table S6) and 50 ng of genomic tumor DNA (with the exception of 14.6–100 ng input DNA used from 4 tumor specimens). For CAPP-Seq libraries from germline DNA, the whole yield of DNA obtained from 200 μL of PBLs was used (input range, 28.8–64.6 ng). Tumor and germline DNA was sheared before library construction using Covaris S2 instrument to obtain approximately 170 bp fragments. Libraries were prepared using unique molecular identifiers as described before (6). Postcapture enrichment was evaluated by qPCR using Power SYBR Green PCR Master Mix (ThermoFisher Scientific) with primers specific for *ARID1A*, *ATRX* (genes included in the selector), *B2M* (as a negative control), and internal quality controls for the NimbleGen SeqCap capture panel. Seven to 12 libraries were pooled and sequenced using 2 × 101 bp mode on HiSeq2000 or HiSeq4000 instruments (Illumina).

CAPP-Seq data analysis

Sequencing data were processed using a custom bioinformatics pipeline, and SNV/indel calling was performed as previously described with minor modifications (6). Briefly, sequencing reads were demultiplexed using a 4 bp sample index and deduplicated using molecular barcodes. For cfDNA samples, background polishing was performed to reduce stereotypical base substitution errors. SNV/indel calling was performed as previously described (6) with the following modifications. We defined a "blacklist" as the genes in our panel that were found to be recurrently mutated in the plasma sequencing data from 24 healthy controls. Alterations were recurrently observed in four genes in our panel: *MLL2*, *APOBR*, *PPR21*, and *DSPP*. One or more alterations were observed in each of these genes in >90% of healthy plasma samples, and thus these genes were removed from consideration for variant calling in tumor or plasma samples in leiomyosarcoma patients. For tumor genotyping, we applied additional requirements to identify high-confidence somatic variant calls, to account for the possible artifacts in DNA extracted from FFPE tissue (25, 26). We required ≥3 supporting duplex reads, a positional depth in tumor and germline ≥ 25% of the selector wide median depth, ≥5% mutant AF in the tumor, ≤1 read in matched germline, and no overlap with the UCSC RepeatMasker track (23, 27).

For CAPP-Seq–based ctDNA analysis, cfDNA samples were sequenced to a median deduplicated depth of 2,031× (Supplementary Table S4), and only somatic mutations that were present in one or more tumor samples were considered.

GRP of plasma specimens

Sequencing libraries were prepared with the TruSeq Chip preparation Kit (Illumina), indexed, and 23 samples were pooled for multiplex sequencing across both lanes of an Illumina HiSeq2500 flow cell. Sequencing was performed in a 1 × 50 bp mode, and at least 10 million reads per sample were required from the leiomyosarcoma plasma specimens.

Sequencing reads were mapped to the GRCh37/hg19 reference genome using BWA-MEM with the default settings (version 0.7.10; ref. 18). The pseudo-autosomal region on chromosome Y was masked in the reference genome. Duplicate reads were removed using SAMtools (version 0.1.18; refs. 19, 28). The sequencing summary statistics are included in Supplementary Table S5. Copy-number variants in cfDNA were identified using the depth-of-coverage Plasma-Seq algorithm version 0.6 (11),

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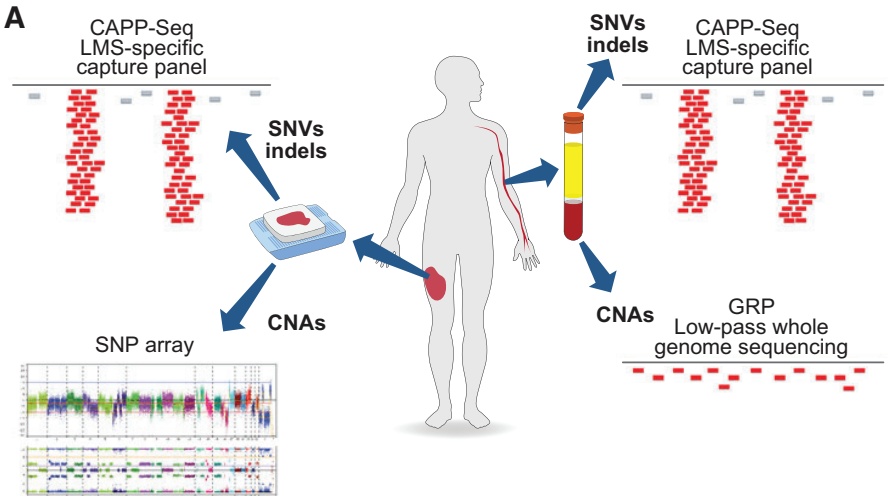

Figure 1.
Study design and a summary of the analyzed specimens. **A,** Types of assays applied to study tumor and plasma specimens. **B,** Summary of all tumor, plasma, and peripheral blood cell specimens analyzed by CAPP-Seq, GRP, and SNP arrays.

