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ORIGINAL ARTICLE

Fractionated small cell-free DNA increases possibility to detect cancer-related gene mutations in advanced colorectal cancer

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colorectal carcinoma, digital PCR, fractionated small cfDNA, liquid biopsy, next-generation sequencing.

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Abstract

Background and Aim: Liquid biopsy is a method that can efficiently detect tumor genetic abnormalities from body fluids such as blood and urine. Detection sensitivity and the available number of mutations in cell-free DNA (cfDNA) are limited. In this study, we develop a highly sensitive and comprehensive method to detect mutations from cfDNA by concentrating tumor fractions of small cfDNA in advanced colorectal cancers.

Methods: Biopsied specimens and 37 serum samples were collected from 27 patients with advanced colorectal carcinoma. A serum-extracted cfDNA was divided into enriched fractionated small cfDNA and unfractionated cfDNA. Both cfDNAs were subjected to digital polymerase chain reaction (PCR) to evaluate their *KRAS, BRAF, CDKN2A*, and *TP53* status. Consequently, their mutant allele frequencies (MAFs) were compared and analyzed by next-generation sequencing (NGS) in conjunction with tissue-derived DNA.

Results: NGS analyses revealed mutations in *TP53* (63%), *KRAS* (63%), *APC* (30%), and *PIK3CA* (22%). Digital PCR could detect mutations in 25 of 27 samples (93%) of unfractionated cfDNA, a rate that increased to 100% when samples were enriched with fractionated small cfDNA (6.8 *vs* 10.7%, $P < 0.001$). NGS also showed increased MAFs in fractionated small cfDNA compared to unfractionated cfDNA (16.3 *vs* 18.8%, $P = 0.012$) and a tendency to detect a greater number of cancer-related genes in fractionated cfDNA.

Conclusions: Fractionated small cfDNA increased MAFs of gene mutations and increases the possibilities to detect cancer-related genes even in advanced cancer patients from whom it is difficult to obtain tissue samples.

Introduction

Colorectal cancer (CRC) is the second leading global cause of cancer death in men and the third leading cause of cancer in women.[1] However, it is also the second leading cause of cancer death in Japan.[2] Advanced CRC patients who cannot be completely resected receive systemic chemotherapy, which has been found to extend the overall survival of patients by 2 years or longer.[3] Recent advancements in therapeutic options, including molecular targeted therapies and immunotherapies,[4,5] have enabled the prescription of more precise medications in accordance with the molecular profile of the patients' tumor.[4,6] For example, activating *KRAS* mutations occurs in approximately 37–45% of CRCs.[7,8] The remaining CRC patients with wild-type *KRAS* (*wt-KRAS*) are bound to benefit from adding anti-epidermal growth factor receptor (EGFR) therapy to their systemic chemotherapy. In other words, and for precision medicine to be accurate and efficient, it necessitates the acquisition of tumor tissues that reveal patients' genetic profiles. However, this process can be a very difficult task in advanced cancer patients with decreased activities of daily living.

Recently, liquid biopsy, a process that identifies the presence of tumor genetic abnormalities using cell-free DNA (cfDNA),[9–11] circulating tumor cells,[12–14] and microRNA[15–17] that are extracted from body fluids such as plasma, serum, and urine, is gaining significant attention because of its less-invasive method to obtain genetic profiles.[11] In fact, and owing to its profound advantages, liquid biopsy is expected to be used clinically in cases such as early tumors detection,[18,19] tumor monitoring,[20–

[24] treatment effect prediction,[20] detection of drug resistance, and as a sensitivity marker.[18,21,25–28] cfDNA normally exists in blood at a length of approximately 170 bp. Furthermore, it is bound to histones, and its half time in blood is reported to be 16 minutes to several hours.[9,10] The detection sensitivity of tumor-derived mutations depends on the proportion of tumor-derived DNAs in cfDNA. Hence, a release of genomic DNA from blood cells can lead to a challenging detection process. Although next-generation sequencing (NGS) has the capacity to detect a wide range of genetic abnormalities in a single assay, NGS sensitivity in detecting mutations is lower than that of dPCR because of sequence errors that may occur at a certain frequency. On the other hand, various technical adjustments have been made to improve liquid biopsy sensitivity, such as its enrichment with methylated cfDNA,[29] enrichment with fractionated small cfDNA,[30,31] preamplification of targeted genes,[32,33] etc. Among them, cfDNA from CRC is reported to have a higher proportion of fractionated small DNA,[31] and hence, mutations in cfDNA could be detected by dPCR with higher sensitivity and by NGS with more comprehensive assays, provided that the fractionated small cfDNA is enriched. In addition, small-sized DNA is rich in tumor-derived cfDNA from hepatocellular carcinoma patients,[34] and cfDNA from lung cancer and melanoma is shorter than that from healthy controls.[35]

The main objective of this research study was to evaluate the sensitivity of liquid biopsy following its enrichment with fractionated small cfDNA that was derived from serum samples of CRC patients. Furthermore, we have also attempted to detect comprehensive genetic mutations by NGS using fractionated small cfDNA that enriched the tumor-derived cfDNA.

