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Analysis of colorectal cancer-related mutations by liquid biopsy: Utility of circulating cell-free DNA and circulating tumor cells

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Abstract

We recruited 56 colorectal cancer patients and compared the mutational spectrum of tumor tissue DNA, circulating cell-free DNA (ccfDNA) and circulating tumor cell (CTC) DNA (ctcDNA) to evaluate the potential of liquid biopsy to detect heterogeneity of cancer. Tumor tissue DNA, ccfDNA, and ctcDNA were extracted from each patient and analyzed using next-generation sequencing (NGS) and digital PCR. To maximize yields of CTC, three antibodies were used in the capture process. From 34 untreated patients, 53 mutations were detected in tumor tissue DNA using NGS. Forty-seven mutations were detected in ccfDNA, including 20 not detected in tissues. Sixteen mutations were detected in ctcDNA, including five not detected in tissues. In 12 patients (35.3%), mutations not found in tumor tissues were detected by liquid biopsy: nine (26.5%) in ccfDNA only and three (8.8%) in ctcDNA only. Combination analysis of the two liquid biopsy samples increased the sensitivity to detect heterogeneity. From 22 stage IV patients with *RAS* mutations in their primary tumors, *RAS* mutations were detected in 14 (63.6%) ccfDNA and in eight (36.4%) ctcDNA using digital PCR. Mutations not detected in primary tumors can be identified in ccfDNA and in ctcDNA, indicating the potential of liquid biopsy in complementing gene analysis. Combination analysis improves sensitivity. Sensitivity to detect cancer-specific mutations is higher in ccfDNA compared with ctcDNA.

KEYWORDS

cell-free nucleic acid, circulating cell free DNA, circulating tumor cell, circulating tumor DNA, liquid biopsy

1 | INTRODUCTION

Accurate genetic analysis is essential for molecular targeted therapy and precision medicine. Conventionally, DNA derived from tumor tissue harvested during surgery or endoscopic biopsy has been the source of the gold standard of DNA information in solid tumors including colorectal cancer (CRC). However, tumors are known to have spatial and temporal heterogeneity, and mutations not identified in primary tumors have been shown to exist in metastatic tumors.[1,2] Additionally, molecular targeted therapy induces clonal evolution.[3,4] Thus, real-time monitoring of genetic information is important for molecular therapy.

Liquid biopsy, which includes circulating cell-free DNA (ccfDNA) and circulating tumor cells (CTC), may help in the detection of this spatial and temporal heterogeneity. ccfDNA is DNA found in the bloodstream, and ccfDNA derives from both normal and malignant cells. Circulating tumor DNA (ctDNA) is ccfDNA derived from malignant cells, and may contain cancer-specific mutations. Both ccfDNA and ctDNA are useful for cancer monitoring and management.[5-9] We previously reported that emerging mutations can be detected from ccfDNA in CRC patients undergoing chemotherapy with epidermal growth factor receptor (EGFR) blockade,[4] that levels of ctDNA increase after self-expanding metallic stent placement in CRC patients with acute bowel obstruction,[10] and that spatial heterogeneity can be detected using ccfDNA.[2] CTC are circulating malignant cells of solid tumor origin that are found in the bloodstream. They are a prognostic biomarker, and the prognosis of patients with high numbers of CTC is poor.[11-13] Detection of CTC offers more specificity for cancer detection compared with ccfDNA[14] because it also includes information about RNA and protein.

To use these liquid biopsy samples for mutational profiling and clinical decision-making, it is necessary to compare the mutational spectrum of liquid biopsy samples and tissue samples. However, in CRC patients, no concordance study has been carried out between tumor tissue DNA, ccfDNA, and DNA derived from CTC (ctcDNA); furthermore, the utility of ccfDNA and ctcDNA combination analysis is unclear. The main reason for this is the difficulty of conducting research into CTC because of their low recovery rate, especially when capturing cells causing epithelial-mesenchymal transition (EMT), and the fact that sufficient DNA cannot be extracted from CTC.[15,16]

In the present study, we evaluated the concordance of the mutational spectrum of tumor tissue DNA, ccfDNA, and ctcDNA using next-generation sequencing (NGS) and digital PCR (dPCR). We also evaluated whether combination analysis of ccfDNA and ctcDNA has a notable impact on genetic analysis. To improve the recovery rate of CTC, we used the multi-antibody method based on antibodies against epithelial cell adhesion molecule (EpCAM), human epidermal growth factor receptor (Her)2, and tumor-associated calcium signal transducer (Trop)2.[17]

2 | MATERIALS AND METHODS

2.1 | Ethical considerations

This was a single institutional prospective, observational study. The Ethics Committee of Nippon Medical School (Tokyo, Japan) approved this study and it was carried out in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants.

2.2 | Cell line authentication

DLD-1 CRC cell line carrying the *KRAS* G13D mutation was obtained from the JCRB Cell Bank (Osaka, Japan). It was authenticated by short tandem repeat profiles by PCR, and was tested for *Mycoplasma* contamination.

2.3 | Collection of tumor cells and detection of mutant DNA by dPCR using DLD-1 cells

Peripheral blood (10 mL) was collected from a healthy individual and spiked with 1000 DLD-1 cells. DLD-1 cells were then isolated from the blood by the LiquidBiopsy platform (Cynvenio Biosystems). After imaging and cell counting, ctcDNA was extracted and analyzed using dPCR to detect the *KRAS* G13D mutation allele.