B

		CAPPseq [somatic SNV/indels]			GRP/SNP array [CNAs]	
Patient ID	Total # of samples	Germline DNA	Longitudinal plasma samples	Tumor samples	Longitudinal plasma samples	Tumor samples
LMS1	5	1	3	1	2	1
LMS2	8	1	3	2	3	4
LMS3	9	1	5	3	5	3
LMS4	10	1	6	3	6	3
LMS5	13	1	5	7	5	7
LMS6	7	1	2	4	2	4
LMS7	8	1	4	2	4	3
Total	**60**	**7**	**28**	**22**	**27**	**25**

with the following modifications: (1) we used sequencing reads of 50 bp; (2) genome was divided into 100,000 windows, where each window contains the same amount of mappable reads; (3) the average length of the bins was 28 kb; (4) data from 189 female and 189 male healthy donors were used as the nontumor controls. Next we applied Plasma-Seq algorithm with these settings to an independent group of 50 healthy donors, to define the genome-wide segmented Z-scores that set the specificity for leiomyosarcoma analysis at 98% (allowing 1 of 50 healthy donors to carry a CNA in cfDNA). The genome-wide Z-score was set at < -5.44 and >5.44 for the significantly under- and overrepresented regions in leiomyosarcoma specimens, respectively.

Copy-number profiling in leiomyosarcoma tumor specimens

Seventy-five nanograms of genomic DNA were used for genome-wide copy-number and allelic ratio profiling with the OncoScan FFPE Assay by the Affymetrix Research Services Laboratory (Affymetrix). Results were visualized and analyzed with NexusExpress for OncoScan3 software using SNP-FASST2 algorithm (BioDiscovery). Specimens with the Median Absolute Pairwise Difference value above 0.3 were excluded from the analysis. Diploid recentering was performed manually in all samples, and copy-number gain and loss was defined as $\log_2$ ratio > 0.25 and <0.25, respectively. A minimum of 200 probes in the segment was used to call CNAs.

Availability of data

GRP sequencing data have been deposited in the European Nucleotide Archive and have study accession number PRJEB22076 (www.ebi.ac.uk/ena/data/view/PRJEB22076). Raw data from CAPP-Seq experiments are available upon request.

Results

Overview of patient cohort

In this study, we report the CAPP-Seq, GRP, and SNP array results from 60 samples from 7 female patients treated for leiomyosarcoma at Stanford University Medical Center (Fig. 1A and B; Supplementary Table S1). These included 28 longitudinal plasma samples (median of 4 samples per patient), 25 tumor specimens (median of 3 samples per patient), and 7 specimens of peripheral blood leukocytes (PBL) for germline DNA analysis. In this proof-of-principle study, we focused primarily on those genomic aberrations that could be detected in matching tumor and plasma specimens.

Development of leiomyosarcoma-specific CAPP-Seq approach for ctDNA monitoring

To assess the feasibility of detecting ctDNA in leiomyosarcoma patients using CAPP-Seq, we designed a custom hybrid capture panel (i.e., "selector") to target the most frequently mutated genomic regions in leiomyosarcoma based on the analysis of matched tumor and germline whole-exome sequencing data from 77 leiomyosarcoma patients from TCGA (data downloaded from the dbGaP web site, under phs000178.v8.p7; Fig. 2A; ref. 17). In addition, our selector included exons with mutations reported in 114 leiomyosarcoma patients in the COSMIC database v74 (29). The final leiomyosarcoma-specific selector targets 184 kb and

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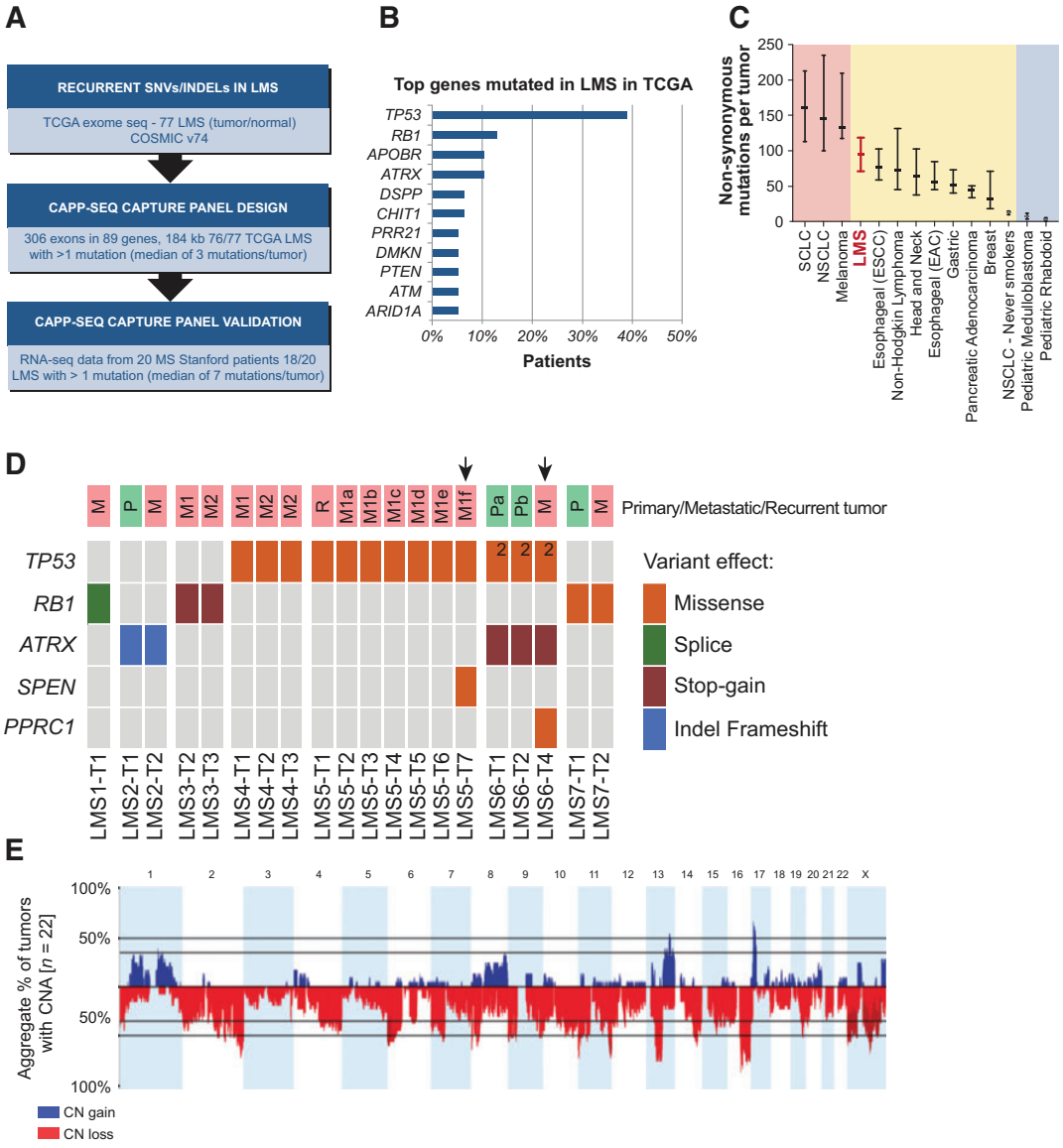