Methods

Patients and tissue samples. We retrospectively reviewed biopsied tissues and serum samples of 27 CRC patients who received surgical resections and/or systemic chemotherapy at Yamanashi University Hospital between January 2009 and September 2019. The patients were included in this study only if both their tissue and serum samples were available. Tissues were obtained from resected or biopsied specimens where tumor components were separated by laser capture microdissection (LCM) using an ArcturusXT Laser Capture Microdissection System (Life Technologies, Carlsbad, CA, USA) from 8 μm-thick sections of formalin-fixed paraffin-embedded (FFPE) samples. DNA extraction from LCM specimens was performed as previously reported.[36] DNA from biopsied specimens was extracted using GeneRead DNA FFPE Kits (QIAGEN, Hilden, Germany) according to the manufacturer's specifications. Quantities and qualities of extracted DNA were assessed by a NanoDrop (Thermo Fisher, Waltham, MA, USA) instrument with the Qubit (Thermo Fisher) platform. The distribution of samples is shown as a flow chart in Figure S1. This study was approved by the Human Ethics Review Committee of Yamanashi University Hospital (Receipt number: 1326 and 1847).

Extraction of unfractionated cfDNA and its enrichment of small fraction component. A total of 37 serum samples were obtained from 27 patients before and during their therapeutic treatments. Furthermore, multiple serum samples were obtained during systemic chemotherapy from two patients, whereas one serum sample was obtained from all patients prior to commencement of the therapy (Figure S1). CfDNA was extracted from between 1.4 and 3 mL of serum with the QIAamp Circulating Nucleic Acid Kit (QIAGEN) and with the QIAvac 24 Plus vacuum manifold. Carrier RNA was added to ACL lysis buffer to enhance the binding of nucleic acids to the QIAamp membrane and thus enhance the respective yields. A fractionated small cfDNA was enriched using SPRIselect beads (Beckman Coulter, CA, USA) in order to obtain DNA sizes of 100–400 bp. The sizes and concentrations of both the unfractionated and the fractionated small cfDNA were subsequently assessed by High Sensitivity DNA Kit (Agilent, Santa Clara, CA, USA) with Agilent 2100 Bioanalyzer on-chip electrophoresis.

Digital polymerase chain reaction analyses. Digital polymerase chain reaction (PCR) was performed on a QuantStudio™ 3D Digital PCR System platform composed of a Gene Amp 9700 PCR machine (including a chip adapter kit), an automatic chip loader, and the QuantStudio™ 3D Instrument (Thermo Fisher Scientific). Consequently, the collected data were analyzed with QuantStudio 3D AnalysisSuite Cloud Software (Thermo Fisher Scientific). Mutation analysis in dPCR was based on a 5′-exonuclease assay using TaqMan®-MGB probes targeting *KRAS* G12V, G12D, G12A, G12S, G12C, G13D, Q61R, *TP53* R248W, Y126*, Y107*, R158H, V272M, R175H, G244D, G245D, *BRAF* V600E, and *CDKN2A* H66R (Thermo Fisher Scientific, Catalog number: A44177). These targets were selected based on the mutations detected in tissues by NGS as indicated below, and one of the tissue-derived mutations was selected for dPCR analysis of cfDNA.

Genetic mutational analysis of colorectal tumor samples. Genetic analysis of tumor specimens was performed by amplifying the extracted DNA (10 ng) using barcode adaptors (Ion Xpress Barcode Adapters 1–96 Kit, Life Technologies) with the Ion AmpliSeq Cancer Hotspot panel v.2 (Thermo Fisher), which contains 207 primer pairs and which targets approximately 2800 hotspot mutations in the following 50 cancer-related genes from the COSMIC database[37]: *ABL1*, *AKT1*, *ALK*, *APC*, *ATM*, *BRAF*, *CDH1*, *CDKN2A*, *CSF1R*, *CTNNB1*, *EGFR*, *ERBB2*, *ERBB4*, *EZH2*, *FBXW7*, *FGFR1*, *FGFR2*, *FGFR3*, *FLT3*, *GNA11*, *GNAS*, *GNAQ*, *HNF1A*, *HRAS*, *IDH1*, *JAK2*, *JAK3*, *IDH2*, *KDR/VEGFR2*, *KIT*, *KRAS*, *MET*, *MLH1*, *MPL*, *NOTCH1*, *NPM1*, *NRAS*, *PDGFRA*, *PIK3CA*, *PTEN*, *PTPN11*, *RB1*, *RET*, *SMAD4*, *SMARCB1*, *SMO*, *SRC*, *STK11*, *TP53*, and *VHL*. Barcoded libraries were amplified using emulsion PCR on Ion Sphere particles, and sequencing was performed on an Ion Chef System and an Ion Proton Sequencer (Life Technologies) using an Ion PI Hi-Q Chef Kit (Life Technologies). Variants were identified using Ion reporter software version 5.10 (Thermo Fisher). Furthermore, and to avoid false-positive variants due to sequencing errors, only variants with a frequency of >4% and >1% (with a sequence read depth of >100) were considered to be true in tissues and cfDNA, respectively.