2.4 | Patients

2.4.1 | Cohort 1

This cohort included previously untreated CRC patients who were treated in our hospital between April 2017 and March 2018. Tumor tissue was collected from each patient, either by primary surgery or by colonoscopic biopsy, as well as 10 mL of whole blood. CTC were isolated by the LiquidBiopsy platform, and captured cells were counted. Afterwards, tumor tissue DNA, ccfDNA, ctcDNA, and DNA derived from white blood cells (wbcDNA) was extracted and analyzed using NGS.

2.4.2 | Cohort 2

This cohort included stage IV CRC patients with *RAS* mutations in their primary tumors who were treated in our hospital between September 2017 and April 2019. All patients had unresectable CRC, and were under treatment or had completed chemotherapy. Peripheral blood (10 mL) was collected from each patient. CTC were isolated by the LiquidBiopsy platform and captured cells were counted. Afterwards, ccfDNA and ctcDNA were extracted and analyzed using dPCR to detect the *RAS* mutation allele.

2.5 | Blood sample collection procedures

Peripheral blood (10 mL) was collected and transferred to BD Vacutainer PPT Plasma Preparation Tubes (BD Biosciences). Plasma, CTC, and WBC were collected from the same patient blood draw using the LiquidBiopsy platform. Samples were stabilized using LiquidBiopsy fixative (Cynvenio Biosystems) within 2 hours. WBC control was collected from 0.4 mL of the original sample and stored at −20°C until use. The sample was centrifuged at 700 × *g* for 10 minutes, and plasma was removed after centrifugation and stored at −80°C until use. The remaining

sample including the pellet was then processed according to the LiquidBiopsy manufacturer's instructions. In brief, LiquidBiopsy Lysis Buffer (Cynvenio Biosystems) was added to the sample, centrifuged, and the supernatant removed. The sample was then labelled with LiquidBiopsy EMT Cocktail (Cynvenio Biosystems) consisting of anti-EpCAM, -Her2, and -Trop2 antibodies, followed by LiquidBiopsy Beads (Cynvenio Biosystems). The sample was stained by LiquidBiopsy CD45, cytokeratin, and DAPI (Cynvenio Biosystems), and processed using Isolation Flow Cells (Cynvenio Biosystems) on the LiquidBiopsy platform. After assay completion, captured cells were processed for imaging using the EVOS FL Cell Imaging System (Thermo Fisher Scientific). DAPI-positive, cytokeratin-positive, and CD45-negative cells were defined as CTC. After imaging and cell counting, the sample was stored at −20°C.

2.6 | DNA extraction from frozen tissue samples

Tissue from the primary tumor was collected by primary surgery or colonoscopic biopsy and preserved at −80°C. DNA was extracted using the QIAamp DNA Mini kit (Qiagen) according to the manufacturer's instructions. Total DNA concentration was measured using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific).

2.7 | DNA extraction from plasma samples

Plasma samples kept at −80°C were recentrifuged at 16 000 × *g* for 10 minutes at 4°C to eliminate debris. Afterwards, ccfDNA was extracted using a QIAamp circulating nucleic acid kit (Qiagen) from 3 mL plasma according to the manufacturer's instructions. Total DNA concentration was measured using a Qubit 2.0 Fluorometer.

2.8 | DNA extraction from CTC and WBC samples

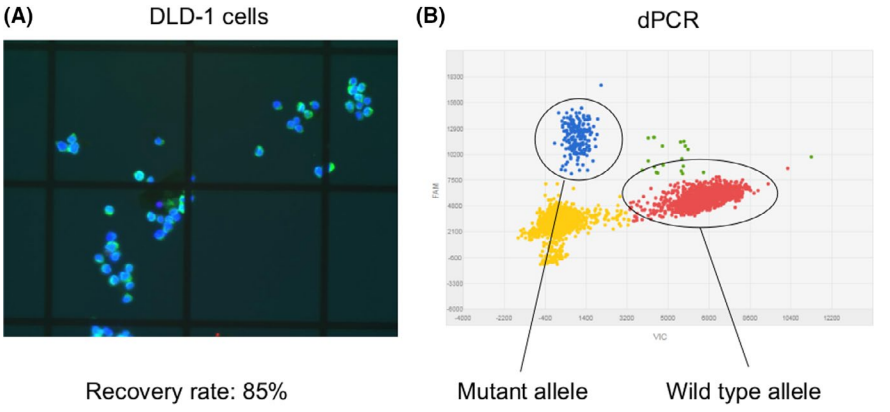
Circulating tumor cell and WBC samples were stored at −20°C as described above. DNA was extracted from each sample using the LiquidBiopsy Reagents and Consumables Kit (Cynvenio Biosystems) according to the manufacturer's instructions. In brief, the pellet was digested using the LiquidBiopsy Digest Mix (Cynvenio Biosystems) and incubated at 55°C for 3 hours and at 70°C for 1 hour. The CTC sample was digested with 6.5 μL LiquidBiopsy Digest Mix, and the WBC sample was digested with 50 μL. Concentrations of all wbcDNA samples were measured using a Qubit 2.0 Fluorometer. ctcDNA concentrations were measured in samples analyzed by dPCR using 1 μL digested sample. Concentration was not measured for samples analyzed by NGS because specifically 6 μL digested sample was required for analysis.