Figure 2.
Mutational landscape of leiomyosarcoma and design of leiomyosarcoma-specific CAPP-Seq selector. **A,** Design and validation of leiomyosarcoma-specific CAPP-Seq capture panel based on TCGA, COSMIC, and Stanford sequencing data. **B,** The most frequently mutated genes in 77 leiomyosarcoma TCGA cases, according to the analysis described in the present study. **C,** The median number of exonic somatic mutations in leiomyosarcoma based on the analysis of TCGA cohort ($n = 77$) compared with selected types of cancer based on the studies reviewed by Vogelstein and colleagues (33). Horizontal bars indicate 25% and 75% quartiles. **D,** SNVs and indels detected by CAPP-Seq in 20 leiomyosarcoma tumor specimens analyzed in the present study. Arrows indicate tumor specimens with subclonal SNVs. Index 2 indicates two different somatic mutations in the same gene. M1 and M2 indicate two different metastatic tumors. Pa, Pb and M1a, M1b, etc. indicate different regions of the same tumor. **E,** Cumulative representation of CNAs identified by SNP array in 22 leiomyosarcoma tumor specimens analyzed in the present study. CN, copy number; EAC, esophageal adenocarcinoma; ESCC, esophageal squamous cell carcinoma; NSCLC, non–small cell lung cancer; SCLC, small cell lung cancer.

includes 306 exons from 89 genes (Supplementary Table S2). This panel covered 98.7% (76/77) of the patients in the TCGA cohort with a median of 3 SNVs/indels per patient. Consistent with prior leiomyosarcoma studies (12, 30–32), the most frequently mutated genes in the TCGA cohort included *TP53*, *RB1*, and *ATRX* (Fig. 2B).

Previous studies have shown a wide spectrum of the prevalence of somatic mutations across human cancer types (33, 34). Based on TCGA data, we calculated that leiomyosarcoma patients carried a median of 96 nonsynonymous mutations per tumor, which classifies leiomyosarcoma as a tumor type with an intermediate level of SNVs and indels, as compared with the other cancer types

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(Fig. 2C; ref. 34). As such, we anticipated that the number of SNVs/indels detectable in ctDNA of leiomyosarcoma patients may be substantially lower than it would be for a cancer with higher mutational burden, such as lung cancer, for which a similar approach to selector design and comparable selector size yielded a panel covering an average of 8 mutations per patient (6).

Performance of CAPP-Seq in leiomyosarcoma patients

To assess the performance of our leiomyosarcoma-focused CAPP-Seq approach, we first analyzed 22 DNA samples obtained from spatially and temporally distinct sites of tumors from 7 leiomyosarcoma patients. We identified a median of 1 (range, 0–4) nonsynonymous SNV/indel in 20 of 22 tumor specimens (Fig. 2D). We identified point mutations in numerous genes previously implicated in leiomyosarcoma including *TP53*, *RB1*, *ATRX*, *SPEN*, and *PPRC1*. *TP53*, *RB1*, and *ATRX* are among the most frequently mutated genes in leiomyosarcoma (Fig. 2B), and all mutations identified in these genes were present in each tumor specimen analyzed from each patient (Fig. 2D), suggesting that these are truncal genetic events, i.e., shared early drivers in the development of leiomyosarcoma. We also found evidence for intrapatient tumor heterogeneity in 2 patients. In LMS5 and LMS6, we identified mutation in *SPEN* and *PPRC1* that were unique to individual metastatic sites in these patients. Mutations in these genes have been less frequently reported in leiomyosarcoma and likely represent subclonal events in these patients (Fig. 2D).