Table 1 Patients' characteristics

Characteristic	Classification	N = 27	%
Age, years, average (range)		70 (50–90)	
Gender	Male	13	48.1
	Female	14	51.9
Stage	I	2	7.4
	II	5	18.5
	III	3	11.1
	IV	17	63.0
Location	Right	16	59.3
	Left	8	29.6
	Rectum	3	11.1

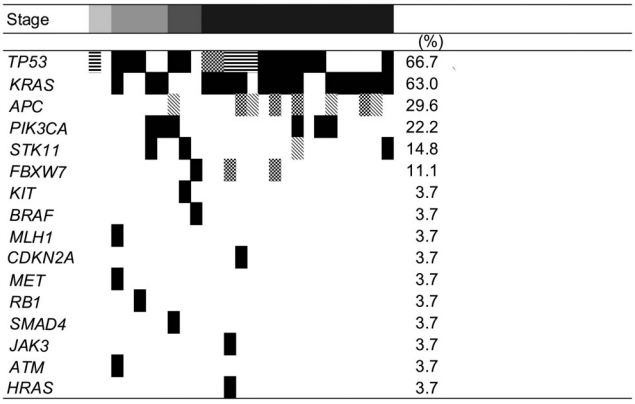

Figure 1 Somatic mutations detected in 50 tissues by cancer-related gene analysis. Samples are distinguished according to the respective clinical stage. The percentage of each gene mutation is shown on the right side of the column. Each column represents one patient, and the black-colored or shaded boxes in each row represent a mutation of each gene. The types of gene mutations are shown in the lower right legend. Stage: (▫) I, (▫) II, (▫) III, (▫) IV. Gene alteration: (▫) nonsense, (▫) nonsense+missense, (▫) missense, (▫) frameshift.

Statistical analysis

Comparisons of mutant allele frequencies (MAFs) and the number of detected mutations between unfractionated and fractionated cfDNA were evaluated using the Wilcoxon signed rank tests and Mann–Whitney U test, respectively, and were considered significant when $P < 0.05$. All statistical analyses of recorded data were performed using the Excel statistical software package (Ekuseru-Toukei 2012; Social Survey Research Information Co., Ltd., Tokyo, Japan).

Results

Patient characteristics and qualitative assessments of extracted DNA. Table 1 depicts the clinical characteristics of all patients included in the study. Among them, 17 patients (63%) presented with distant metastasis, 3 of whom had only pulmonary metastasis (Table S1), and 11 patients (41%) had tumors in their left-sided colon. Median quantities and concentrations of extracted DNA from FFPE samples were 110 ng (range, 4.9–618 ng) and 3.7 ng/μL (range, 0.16–20.6), respectively, while those of extracted cfDNA from 1 ml of serum were 73 ng (range, 5.5–12 700 ng). In NGS analyses, target regions of 50 cancer-related genes included 22 027 bases, and the average (±SD) sequenced read depths were 18 929 (±13 490) and 16 833 (±12 799) in unfractionated and fractionated cfDNA, respectively.

Detected mutations in tissue samples and mutation detection in cfDNA from serum. The four most frequent mutations in tissue samples were identified in *TP53* (63%), *KRAS* (63%), *APC* (30%), and *PIK3CA* (22%) followed by those in *STK11* (15%) and *FBXW7* (11%; Fig. 1). Besides *KRAS* and *PIK3AC*, mutations in *BRAF* and *HRAS*, which are related to EGFR-RAS signaling, were detected in one case (3.7%). A mutation in driver genes from cfDNA was detected by dPCR in all 27 cases. Among *KRAS* mutations detected in 17 cases, amino acid alteration of G12D was the most common observation detected in 5 cases (29%) followed by G12V (4 cases, 24%), G12C (2 cases, 12%), G12S (2 cases, 12%), G12A (1 case, 6%), and Q61R (1 case, 6%). MAFs and the number of mutant alleles in cfDNA by dPCR were 0–63.4% and 0–3 030 933 copies/mL of serum, respectively, whereas detection sensitivities amounted to 85% and 63% when cut-off values were set at 0.1% and 0.5%, respectively (Table 2).