2.9 | Next-generation sequencing

Tumor tissue DNA, ccfDNA, and wbcDNA were diluted to 830 ng/mL, as measured by a Qubit 2.0 Fluorometer. If the concentration of the DNA sample was lower than 830 ng/mL, it was not diluted. Nuclease-free water (6 μL) was added to 6 μL digested ctcDNA sample, then amplified by PCR using the Ion AmpliSeq Cancer Hotspot Panel v2 (Thermo Fisher Scientific) which targets 207 amplicons for 2885 mutations in 50 cancer-associated genes. Barcoded libraries were constructed using an Ion AmpliSeq Library Kit 2.0 (Thermo Fisher Scientific) according to the manufacturer's instructions. Barcoded libraries were then amplified and loaded on an Ion 318 Chip using the Ion Chef System (Thermo Fisher Scientific), and sequenced on an Ion Torrent PGM System (Thermo Fisher Scientific).

2.10 | Sequencing data analysis

Sequence data on the Ion Torrent PGM System were automatically transferred and analyzed using Torrent Suite ver. 5.8.0 (Thermo Fisher Scientific). Work flow included mapping to the hg19 reference genome and variant calling. The Torrent Suite uses the Torrent Browser, which includes the Torrent Mapping Alignment Program and Torrent Variant Caller for alignment and variant detection. Variant calling was done with CHP2 Panel Somatic PGM using low stringency settings. Same parameters were used for variant calling in tumor tissue DNA,



Spiked cells	Recovered cells	Recovery rate (%)	ctcDNA (ng/ml)	Variant allele frequency (%)
1000	850	85	25800	9.676

FIGURE 1 A, Image of captured DLD-1 cells. A total of 85% (850/1000) of cells was recovered by the LiquidBiopsy platform (Cynvenio Biosystems). B, *KRAS* G13D mutation allele was successfully detected by digital PCR with a variant allele frequency of 9.68%. ctcDNA, circulating tumor cell DNA

TABLE 1 Summary of patient characteristics in cohort 1

Total	N = 34
Age (y)	68 (41-85)
Gender	
Male	23
Female	11
Primary tumor location	
Cecum	2
Ascending colon	5
Transverse colon	3
Descending colon	4
Sigmoid colon	11
Rectum	9
TNM stage	
Stage II	4
Stage III	7
Stage IV	23
Tissue collection	
Operation	25
Biopsy	9
CEA (ng/mL)	11.8 (1.5-3351.8)
CA19-9 (U/mL)	12.8 (2-12 000)
ccfDNA (ng/mL)	195 (112-30 300)
Collected CTC (no. cells)	31 (3-94)

Note: Data are shown as the median (range).
Abbreviations: CA19-9, carbohydrate antigen 19-9; ccfDNA, circulating cell-free DNA; CEA, carcinoembryonic antigen; CTC, circulating tumor cells.

ccfDNA, ctcDNA and wbcDNA. Mutations detected in wbcDNA were defined as germline mutations or miscalls, and were excluded from mutations found in tumor tissue DNA, ccfDNA, and ctcDNA.

2.11 | Mutation detection by dPCR

Each DNA sample was diluted to 1000 ng/mL, as measured by a Qubit 2.0 Fluorometer (Thermo Fisher Scientific). PCR reaction mixtures contained 9 μL QuantStudio 3D Digital PCR Master Mix (Thermo Fisher Scientific), 0.45 μL TaqMan assay, and 8.55 μL diluted DNA. Fifteen μL of the 18 μL reaction mixture was loaded in a QuantStudio 3D Digital PCR 20K chip (Thermo Fisher Scientific) and amplified using the GeneAmp PCR system 9700 (Thermo Fisher Scientific) as follows: 96°C for 10 minutes, then 39 cycles of 56°C for 2 minutes, 98°C for 30 seconds, and a final extension step at 60°C for 2 minutes. Commercial primers (wet lab-validated Custom TaqMan SNP Genotyping Assays) were used.

2.12 | Statistical analysis

All statistical analyses were carried out using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R version 3.0.2 (R Foundation for Statistical Computing). We carried out comparisons using Fisher's exact test for categorical variables and the Mann-Whitney *U* test for quantitative variables. *P*-value <.05 was considered significant.

3 | RESULTS

3.1 | Collection of tumor cells and detection of mutant DNA by dPCR using DLD-1 cells

A total of 85% (850/1000) of spiked tumor cells (DLD-1) were recovered by the LiquidBiopsy platform. Concentration of ctcDNA was 25 800 ng/mL, and the *KRAS* G13D mutation allele was successfully detected by dPCR. Variant allele frequency (VAF) was 9.68%. Data are shown in Figure 1.