We next applied leiomyosarcoma-focused CAPP-Seq to a total of 28 serial plasma samples from the 7 patients, as well as plasma samples from 24 healthy controls to assess specificity of ctDNA detection. Data from cfDNA of healthy donors were used to characterize selector-specific error profiles and perform digital error suppression as described before (6). CAPP-Seq demonstrated a baseline sensitivity of ctDNA detection of 86% (defined as detection of ctDNA at the first blood draw in a patient with known disease, Table 1; Supplementary Fig. S1) with a specificity of 98.91% (determined using plasma from the 24 healthy donors; Supplementary Fig. S2). The overall sensitivity of ctDNA detection across all analyzed samples was 68% (19/28 positive samples) with a median VAF of 0.27% (range, 0%–31.89%), indicating that ctDNA levels are relatively low in leiomyosarcoma as compared with other solid tumors (7). The CAPP-Seq protocol was initially optimized for the input of 32 ng cfDNA, but it has been previously shown to perform well with the input as low as 4 ng cfDNA (5). We confirm this in leiomyosarcoma patients, as for 7 of 28 samples, we used less than 32 ng of cfDNA (range, 18.3–29.6 ng; Supplementary Table S6), and ctDNA detection by CAPP-Seq was not correlated with the amount of input cfDNA (two-tailed Fisher exact test, $P = 0.65$).

Spectrum of CNAs and performance of GRP in leiomyosarcoma patients

To obtain a more comprehensive picture of ctDNA profiles in leiomyosarcoma, we sought to increase the number of molecular markers queried in the cfDNA by including CNAs as an additional class of genomic alterations. We decided to study CNAs in the same group of patients because genomic instability resulting in complex chromosomal aberrations has been previously described in leiomyosarcoma (13). The analysis of 72 leiomyosarcoma cases within the AACR Genie project identified 15 recurrent CNAs in this entity (GENIE cBioPortal data accessed on June 3, 2017).

Table 1. Summary of SNV/indels and CNAs detected in ctDNA of leiomyosarcoma patients

	ctDNA monitoring	
Plasma sample ID	CAPP-Seq SNV/indels	Number of CNA regions per GRP
LMS1-C1	*RB1* splice site	—
LMS1-C2	—	Not analyzed
LMS1-C3	*RB1* splice site	—
LMS2-C1	*ATRX* indel	—
LMS2-C2	*ATRX* indel	—
LMS2-C3	*ATRX* indel	3
LMS3-C1	*RB1* K154*	194
LMS3-C2	—	—
LMS3-C3	*RB1* K154*	1
LMS3-C4	*RB1* K154*	16
LMS3-C5	*RB1* K154*	4
LMS4-C1	*TP53* V272L	—
LMS4-C2	*TP53* V272L	—
LMS4-C3	—	—
LMS4-C4	*TP53* V272L	3
LMS4-C5	*TP53* V272L	—
LMS4-C6	*TP53* V272L	41
LMS5-C1	*TP53* L344P	40
LMS5-C2	*TP53* L344P	5
LMS5-C3	—	1
LMS5-C4	*TP53* L344P	130
LMS5-C5	*TP53* L344P	—
LMS6-C1	—	—
LMS6-C2	—	—
LMS7-C1	*RB1* D604G	2
LMS7-C2	—	—
LMS7-C3	—	—
LMS7-C4	—	—

This roughly classifies leiomyosarcoma as having intermediate levels of CNAs, compared with the pan-cancer analysis of genomic aberrations published previously by Ciriello and colleagues (35). Copy-number calls from 22 leiomyosarcoma tumor specimens from 7 patients were filtered for the presence of large segmental CNAs (covered by $\geq$ 200 probes) with a copy-number gain and loss defined as $\log_2$ ratio > 0.25 and < 0.25, respectively. With these criteria, we identified extensive CNAs across all 22 tumor samples (median of 80 CNAs per tumor; range, 25–198; Fig. 2E; Supplementary Table S7; Supplementary Fig. S3). Consistent with previous leiomyosarcoma studies, the most frequent CNAs included loss of chromosome 13q with *RB1* locus and gain of chromosome 17p encompassing the *MYOCD* gene (12, 32). CNAs in leiomyosarcoma were highly heterogeneous, and the vast majority of CNAs called with the above-described criteria appear to be subclonal events (Supplementary Fig. S3). The only consistently truncal CNA, found across all tumors specimens analyzed in patients LMS2, LMS3, LMS4, LMS5, and LMS7, was a 3.5 Mb copy-number loss on chromosome 11q24.3-q25 (Supplementary Fig. S3). This genomic region includes 24 genes (Supplementary Fig. S4), including *ADAMTS8* gene (ADAM metallopeptidase with thrombospondin type 1 motif 8, also known as *METH2*) that has been previously described as a tumor suppressor in multiple types of cancer (36).

GRP was performed at a median depth of 0.21× across the whole genome on 27 plasma specimens, all of which had been profiled also by CAPP-Seq (Supplementary Table S5). We also analyzed GRP data obtained from 378 healthy donors (189 males and 189 females) to calibrate the analysis algorithm, and the data from additional 50 healthy donors were used to set the genome-wide z-score cut-off at ±5.44 that allowed for 98% specificity.

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Tumor-matched CNAs were detected in plasma specimens of 71% (5/7) of patients, with the overall sensitivity of 44% (12/27) across all samples (Table 1; Supplementary Fig. S1). Based on ctDNA quantification by CAPP-Seq, ctDNA detection by GRP was achieved at allele fractions as low as 0.23% (sample LMS3-C3). GRP libraries were prepared with 4.25 to 87.5 ng of input cfDNA (Supplementary Table S6), but the detection of ctDNA by GRP was again independent of the amount of input cfDNA (two-tailed Fisher exact test, $P = 0.25$).