Because the cfDNA-positive rates in CRC patients with only pulmonary metastasis were reported to be low,[38] we compared MAFs of cfDNA with only pulmonary metastasis with those of other metastasis sites. As predicted, the MAFs of cfDNA with only pulmonary metastasis were lower than the latter ($P = 0.037$, Figure S3).

Enriched small fraction of cfDNA raised MAFs of driver genes by dPCR. We enriched small fractions of cfDNA with SPRIselect beads to enhance mutation detection sensitivity.[31] On-chip electrophoresis by Agilent 2100 Bioanalyzer™ exhibited the absence of large-sized cfDNA (Fig. 2a) and an increase in the proportion of small cfDNA from 3.0% in unfractionated cfDNA to 25.9% in fractionated small cfDNA ($P < 0.001$, Fig. 2b).

To confirm the significance of small cfDNA, we consequently analyzed the relationship between existing metastasis and the amount of cfDNA sized 90–150 bp. The average concentration of small cfDNA sized 90–150 bp was 4.4 ng without metastasis and 131.9 ng per 1 mL of serum with metastasis, without any statistical significance ($P = 0.33$). On the contrary, MAFs of a driver gene by dPCR in fractionated small cfDNA were higher than those in unfractionated cfDNA (6.8% *vs* 10.7%, $P < 0.001$, Fig. 3, Table S2), thus suggesting that tumor-derived cfDNA was enriched in small cfDNA.

NGS analysis of fractionated small cfDNA for the detection of cancer-related genes. Comparison of detected mutations in tissue-derived DNA, unfractionated cfDNA, and fractionated small cfDNA samples using deep-sequencing analysis of 50 cancer-related genes is shown in Figure 4a. MAFs of fractionated small cfDNA detected by NGS

Table 2 Mutation detection by digital PCR

Case	Stage	Gene	AA mutation	Tissue MAF (%)	Serum MAF (%)	No. of mutant allele read (copies/mL)†
Case_1	IV	*KRAS*	p.G13D	18.7	0.2	186
Case_2	III	*BRAF*	p.V600E	27.4	0.1	14
Case_3	II	*KRAS*	p.G12V	48.0	0.1	425
Case_4	IV	*KRAS*	p.G12V	50.0	0.0	0
Case_5	IV	*TP53*	p.Y126*	72.0	0.3	336
Case_6	III	*TP53*	p.R248W	88.6	0.0	0
Case_7	IV	*KRAS*	p.G13D	8.4	0.4	151
Case_8	IV	*KRAS*	p.G12S	21.5	0.5	25
Case_9	IV	*KRAS*	p.G12D	36.0	0.3	54
Case_10	II	*KRAS*	p.G12D	49.3	0.8	249
Case_11	II	*KRAS*	p.G12V	55.0	0.3	355
Case_12	IV	*KRAS*	p.Q61R	69.4	0.7	184
Case_13	IV	*KRAS*	p.G12S	6.8	1.8	372
Case_14	IV	*KRAS*	p.G12D	26.9	1.8	547
Case_15	IV	*KRAS*	p.G12C	37.5	1.4	756
Case_16	I	*TP53*	p.Y107*	52.6	0.8	89
Case_17	IV	*KRAS*	p.G12D	74.2	1.3	672
Case_18	II	*TP53*	p.R158H	77.9	1.1	259
Case_19	IV	*TP53*	p.V272M	28.7	2.0	53
Case_20	IV	*KRAS*	p.G12V	30.2	2.5	399
Case_21	IV	*KRAS*	p.G12D	36.6	37.4	3 030 933
Case_22	IV	*KRAS*	p.G12A	43.0	17.1	6948
Case_23	I	N/A	N/A	N/A	N/A	N/A
Case_24	II	*TP53*	p.R175H	91.0	43.7	103
Case_25	III	*TP53*	p.G244D	34.5	49.9	170 551
Case_26	IV	*KRAS*	p.G12C	60.9	63.4	7118
Case_27	IV	*TP53*	p.G245D	81.0	53.0	10 368

†Serum.

AA, amino acid; MAF, mutant allele frequency; N/A, not available.