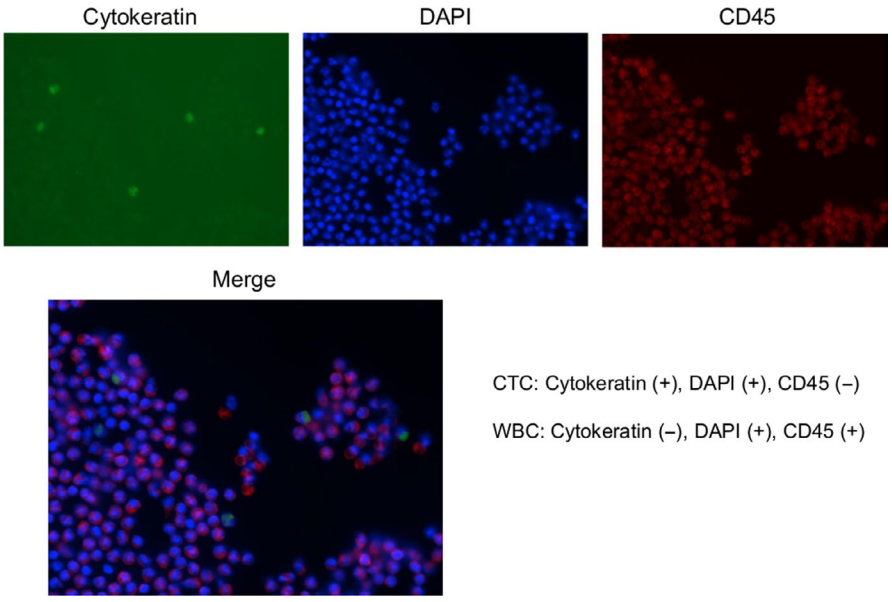


FIGURE 2 Collected and stained circulating tumor cells (CTC) and white blood cells (WBC) from cohort 1. Cytokeratin-positive, DAPI-positive, and CD45-negative cells were defined as CTC. Cytokeratin-negative, DAPI-positive, and CD45-positive cells were defined as WBC

TABLE 2 Detailed NGS data of each patient in cohort 1

	Tumor tissue DNA		ccfDNA		ctcDNA	
Case	No. mutations	Gene (COSMIC)	No. mutations	Gene (COSMIC)	No. mutations	Gene (COSMIC)
1	3	KRAS (532) PIK3CA (776) SMAD4 (14 122)	1	SMAD4 (14 122)	0	
2	3	APC (13 113) KRAS (520) TP53 (10 667)	0		0	
3	2	APC (18 862) KRAS (554)	0		0	
4	1	TP53 (99 020)	0		2	APC (19 652)FLT3 (19 692)
5	4	APC (19 048) KRAS (521) SMAD4 (14 140) TP53 (10 656)	1	VHL (25 719)	0	
6	1	TP53 (10 663)	1	TP53 (10 663)	0	
7	2	APC (13 727) APC (18 779)	2	APC (13 727) APC (18 779)	0	
8	2	APC (19 072) KRAS (521)	2	APC (19 072) KRAS (521)	1	KRAS (521)
9	1	TP53 (44 580)	1	TP53 (44 580)	0	
10	1	PIK3CA (763)	2	PIK3CA (763) TP53 (99 602)	0	
11	0		2	APC (13 113) NOTCH1 (13 047)	0	
12	2	APC (19 349) KRAS (532)	1	KRAS (532)	0	
13	2	APC (25 826) TP53 (43 750)	2	APC (25 826) TP53 (43 750)	1	APC (25 826)
14	1	TP53 (99 667)	1	TP53 (99 667)	3	TP53 (99 667) CDKN2A (13 252) FLT3 (19 692)
15	2	KRAS (521) TP53 (10 659)	2	KRAS (521) SMARCB1 (1090)	0	
16	3	KRAS (520) TP53 (43 807) TP53 (99 617)	0		0	
17	1	TP53 (99 925)	4	TP53 (99 925) TP53 (43 756) NRAS (563) KRAS (520)	0	
18	2	KRAS (520) TP53 (10 654)	2	KRAS (520) TP53 (10 654)	2	KRAS (520) TP53 (10 654)
19	0		0		0	
20	0		2	KRAS (517) TP53 (44 032)	0	
21	1	TP53 (10 650)	0		0	
22	1	TP53 (10 648)	0		0	
23	1	BRAF (476)	2	BRAF (476) PDGFR (736)	0	
24	0		0		0	

(Continues)

TABLE 2 (Continued)

Case	Tumor tissue DNA		ccfDNA		ctcDNA	
	No. mutations	Gene (COSMIC)	No. mutations	Gene (COSMIC)	No. mutations	Gene (COSMIC)
25	1	KRAS (520)	1	KRAS (520)	1	KRAS (520)
26	4	BRAF (27 639) HRAS (480) FBXW7 (22 932) APC (18 852)	5	BRAF (27 639) HRAS (480) FBXW7 (22 932) APC (18 852) KRAS (520)	1	APC (18 852)
27	1	KRAS (516)	5	KRAS (516) BRAF (467) TP53 (43 747) TP53 (45 397) TP53 (44 032)	1	TP53 (45 050)
28	0		4	PDGFRA (736) APC (18 700) TP53 (99 024) SMARCB1 (1090)	0	
29	2	APC (13 123) TP53 (10 647)	1	TP53 (10 647)	0	
30	3	APC (13 127) KRAS (532) TP53 (10 663)	2	APC (13 127) KRAS (532)	3	APC (13 127) KRAS (532) TP53 (10 663)
31	1	TP53 (11 218)	1	TP53 (11 218)	1	TP53 (11 218)
32	2	APC (18 764) TP53 (99 024)	0		0	
33	3	PIK3CA (746) APC (13 125) PTEN (5033)	0		0	
34	0		0		0	
Total	53		47		16	

Note: Using NGS, we detected 53 mutations in tumor tissue DNA, 47 mutations in ccfDNA and 16 mutations in ctcDNA in 34 patients. Twenty mutations in nine patients (26.5%) detected in ccfDNA were not detected in tumor tissue DNA. Five mutations in three patients (8.8%) detected in ctcDNA were not detected in tumor tissue DNA.