Combination of CAPP-Seq and GRP increases the number of reporters that can be monitored in ctDNA

Individually, CAPP-Seq demonstrated a higher sensitivity for ctDNA detection than GRP, but both methods showed highly concordant ctDNA profiles in leiomyosarcoma patients. Interestingly, there was a high correlation between ctDNA levels detected by CAPP-Seq (quantified as VAF) and GRP (represented as a percentage of the whole genome affected by CNAs) across all samples ($R^2 = 0.91$, $P < 0.0001$; Supplementary Fig. S5) indicating the utility of both approaches for ctDNA monitoring in leiomyosarcoma. In patients LMS3 and LMS7, all of the plasma samples were concordantly positive or negative for ctDNA by both methods (Table 1; Fig. 3C and F; Supplementary Fig. S1). However, in patients LMS1, LMS2, LMS4, and LMS5, we demonstrated that combining CAPP-Seq and GRP might prevent false-negative results in ctDNA monitoring (Fig. 3A, B, D, and E). Overall, 9 of 28 plasma samples were ctDNA-positive by only one of the methods (8 samples positive only by CAPP-Seq and one sample positive only by GRP).

The number of SNVs/indels detected in tumors of leiomyosarcoma patients enrolled in this study was lower than expected (a median of 1 detected mutation vs. 3 expected mutations per tumor). This contrasted with prior CAPP-Seq studies in lung cancer and lymphoma where it was possible to track multiple

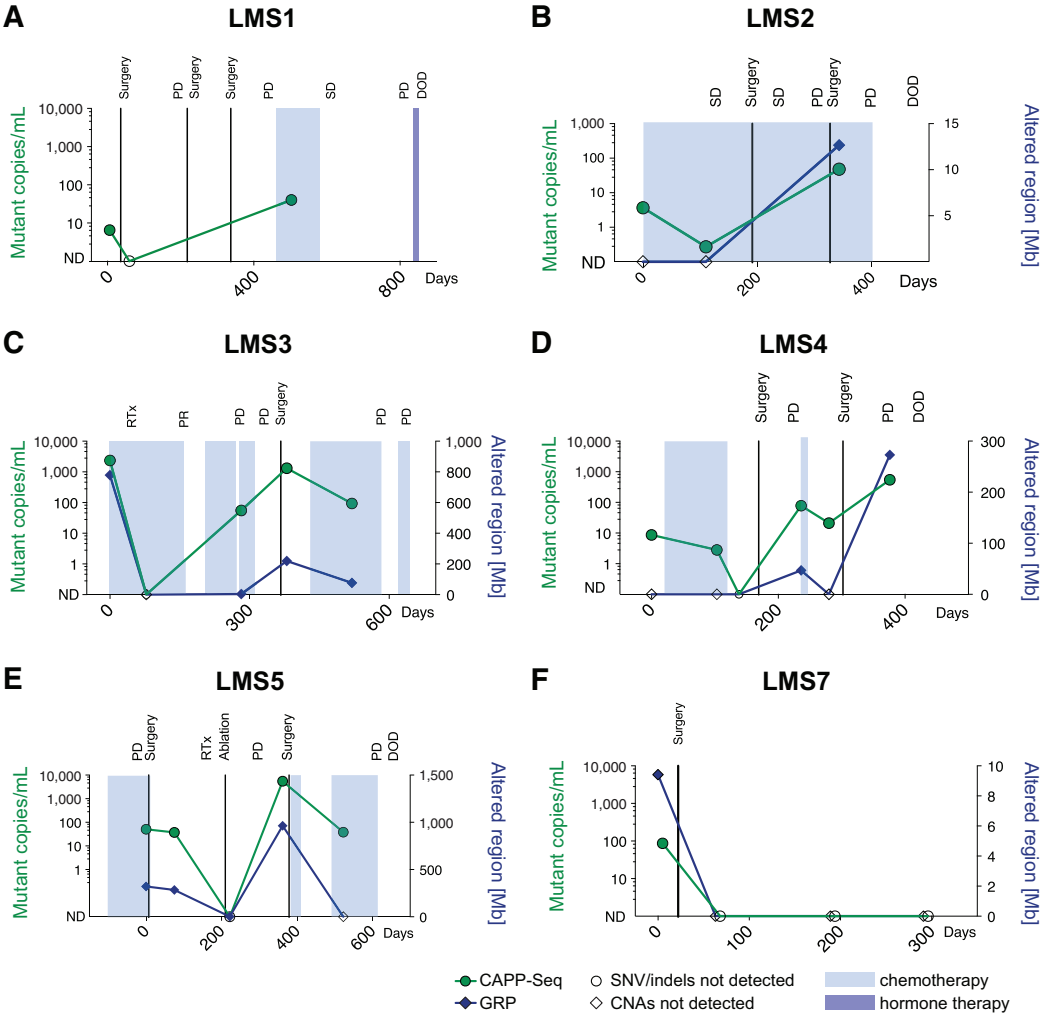

Figure 3.

Patient-specific ctDNA profiles characterized by CAPP-Seq and GRP. The levels of ctDNA decreased in selected patients in response to surgery (LMS1 and LMS7) or chemotherapy (LMS2, LMS3, LMS4). In patients LMS1, LMS2, LMS3, LMS4, and LMS5, the increasing levels of ctDNA correlated with progression of disease throughout the treatment. DOD, dead of disease; Mb, megabases; PD, progressive disease; SD, stable disease.