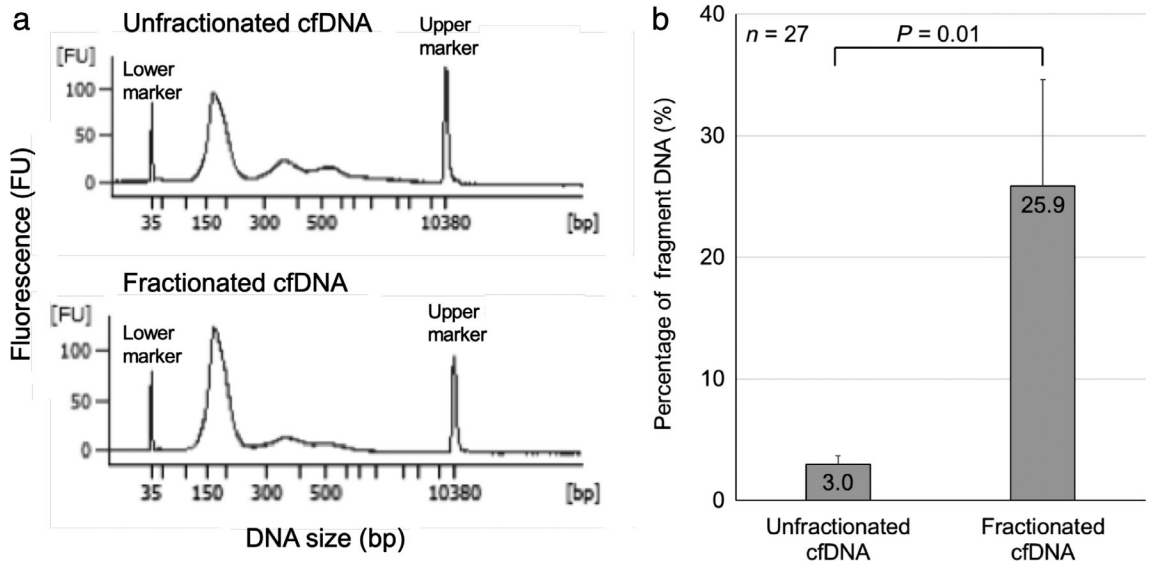

Figure 2 Fractionation of small cfDNA. (a) The size and concentration of the unfractionated and fractionated small cfDNA were assessed by Agilent 2100 bioanalyzer on-chip electrophoresis. The horizontal axis represents the DNA size, whereas the vertical axis represents the DNA concentration (FU). Fractionation of cfDNA increased the proportion of small cfDNA. (b) Proportions of small cfDNA between unfractionated and fractionated cfDNA.

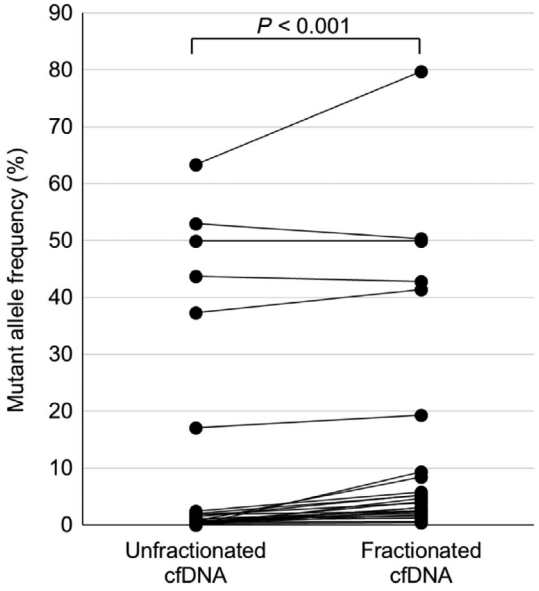

Figure 3 Mutant allele frequencies (MAFs) of driver gene mutations detected by dPCR. MAFs of driver genes detected by dPCR were higher in fractionated small-sized cfDNA than in unfractionated cfDNA.

were higher than those of unfractionated cfDNA (Fig. 4b). All the MAFs detected by NGS were shown in Figure S2A, which showed too many dots with MAFs below 1%, which seemed to be erroneous reads, although some true variants were included in them. We set the MAFs cut-off values as >1% or >2% to remove erroneous reads. The average number of mutations detected in fractionated small cfDNA was higher than those in unfractionated cfDNA (1.8 *vs* 1.0 per case, $P = 0.068$, and 0.78 *vs* 0.56 per case, $P = 0.056$) when cut-off values of MAFs was set at 1% (Fig. 4c) and 2% (Figure S2B), respectively.

Clinical courses of two CRCs with concurrent genetic analysis. Clinical courses of two CRC cases (Cases 19 and 20 in Table 2) who received systemic chemotherapy are shown in Figure 5 with the change of MAFs in genes that were detected in tissues. Progressing disease was observed in case 19 as shown in CT images acquired during chemotherapy. Although carcinoembryonic antigen (CEA) levels in serum were not elevated throughout the course of treatment, MAFs in *PIK3CA* by NGS demonstrated an abrupt elevation in the fractionated small cfDNA (Fig. 5a). Similarly, sensitive reactions of MAFs in *KRAS* were monitored during the clinical course and triggered a partial response by chemotherapy in case 20 (Fig. 5b).

Moreover, *PIK3CA* MAF in unfractionated cfDNA was well below the cut-off value (0.8%), whereas that in fractionated cfDNA was 1.2%, which was high enough to differentiate potential sequence errors introduced by NGS.