Abbreviations: ccfDNA, circulating cell-free DNA; COSMIC, catalogue of somatic mutations in cancer; ctcDNA, circulating tumor cell DNA; NGS, next-generation sequencing.

3.2 | Cohort 1

3.2.1 | Patient characteristics and collection quantity of CTC

Thirty-four patients (stage II: n = 4, stage III: n = 7, stage IV: n = 23) were enrolled in cohort 1. Patient characteristics are summarized in Table 1. All blood samples were collected before treatment (operation or chemotherapy). Of the 34 patients, 25 had primary tumor resection from which tumor tissue was collected. From the remaining nine patients, tumor tissue was collected by colonoscopic biopsy. Median number of collected CTC was 31 cells (range, 3-94). Figure 2 shows images of stained CTC.

3.2.2 | Next-generation sequencing

A total of 53 somatic hot spot mutations was detected in tumor tissue DNA, with a median number of one per patient (range, 0-4). The most frequent mutation was within *TP53* (n = 18), followed by mutations in *APC* (n = 13) and *KRAS* (n = 12). A total of 47 somatic hot spot mutations was detected in ccfDNA, with a median number of one per patient (range, 0-5). The most frequent mutation was within *TP53* (n = 15), followed by mutations in *KRAS* (n = 10) and *APC* (n = 8). In ctcDNA, a total of 16 somatic hot spot mutations was detected in only 10 of the 34 patients. Median number of detected mutations was 0 per patient (range, 0-3). The most frequent mutation was within *TP53* (n = 5), followed by mutations in *KRAS* and *APC* (n = 4 each). Detailed data of each patient are shown in Table 2.

Ten mutations were concordant between all three samples (tumor tissue DNA, ccfDNA, and ctcDNA). Of the 53 mutations detected in tissue DNA, 27 (50.9%) were detected in ccfDNA and 11 (20.8%) were detected in ctcDNA. Twenty mutations were specific to ccfDNA and five were specific to ctcDNA. The concordance Venn diagram of the three samples is shown in Figure 3.

FIGURE 3 A, Image of the concordance Venn diagram of mutations detected in tumor tissue DNA, circulating cell-free DNA (ccfDNA), and circulating tumor cell DNA (ctcDNA) using next-generation sequencing in cohort 1. Twenty mutations were specific to ccfDNA and five were specific to ctcDNA, not being identified in tumor tissue DNA. B, Focusing on the three major mutations in colorectal cancer (*TP53*, *KRAS*, *APC*) only, specific mutations not identified in tumor tissue DNA were detected in liquid biopsy samples

In 12 patients (35.3%), mutations not found in tumor tissue DNA were detected by liquid biopsy; nine patients (26.5%) had mutations detected by ccfDNA only and three (8.8%) by ctcDNA only.

Positive predictive value (PPV), negative predictive value (NPV), sensitivity and specificity of the three major mutations (*TP53*, *APC* and *KRAS*) between tumor tissue DNA and liquid biopsy samples (ccfDNA and ctcDNA) are shown in Table 3.

3.3 | Cohort 2

3.3.1 | Patient characteristics and collection quantity of CTC

Twenty-two patients were enrolled in cohort 2, and patient characteristics are summarized in Table 4. All patients had stage IV CRC with *RAS* mutations in their primary tumor. Thirteen patients were undergoing chemotherapy, and the remaining nine were terminal patients who had completed chemotherapy and were receiving best supportive care (BSC). Median number of collected CTC was 35 cells (range, 0-383).

3.3.2 | Detection of the RAS mutant allele by dPCR

From 22 patients, *RAS* mutant allele was detected in ccfDNA of 14 patients (63.6%) and in ctcDNA of eight patients (36.4%). In these eight patients, *RAS* mutant allele was detected in ctcDNA and was also detected in ccfDNA. VAF was higher in ccfDNA compared with ctcDNA. Detailed patient data are shown in Table 5.

From ccfDNA, patients with high levels of carcinoembryonic antigen (CEA) were significantly more likely to have *RAS* mutation alleles detected than those with lower CEA levels ($P = .046$). Patients receiving BSC showed a tendency for increased detection of the *RAS* mutant allele, but the difference was not significant ($P = .07$). Carbohydrate antigen 19-9 (CA19-9) levels and ccfDNA concentration did not correlate with *RAS* mutant allele detection ($P = .14$ and $P = .36$, respectively).

From ctcDNA, patients receiving BSC, those with high CEA levels, large numbers of CTC, and high ratio of CTC to WBC were associated with a significantly higher likelihood of detecting the *RAS* mutant allele ($P = .03$, $P = .008$, $P = .005$, and $P = .01$, respectively). CA19-9 levels and the ctcDNA concentration did not correlate with *RAS* mutant allele detection ($P = .32$ and $P = .29$, respectively, Table 6).