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markers per patient due to more recurrently mutated genomic regions in these diseases (5, 14). Counting each tumor-matched CNA detected in plasma as an additional reporter that confirms the presence of ctDNA, the average number of reporters detected per plasma sample increased from 1 to 17 (range, 0–195). These results show that the combination approach to liquid biopsy in leiomyosarcoma patients substantially increases the number of molecular markers that can be monitored in plasma. Both approaches cross-validate each other's results, which increases the confidence of detecting tumor-specific aberrations in plasma of leiomyosarcoma patients. Combination of CAPP-Seq and GRP resulted in the overall sensitivity of ctDNA detection of 71% (20/28) across all plasma samples.

Clinical utility of ctDNA monitoring in leiomyosarcoma

Our results demonstrate the feasibility of ctDNA-based assessment of treatment response in leiomyosarcoma patients. In 2 patients, LMS1 and LMS7, the ctDNA signal became undetectable after surgery for a primary extremity tumor and a metastatic liver tumor, respectively (Fig. 3A and F). ctDNA levels in 3 patients (LMS2, 3, and 4) reflected the initial response to chemo- and radiotherapy (Fig. 3B–D). In LMS2, we observed a decrease of ctDNA level from 3.66 to 0.27 mutant copies per mL of plasma after the first 110 days of temozolomide treatment. In patient LMS3, ctDNA signal dropped to undetectable levels by CAPP-Seq and GRP after the first two cycles of gemcitabine and docetaxel treatment combined with radiotherapy. In patient LMS4, we showed a gradual decrease of ctDNA levels in the first three plasma samples collected before, during, and after completion of aldoxorubicin treatment. All patients analyzed in this study eventually progressed based on imaging studies, which was reflected by the increasing levels of ctDNA in all but 1 patient (Fig. 3). The single patient without evidence of ctDNA during progression (patient LMS7) had metastases in lymph nodes and lung but no metastases in soft tissue. Interestingly, we found a highly significant correlation between presence of ctDNA and the location of metastatic tumors in leiomyosarcoma patients. There was a lower rate of ctDNA-positive plasma samples in patients with metastases located in lymph nodes or lung only, compared with patients with metastatic tumors present also in soft tissues, liver, and bone (Table 2, $P < 0.0001$, two-tailed Fisher exact test). Considering all the plasma samples analyzed from the 7 patients in this cohort, 12.5% (1/8) of the samples collected at time points when patients had only lymph node and/or lung metastases had detectable ctDNA, compared with 95% (18/19) of samples collected at time points when patients had also soft tissue, liver, and/or bone metastases. These results suggest that lymph node and lung nodules in leiomyosarcoma patients may shed less DNA into circulation than metastases present in other sites. Although the reason underlying this observation is unclear based on the results from the present study, we hypothesize that it is most likely due the differences in aggregate tumor size. All nodules found in lymph nodes and lung were small and growing slowly, which may indicate their relatively low activity. Larger studies are required to determine whether the size and/or site of metastatic deposits might have an effect on the ctDNA burden in leiomyosarcoma patients.

Table 2. Correlation between ctDNA signal detected by CAPP-Seq/GRP and the location of metastatic sites in leiomyosarcoma patients

	Metastatic sites	
	Lymph node/lung only	Soft tissue/liver/bone/lung
ctDNA positive	1	18
ctDNA negative	7	1

NOTE: Two-tailed Fisher exact test $P < 0.0001$.

Leiomyosarcoma tumor heterogeneity reflected by ctDNA

Overcoming tumor heterogeneity is a major challenge for the personalized treatment of cancer (37). Cancers are known to vary in their mutational profile at different sites within an individual lesion, as well as across different tumor deposits in metastatic patients (38–40). A major advantage of ctDNA compared with tissue biopsies is that ctDNA analysis simultaneously integrates contributions from multiple tumor deposits, enabling a more comprehensive analysis of tumor heterogeneity (15, 41–43). Thus, we sought to explore the utility of our approach for the study of tumor heterogeneity in leiomyosarcoma. We profiled multiple tumor specimens per patient (median of 3; range, 1–7) and found evidence for intrapatient heterogeneity of mutations across different lesions in 2 of 5 of the patients in whom more than 1 tumor sample was sequenced (Fig. 2D). However, these subclonal SNVs were not detected in ctDNA, likely due to the low overall ctDNA levels in these patients. On the other hand, we have observed substantial intrapatient tumor heterogeneity by profiling CNAs (Supplementary Fig. S3). Multiple aberrations were present only in a single sample or in a single region of the same tumor, and some of these subclonal CNAs were detectable in ctDNA. For example, among the 5 plasma specimens from one of these patients (LMS5), we detected a copy-number gain of chromosome 1q21.3-q42.12 in the first and the fourth plasma samples. We did not detect this aberration in the metastatic tumor removed shortly after the first blood sample collection, as we examined only one region of the multifocal abdominal recurrence. But one of six subsequent metastatic tumors removed shortly after the fourth blood sample collection harbored the identical chromosomal gain as detected in ctDNA (Fig. 4). As has previously been shown for lymphoma and lung cancer (14, 15, 34), the analysis of ctDNA enables detection of subclonal alterations also in leiomyosarcoma.