Discussion

Results of this study indicate that the fractionation of cfDNA from CRC patients offers sensitive genetic detection by dPCR and NGS analysis, which would provide a less-invasive method to obtain the genetic tumor profiles. The sensitivity of cfDNA in detecting tumor mutations is currently reported to be 51–97% with digital PCR (dPCR)[13,18,19,24–27,39] and 35–86% with NGS.[19,22,23,28,40,41] In our study, the sensitivity in detecting mutations in cfDNA from serum using dPCR was 85% and 93% when cut-off values of MAFs were set at >0% and >0.1%, respectively. These results are consistent with current literature findings. On the contrary, the sensitivity in detecting cfDNA mutations from the serum using NGS was 14% and 25% when cut-off values of MAFs were set at >2% and >1%, respectively. Therefore, NGS sensitivity directly relies on MAFs cut-off. In fact, a great number of sequences with incorrect (erroneous) variants was identified when variants with MAFs below or around 1% are investigated.

To improve the sensitivity of cfDNA mutation detection, we enriched fractionated small nucleic acids from cfDNA. This process facilitated a greater number of MAFs of driver mutations by dPCR and NGS, thus leading to higher possibilities to detect cancer-related gene alterations by NGS. Despite the fact that dPCR is highly sensitive, it can only detect a few targets, whereas although NGS is less sensitive, it facilitates a comprehensive gene analysis. cfDNA from tumor cells has been reported to demonstrate altered fragmentation profiles compared to cfDNA from healthy individuals.[42] Moreover, the proportion of small, fragmented DNA in cfDNA has been found to be significantly higher in patients with lung cancer, CRC, and cholangiocarcinoma.[31] In addition, Jiang analyzed plasma-derived cfDNA size and concluded that cfDNAs derived from patients with HCC are smaller in size than those from healthy controls, and small cfDNA reflects CNA relating to primary tumors. Underhill analyzed the size of tumor-derived cfDNA and normal cell-derived cfDNA using a xenograft model of several tumors and concluded that tumor-derived cfDNA is shorter than normal cfDNA and size selection of cfDNA-elevated MAFs of EGFR T790M mutation in 3 of the 15 lung cancer cases. Our results are consistent with reports using a larger sample size of CRC patients than before and demonstrate a distinct possibility to detect a greater number of cancer-related genes that can, in turn, be targeted by related molecular agents.

Multiple clinical implications are fostered by the findings of this study. First, our sensitive liquid biopsy can facilitate efficient monitoring of the therapeutic outcomes in a more rapid and accurate manner compared to ordinary tumor markers such as CEA and CA19-9. In fact, and as shown earlier, conventional tumor marker responses during the systemic therapy in our two cases shown in Figure 5 were very slow despite the change observed in their CT images. On the other hand, very rapid responses were observed when using liquid biopsy, especially in fractionated small cfDNAs. Second, efficient mutations detection in *KRAS*, *BRAF*, *NRAS*, and *PIK3CA* by liquid biopsy with our sensitive method can be a resistance marker for molecular targeted drugs, which are widely used in conjunction with systemic chemotherapy in CRC.[43] Therefore, and with this method, it is possible to obtain a genetic profile of the tumor even in advanced cancer patients with decreased physical strength. Third, fractionated small cfDNA increased the possibility of detecting cancer-related gene mutations, including actionable gene mutations, which can be a potential molecular target for drugs.