4 | DISCUSSION

In the present study, we showed three novel and valuable findings. First, mutations not found in primary tumors of CRC patients could be detected using liquid biopsy, and combination analysis of ccfDNA and ctcDNA increased the number of mutations detected. Second, sensitivity of detecting cancer-specific mutations was higher in ccfDNA compared with ctcDNA. Third, the multi-antibody CTC capturing method is a promising technique for detecting large numbers of CTC.

We clearly showed that mutations not found in CRC tumor tissue DNA could be detected in ccfDNA or ctcDNA, and that a combination of the two types of liquid biopsy sample allowed more precise analysis. We compared NGS data of three distinct patient-matched samples (tumor tissue DNA, ccfDNA, and ctcDNA) from 34 CRC patients, and detected 20 mutations in ccfDNA and five in ctcDNA that were not identified in tumor tissue DNA. From 12 of the 34 patients, mutations not found in tumor tissue DNA were detected by liquid biopsy: in ccfDNA from nine patients and in ctcDNA from three. These mutations were within *KRAS* (n = 3), *NRAS* (n = 1), and *BRAF* (n = 1),

TABLE 3 PPV, NPV, sensitivity and specificity of the three major mutations (*TP53*, *APC* and *KRAS*) between tumor tissue DNA and liquid biopsy samples (ccfDNA and ctcDNA) in cohort 1

TP53		
	ccfDNA mutation (+)	**ccfDNA mutation (–)**
Tissue DNA mutation (+)	8	9
Tissue DNA mutation (–)	4	13
PPV: 47.1%		NPV: 76.5%
Sensitivity: 66.7%		Specificity: 59.1%
	ctcDNA mutation (+)	**ctcDNA mutation (–)**
Tissue DNA mutation (+)	4	13
Tissue DNA mutation (–)	1	16
PPV: 23.5%		NPV: 94.1%
Sensitivity: 80.0%		Specificity: 55.2%
APC		
	ccfDNA mutation (+)	**ccfDNA mutation (–)**
Tissue DNA mutation (+)	5	7
Tissue DNA mutation (–)	3	19
PPV: 41.7%		NPV: 86.4%
Sensitivity: 62.5%		Specificity: 73.1%
	ctcDNA mutation (+)	**ctcDNA mutation (–)**
Tissue DNA mutation (+)	3	9
Tissue DNA mutation (–)	1	21
PPV: 25.0%		NPV: 95.5%
Sensitivity: 75.0%		Specificity: 70.0%
KRAS		
	ccfDNA mutation (+)	**ccfDNA mutation (–)**
Tissue DNA mutation (+)	7	5
Tissue DNA mutation (–)	3	19
PPV: 58.3%		NPV: 86.4%
Sensitivity: 70.0%		Specificity: 79.2%
	ctcDNA mutation (+)	**ctcDNA mutation (–)**
Tissue DNA mutation (+)	4	8
Tissue DNA mutation (–)	0	22
PPV: 33.3%		NPV: 100%
Sensitivity: 100%		Specificity: 73.3%

Abbreviations: ccfDNA, circulating cell-free DNA; ctcDNA, circulating tumor cell DNA; NPV, negative predictive value; PPV, positive predictive value.

and related to resistance of epidermal growth factor receptor blockade, which may contribute to chemotherapy selection. Some previous studies, including our own,[2] reported that mutations not found in primary tumors could be detected in ccfDNA of CRC patients.[18,19] However, detection of mutations in ctcDNA that were not found in primary tumors has only been reported in breast cancer patients.[20,21] Recently, cancer has been considered to be a more heterogeneous disease than previously thought,[22] and discordance between primary and metastatic tumors has been reported.[23] Thus, the detection of spatial and temporal heterogeneity using liquid biopsy will have a great impact,[24] and the present study shows the potential of analyzing both ccfDNA and ctcDNA using combination liquid biopsy.

TABLE 4 Summary of patient characteristics in cohort 2

Total	**N = 22**
Age (y)	73 (44-86)
Gender	
Male	13
Female	9
Primary tumor location	
Cecum	1
Ascending colon	0
Transverse colon	3
Descending colon	1
Sigmoid colon	10
Rectum	7
TNM stage	
Stage IV	22
Therapeutic statement	
First-line chemotherapy	8
Second-line chemotherapy	5
Best supportive care	9
CEA (ng/mL)	56.4 (2.4-12 604.1)
CA19-9 (U/mL)	44.9 (2-12 000)
ccfDNA (ng/mL)	326 (112-14 533)
Collected CTC (no. cells)	35 (0-383)
ctcDNA (ng/mL)	7360 (336-110 000)

Note: Data are shown as the median (range).
Abbreviations: CA19-9, carbohydrate antigen 19-9; ccfDNA, circulating cell-free DNA; CEA, carcinoembryonic antigen; CTC, circulating tumor cells; ctcDNA, circulating tumor cell DNA.

Sensitivity to detect cancer-specific mutation is higher in ccfDNA than in ctcDNA. In both cohorts 1 and 2 of the present study, concordance rate of ccfDNA between tumor tissue DNA was superior to that of ctcDNA. In cohort 2, the *RAS* mutant allele was detected in ccfDNA of all patients in whom the allele was detected in ctcDNA, and VAF of ccfDNA was significantly higher than that of ctcDNA. According to the results of previous studies, detection rates of *RAS* mutations using ccfDNA[25-28] are considered to be higher than that using ctcDNA.[29,30] However, the present study is the first to show the superiority of ccfDNA by direct comparison.