Discussion

ctDNA monitoring offers a great promise for improved diagnosis, prognosis, treatment selection, and surveillance of cancer patients. A personalized approach through tumor-specific phylogenetic ctDNA profiling has been demonstrated to predict resistance to therapy and development of recurrent tumors in lung cancer patients (4). Clinical utility of CAPP-Seq has been demonstrated for improved prognostication in lymphomas (14) and for studying heterogeneity of resistance mechanisms to EGFR inhibitors in lung cancer patients (15). Plasma genotyping for EGFR-sensitizing mutations is already approved by the European Medicines Agency and the FDA for selection of patients for treatments with gefitinib, erlotinib, and osimertinib when tumor genotyping is not feasible (44, 45). In prostate cancer patients, androgen receptor copy-number gain in ctDNA was associated with worse overall survival in response to abiraterone (46). These examples show that ctDNA analysis may provide clinically useful information. However, given a wide variety of DNA abnormalities in different entities,

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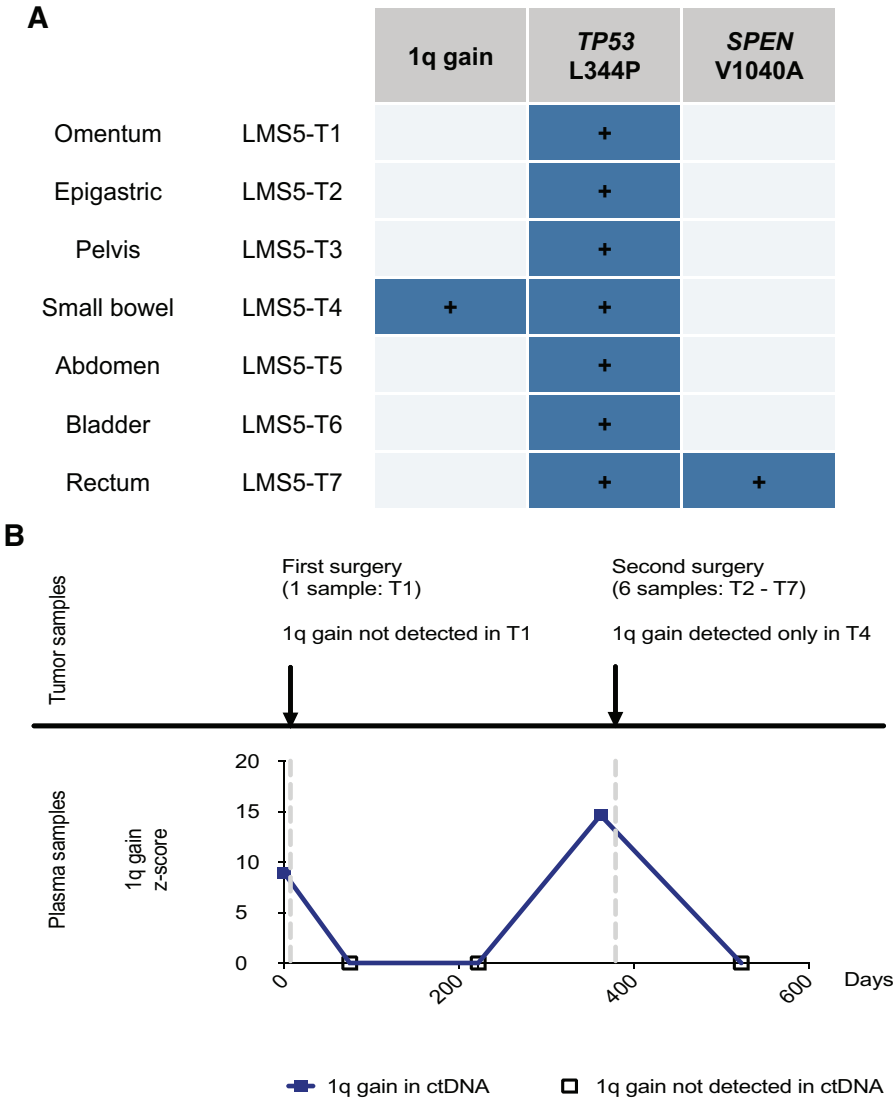

Figure 4.
Tumor heterogeneity detected by ctDNA in patient LMS5. **A,** CNA and SNV profiles of tumor specimens from patient LMS5. Copy-number gain spanning genomic region 1q21.3–1q42.12 has been identified by SNP arrays only in specimen T4 from small bowel. Point mutation in TP53 is a truncal event detected across all recurrent and metastatic tumor specimens, whereas SPEN mutation was a subclonal event detected only in a single metastatic tumor specimen. **B,** GRP-based ctDNA profile of region 1q21.3–1q42.12 across 5 longitudinal plasma samples from patient LMS5. Copy-number gains in this region have been detected in ctDNA plasma sample #1 (collected 7 days before the excision of tumor T1) and plasma sample #4 (collected 17 days before the second surgery of tumors T2–T7).

ctDNA testing needs to be tailored to the genomic profiles of specific types of tumors to achieve clinically relevant sensitivity and specificity.

Here, we present a combinatorial approach to ctDNA monitoring, using leiomyosarcoma as a disease with an intermediate level of both point mutations and copy-number aberrations. We describe the application of two previously validated next-generation sequencing-based methods for ctDNA detection in a group of 7 patients with leiomyosarcoma for whom sequential blood samples were collected. The first method, CAPP-Seq, relies on deep sequencing of a tumor type–specific panel covering the most recurrent SNVs and indels in that disease. CAPP-Seq is an ultrasensitive method for ctDNA detection that is most effective in tumor types with a relatively high rate of recurrent mutations that can be covered by a capture panel of a limited size to ensure cost-effectiveness of deep sequencing, as has been previously demonstrated in lung cancer and lymphoma (5, 14, 15). The second method, GRP, is routinely used for noninvasive prenatal screening and relies on low pass whole-genome sequencing of cfDNA. GRP has been reported to detect ctDNA in asymptomatic patients with ovarian carcinoma, Hodgkin lymphoma, and follicular lymphoma (9).