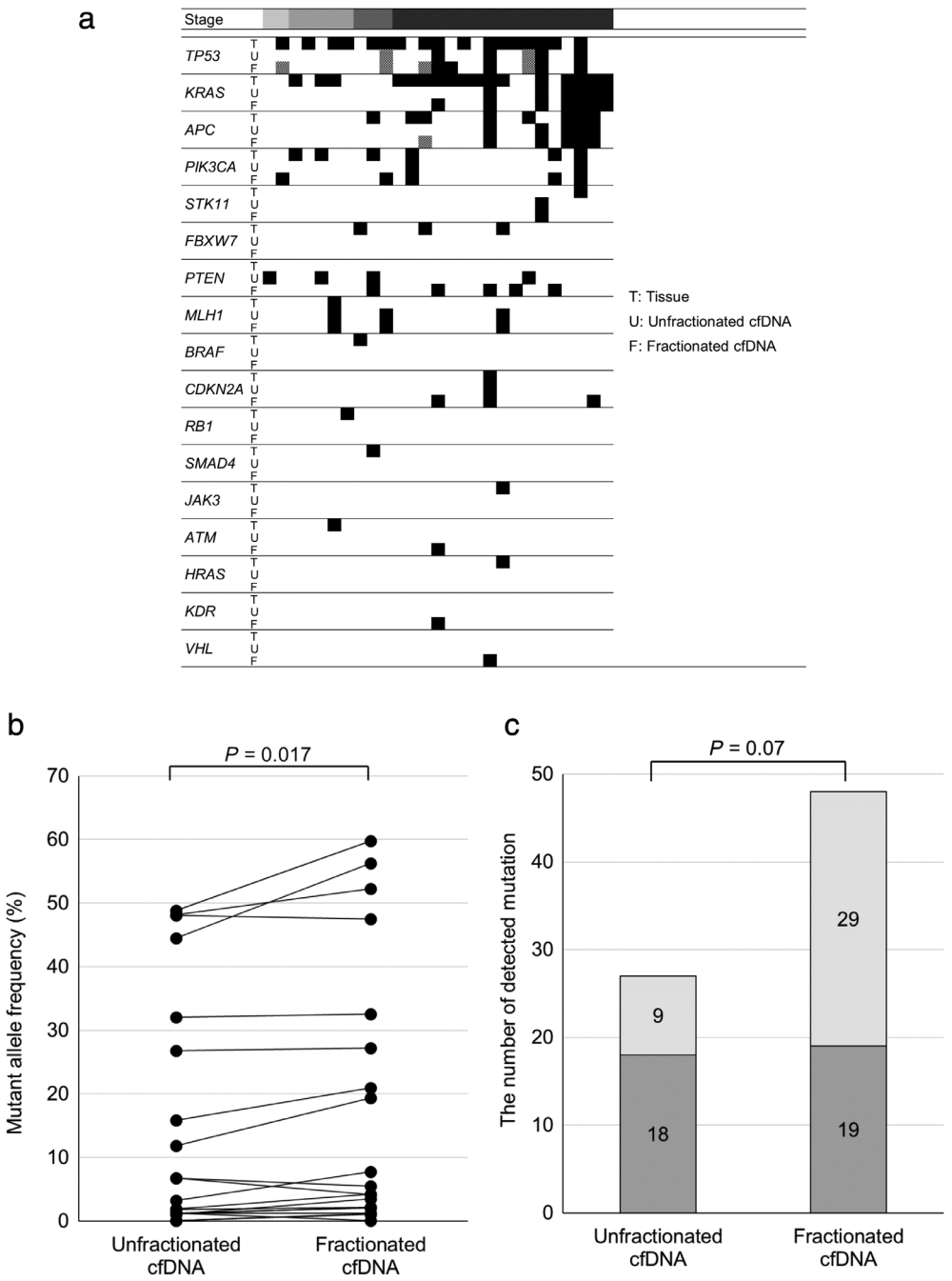

Figure 4 Next-generation sequencing (NGS) analysis of cfDNA. (a) Comparison of detected mutations among tissues, unfractionated cfDNA, and fractionated small cfDNA. Black boxes represent mutations detected by NGS in tissues or the same mutations in cfDNA as in tissues, whereas shaded boxes represent genetic mutations in cfDNA, which were different from the tissue mutation. (b) Mutant allele frequencies (MAFs) of detected genes by NGS in fractionated, small cfDNA were higher compared to those in unfractionated cfDNA. (c) Comparison of the number of detected gene mutations with MAFs that were no less than 1% between unfractionated and fractionated small cfDNA. T, tissue; U, unfractionated cfDNA; F, fractionated cfDNA. Stage: (▫) I, (▫) II, (▫) III, (▫) IV. Gene alteration: (■) same mutation, (▩) different mutation.