Sensitivity of detecting mutations is poor in patients undergoing chemotherapy. The *RAS* mutant allele was detected in ccfDNA in only six of the 13 patients receiving chemotherapy (46.2%) and in ctcDNA in only two of the 13 patients receiving chemotherapy (15.4%). The allele was detected in ccfDNA in eight of the nine patients receiving BSC (88.9%) and in ctcDNA in six of the nine patients receiving BSC (66.7%). These results indicate that

TABLE 5 Detailed data of *RAS* mutant allele detection by dPCR of each patient in cohort 2

Case	RAS mutation (COSMIC)	Therapeutic statement	CEA (ng/mL)	CA19-9 (U/mL)	No. CTC, WBC (/10 mL whole blood)	Ratio (CTC/WBC)	Sample	DNA concentration (ng/mL)	dPCR (RAS mutant allele)	VAF (%)
1	G12V (520)	BSC	119	12 000	128, 1449	0.088	ccfDNA	380	+	20.69
							ctcDNA	2180	+	1.23
2	G12D (521)	First-line	15.2	31.5	36, 927	0.039	ccfDNA	153	−	0
							ctcDNA	15 900	−	0
3	G13C (527)	First-line	5.0	39.5	0, 1341	0	ccfDNA	222	−	0
							ctcDNA	12 420	−	0
4	G12D (521)	BSC	12 604.1	2.0	117, 2007	0.058	ccfDNA	2813	+	32.05
							ctcDNA	4720	+	0.63
5	G13D (532)	Second-line	17.1	458.3	45, 1494	0.030	ccfDNA	727	+	17.24
							ctcDNA	3540	+	0.19
6	G12D (521)	First-line	2.4	2.4	8, 756	0.011	ccfDNA	390	−	0
							ctcDNA	2940	−	0
7	G13D (532)	First-line	2.7	6.9	21, 1098	0.019	ccfDNA	483	+	1.60
							ctcDNA	14 660	−	0
8	G13D (532)	First-line	6.6	47.2	18, 1260	0.014	ccfDNA	1240	+	1.66
							ctcDNA	8660	−	0
9	G13D (532)	Second-line	68.9	45.4	6, 792	0.008	ccfDNA	186	−	0
							ctcDNA	67 000	−	0
10	G12D/G13D (521)/(532)	BSC	83.6	2292.3	36, 2799	0.013	ccfDNA	595	−/−	0/0
							ctcDNA	1670	−/−	0/0
11	G12D (521)	Second-line	5.0	2.0	21, 891	0.024	ccfDNA	433	−	0
							ctcDNA	930	−	0
12	G12V (520)	BSC	12.2	12 000	35, 1521	0.023	ccfDNA	240	+	2.35
							ctcDNA	782	−	0
13	G12C (516)	Second-line	10.9	9.8	0, 477	0	ccfDNA	221	−	0
							ctcDNA	336	−	0
14	G12D (521)	BSC	737	652.5	28, 1296	0.027	ccfDNA	787	+	23.27
							ctcDNA	4440	−	0
15	G12V (520)	BSC	2325.9	360.9	27, 1422	0.019	ccfDNA	161	+	22.79
							ctcDNA	110 000	+	0.79

(Continues)

TABLE 5 (Continued)

Case	RAS mutation (COSMIC)	Therapeutic statement	CEA (ng/mL)	CA19-9 (U/mL)	No. CTC, WBC (/10 mL whole blood)	Ratio (CTC/WBC)	Sample	DNA concentration (ng/mL)	dPCR (RAS mutant allele)	VAF (%)
16	G12D (521)	BSC	191	48.6	88, 2736	0.032	ccfDNA	14 533	+	4.03
							ctcDNA	1946	–	0
17	G12D (521)	BSC	43.9	44.4	67, 2259	0.030	ccfDNA	153	+	17.72
							ctcDNA	42 000	+	0.10
18	G12V (520)	BSC	4840.7	12 000	201, 2574	0.078	ccfDNA	378	+	44.23
							ctcDNA	6060	+	1.11
19	G12D (521)	Second-line	95.6	12.6	55, 846	0.065	ccfDNA	221	–	0
							ctcDNA	12 500	–	0
20	G12V (520)	BSC	841.1	2.0	383, 3816	0.100	ccfDNA	274	+	21.882
							ctcDNA	55 800	+	5.468
21	A146V (19 900)	First-line	26.4	11.4	20, 1197	0.017	ccfDNA	112	+	1.348
							ctcDNA	47 600	–	0
22	G12V (520)	First-line	3414	4039.2	70, 1242	0.056	ccfDNA	198	+	15.743
							ctcDNA	79 800	+	0.388

Note: Using dPCR, *RAS* mutant allele was detected from 14 patients (63.6%) in ccfDNA and from eight patients (36.4%) in ctcDNA. In all eight patients *RAS* mutant allele was detected in ctcDNA; it was also detected in ccfDNA, and VAF was higher in ccfDNA compared with ctcDNA.

Abbreviations: CA19-9, carbohydrate antigen 19-9; ccfDNA, circulating cell-free DNA; CEA, carcinoembryonic antigen; COSMIC, catalogue of somatic mutations in cancer, CTC, circulating tumor cells; ctcDNA, circulating tumor cell DNA; dPCR, digital PCR; VAF, variant allele frequency, WBC, white blood cells.