In the previous CAPP-Seq studies in lung cancer, a selector designed to detect a median of 8 mutations per tumor yielded a median of 6 mutations per tumor when applied to an independent group of patients (6). This number of expected reporters tracked in plasma was high enough to reliably quantify ctDNA with a very high specificity and sensitivity. In the present study, we applied a similar approach to design a leiomyosarcoma-specific CAPP-Seq selector based on TCGA data from 77 leiomyosarcoma cases as described previously for lung cancer (5). Our leiomyosarcoma-specific capture panel was expected to detect 3 mutations per leiomyosarcoma tumor. Similar to the lung cancer CAPP-Seq study, we detected a lower than expected number of mutations also in leiomyosarcoma patients (a median of 1 mutation per tumor). The discrepancy between the expected and detected

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number of SNV/indels in leiomyosarcoma patients is most likely a combination of (1) the selector design being over-fit due to the relatively small number of leiomyosarcoma exomes used to design the panel, (2) the small size of our pilot cohort, (3) the heterogeneity of somatic mutations in this disease, and (4) more stringent analysis criteria applied in CAPP-Seq analysis as opposed to TCGA dataset. Because of the lower than expected number of SNV/indels detected in leiomyosarcoma tumors, we employed a combination approach to simultaneously detect CNAs in tumor and plasma specimens. Separate sequencing of libraries for SNV/indel and CNA analyses may not be practical in the clinical setting. We propose that this strategy could be further improved by developing more robust experimental and bioinformatics methods that would combine targeted detection of SNV/indels with a genome-wide interrogation of copy-number changes in a single experiment.

To obtain the highest level of confidence in the measurement of leiomyosarcoma ctDNA in plasma samples, we required each aberration to be present in patient-matched tumor and plasma specimens, and required each somatic SNV/indel to have duplex support. Our data show that ctDNA could be measured in 86% (6/7) of leiomyosarcoma patients with active disease at >98% specificity and suggest that the levels of ctDNA correlate with the response to treatment. We demonstrate that the orthogonal analysis of ctDNA using two separate methods increased the number of molecular markers that can be monitored in plasma. By CAPP-Seq, we measured the VAF of each mutation, which can be translated into mutant copies of ctDNA per mL of plasma, an absolute quantification of ctDNA levels. By GRP, it is not possible to perform an absolute measurement of the abundance of ctDNA. Therefore, to represent the GRP data, we used the extent of ctDNA with tumor-matched CNAs measured as Mb or the fraction of genome altered by CNAs. Although detection of CNAs in ctDNA is in principle limited by the mutant allele fraction and the original profile of CNAs in the tumor, GRP provided a good proxy for ctDNA load compared with CAPP-Seq results (Supplementary Fig. S5). We propose that a similar strategy may also prove beneficial in other types of tumors that are characterized by moderate levels of both SNVs/indels and CNAs, such as ovarian, breast, or head and neck squamous cell carcinomas (35). Cohen and colleagues have recently demonstrated a different combinatorial strategy for an improved sensitivity of liquid biopsy testing in early stage pancreatic cancer patients by integrated detection of genomic alterations and measurement of protein biomarkers, including carcinoembryonic antigen and carbohydrate antigen 19-9 in plasma specimens (47). This approach increased sensitivity because a large portion of patients was positive for only a single marker.

This proof-of-principle study shows for the first time the feasibility of ctDNA monitoring in leiomyosarcoma patients. Our report provides a rationale for prospective studies in a larger cohort of leiomyosarcoma patients to evaluate whether ctDNA testing may complement current imaging-based regimens for evaluation of leiomyosarcoma patients and drive better-informed clinical decisions on the postoperative treatment, allowing for early detection of residual disease or disease recurrence in asymptomatic patients. In the future, ctDNA-based assessment of treatment response in leiomyosarcoma patients could also lead to a significant improvement of the efficiency of clinical trials. We hypothesize that ctDNA testing may also be useful for a noninvasive distinction between leiomyosarcoma and the much more common benign leiomyomas in the uterus. Treatment of sarcomas poses a significant clinical challenge because imaging-based methods may not accurately assess tumor response to treatment. We hypothesize that a ctDNA-based assay for evaluation of response to therapy may allow for more economical monitoring and could spare patients an exposure to radiation. The cost of next-generation sequencing is steadily decreasing as the technology matures, and we envision that ctDNA assay may, in the future, become complimentary to standard imaging.

In summary, we report that ctDNA analysis may have significant potential to improve monitoring of leiomyosarcoma patients. Although CAPP-Seq outperformed GRP in the number of samples that were called positive for the presence of ctDNA, we show that integrated detection of multiple classes of genomic alterations substantially increased the number of reporters that can be tracked in ctDNA of leiomyosarcoma patients. We propose that such combination approach to liquid biopsies may be applicable also to many other types of tumors that harbor moderate levels of different types of genomic aberrations.

Disclosure of Potential Conflicts of Interest

A.M. Newman is a consultant/advisory board member for Roche. A.A. Alizadeh holds ownership interest (including patents) in CiberMed, and is a consultant/advisory board member for Roche. M. Diehn holds ownership interest (including patents) in CiberMed, and is a consultant/advisory board member for Roche. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Przybyl, J.J. Chabon, L. Spans, K.N. Ganjoo, S. Vennam, A.M. Newman, M. Debiec-Rychter, A.A. Alizadeh, M. Diehn, M. van de Rijn

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