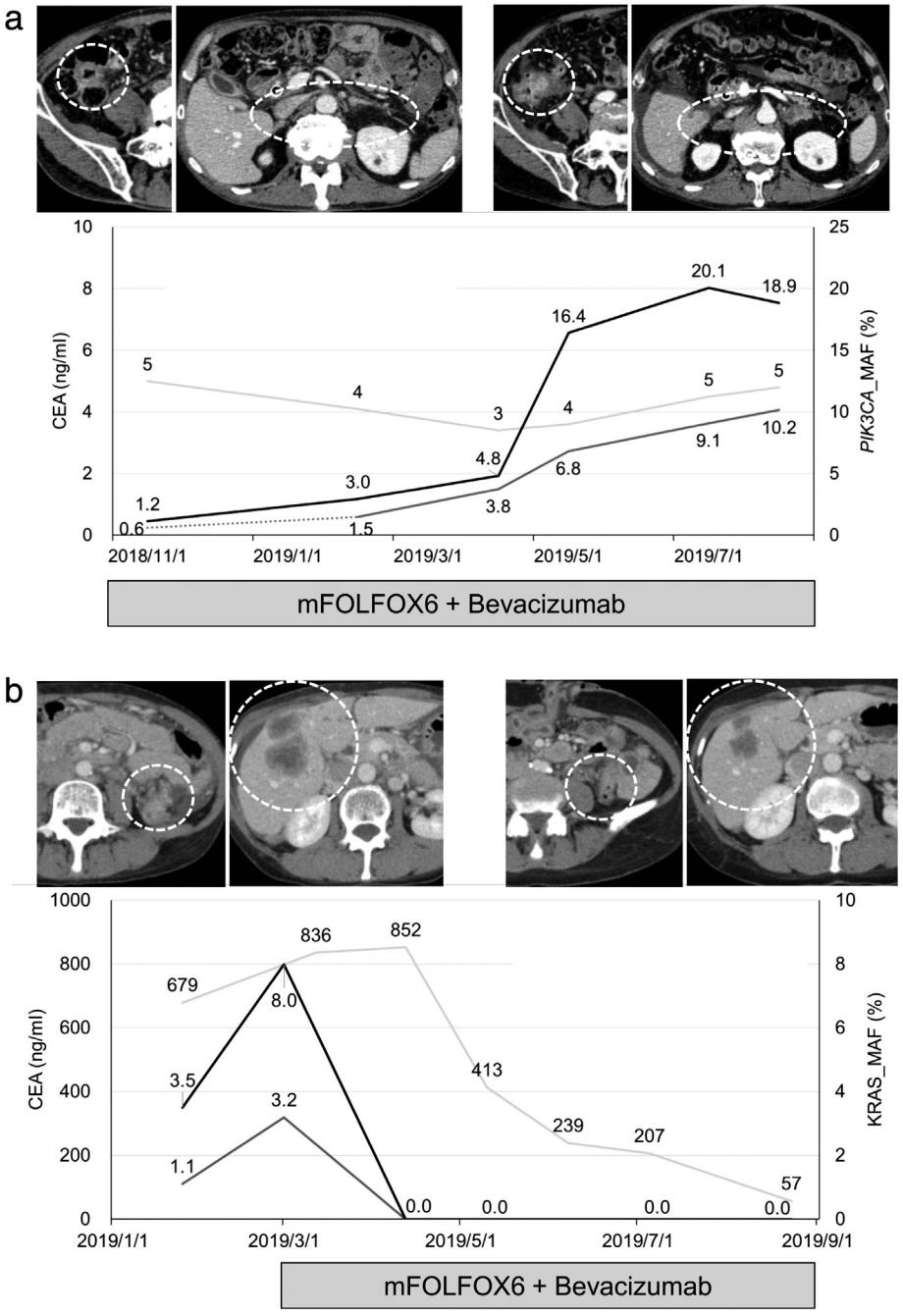

Figure 5 Clinical course of two patients during systemic chemotherapy. (a) MAFs of *PIK3CA* before and after disease progression using unfractionated cfDNA, fractionated cfDNA, and a tumor marker CEA along with the corresponding CT images in case 19, who was a 67-year-old male with extraregional lymph node metastasis. CEA level did not change significantly when the cancer progressed, but MAFs in *PIK3CA* increased rapidly, especially by fractionated cfDNA. (b) MAFs of *KRAS* before and after tumor reduction using unfractionated cfDNA, fractionated cfDNA, and tumor markers along with the corresponding CT images in case 20, who was a 51-year-old female with hepatic metastasis. The MAFs of *KRAS* decreased approximately 2 months before cancer shrinkage, with a decrease in CEA. Monitored genes were chosen from those detected in tissues. Top: (——) CEA, (——) unfractionated cfDNA (PIK3CA), (——) fractionated cfDNA (PIK3CA). Bottom: (——) CEA, (——) unfractionated cfDNA (KRAS), (——) fractionated cfDNA (KRAS).

This study has several limitations. First, the design is retrospective, and hence, only a small number of cases were recruited from a single center. Second, sequencing errors with derived incorrect readings could not be completely eliminated in deep-sequencing analysis by NGS. Therefore, we discarded variants with MAFs that were less than 1% in our analysis while at the same time eliminating sequence reads that had an inferior quality. We are now aware that we should use more sophisticated methods in order to differentiate between true and incorrect variants.

In conclusion, we demonstrated elevated MAFs of driver genes by means of small cfDNA fractionation, which could increase the possibility of detecting cancer-related genes. We believe that these findings will help the scientific community to improve detecting molecular targetable genes using liquid biopsy even in patients whose physical strength has significantly declined owing to cancer progression.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's website:

Figure S1 Flow chart of this study.

Figure S2 (A) Dot spots of all MAFs detected by NGS between unfractionated and fractionated small cfDNA. (B) Comparison of the number of detected gene mutations with MAFs no less than 2% or 0.5% between unfractionated and fractionated small cfDNA.

Figure S3 Comparison of MAFs of unfractionated cfDNA in patients with only pulmonary metastasis and those with metastasis at other sites.

Table S1 Detailed clinical information of all cases.

Table S2 Comparison of MAF(%) by dPCR.