TABLE 6 Analysis of *RAS* mutant allele detection by dPCR in cohort 2

RAS mutant allele detection	ccfDNA			ctcDNA		
	+ (N = 14)	− (N = 8)	*P*-value	+ (N = 8)	− (N = 14)	*P*-value
Therapeutic statement						
Under treatment (N = 13)	6	7	.07	2	11	.03
BSC (N = 9)	8	1		6	3	
CEA (ng/mL)	155.0 (2.7-12 604.1)	13.1 (2.4-95.6)	.046	1583.5 (17.1-12 604.1)	13.7 (2.4-737)	.008
CA19-9 (U/mL)	204.8 (2-12 000)	31.5 (2-2292.3)	.14	409.6 (2-12 000)	35.5 (2-12 000)	.32
DNA concentration (ng/mL)	379 (112-14 533)	222 (153-595)	.36	5390 (2180-110 000)	3690 (336-67 000)	.29
No. CTC				94 (27-383)	21 (0-88)	.005
Ratio (CTC/WBC)				0.057 (0.019-0.100)	0.018 (0-0.065)	.01

Note: Data are shown as the median (range).
From ccfDNA, *RAS* mutant allele was detected in 14 patients (63.6%) using dPCR. Patients with high levels of carcinoembryonic antigen (CEA) were significantly more likely to have *RAS* mutant alleles detected than those with lower CEA levels ($P = .046$).
From ctcDNA, *RAS* mutant allele was detected in eight patients (36.4%) using dPCR. Patients receiving BSC, those with high CEA levels, large numbers of CTC, and high ratio of CTC to WBC were associated with a significantly higher likelihood of detecting the *RAS* mutant allele ($P = .03$, $P = .008$, $P = .005$. $P = .01$, respectively).
Abbreviations: BSC, best supportive care; CA19-9, carbohydrate antigen 19-9; ccfDNA, circulating cell-free DNA; CTC, circulating tumor cells; ctcDNA, circulating tumor cell DNA; dPCR, digital PCR; WBC, white blood cells.

genetic analysis from liquid biopsy before starting chemotherapy is desirable.

The multi-antibody CTC capturing method can increase the detection rate and yield of CTC. We obtained a recovery rate of 85% using the cell line, with median numbers of 31 CTC collected in cohort 1 and 35 in cohort 2. Currently, the CellSearch System, which uses anti-EpCAM, is the only platform approved by the FDA for monitoring patients with metastatic breast cancer, prostate cancer, and CRC.[31] However, the CTC detection rate and counts for this system are typically low. For example, in 413 metastatic CRC patients and 239 preoperative non-metastatic CRC patients, median CTC per 7.5 mL peripheral blood was 0.[13,32] Additionally, Mostert et al[30] reported that the *RAS* mutation was detected in ctcDNA in only one of nine patients with the mutation in their primary tumor using CellSearch, and that the median CTC count was 1. We detected *RAS* mutation in eight of 22 patients. This may be because the CellSearch System fails to capture CTC involved in EMT because EpCAM is downregulated in tumor cells undergoing EMT.[33] Moreover, the cell recovery rate was significantly higher using multi-antibodies compared with EpCAM antibody alone in 10 different breast cancer cell lines and 32 stage IV breast cancer patients.[20] A multi-antibody method based on various antibodies in addition to anti-EpCAM has been reported,[34] but the best combination of antibodies should be investigated in further studies.

We confirmed that cancer-specific mutations can be identified from ctcDNA, which is important because CTC detection by imaging alone can lead to false-positives. Epithelial cells are reported to be present in the blood of 3.1% of healthy individuals with no previous history of cancer,[35] as well as in patients with disorders such as inflammatory bowel disease.[36] In the present study, cancer-specific mutations were not detected in ctcDNA in some patients, indicating that false-positive cases might be included. However, in cohort 2, sensitivity of *RAS* mutation detection in patients with higher numbers of CTC was greater than in those with fewer CTC. Thus, we believe in the reliability of our imaging and counting criteria. Considering the accuracy of genetic testing using ctcDNA in this study, further development of CTC recovery systems is expected.

The present study had several limitations. First, it included only a small number of patients recruited from a single institution. Second, we evaluated only 50 oncogenes and tumor suppressor genes included in the Ion AmpliSeq Cancer Hotspot Panel v2. Finally, NGS of ctcDNA remains a challenge. We did not obtain satisfactory results from the NGS of ctcDNA. A single cell contains only 6-7 pg DNA, and by increasing the yield of CTC, ctcDNA can provide further information.

In conclusion, mutations not found in CRC primary tumors were detectable in ccfDNA and ctcDNA, showing the potential of liquid biopsy samples to provide a complementary role in genetic analysis. Combination analysis of ccfDNA and ctcDNA increased the sensitivity to detect heterogeneity. However, the sensitivity of detecting cancer-specific mutations using ctcDNA is inferior compared with ccfDNA. The technique to capture CTC using multi-antibodies appears to increase the detection rate and yield of CTC, but future studies should investigate the best combination of antibodies to extract more CTC with higher specificity.

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DISCLOSURE

Authors declare no conflicts of interest for this article.

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