Clinical impact of circulating tumor DNA analysis for monitoring treatment response in colorectal cancer patients

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Directed by Professor JONG RAK CHOI

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ABSTRACT

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Colorectal cancer (CRC) patients receiving adjuvant therapy and undergoing surgery require non-invasive and sensitive biomarkers for monitoring treatment outcomes. Currently, conventional tumor biomarkers are widely used as carcinoembryonic antigen (CEA). However, this marker has several limitations including suboptimal sensitivity and specificity. Circulating tumor DNA (ctDNA) could be a useful biomarker for monitoring patients with CRC. This study prospectively enrolled 105 CRC patients and collected 263 plasma samples with variety of clinical features. We designed a customized panel targeting

14 genes related to CRC for monitoring treatment responses in CRC patients. NGS of ctDNA was performed average depth of 30,000 x, and the sequencing results were assessed to examine potential correlations with clinicopathologic characteristics and clinical outcomes. The average variant allele frequency (VAF) was calculated for tier 1/2 mutations, and significantly associated with tumor characteristics ($P < 0.01$). Analysis of ctDNA identified a total of 140 alterations in 56.2 % of the patients (59/105). The average number of ctDNA somatic mutations per sample was 1.35. The results showed that *APC* (36.0 %), *TP53* (35.0 %), and *KRAS* (23.0 %) were the most frequently mutated genes. The presence of *KRAS* mutations in 39 patients, whose tissue biopsies were available, was compared between the tissue and ctDNA samples. Out of 39 patients, 30 exhibited consistent results, In the cases in which ctDNA mutations did not match those detected in tissues samples, the differences were associated with the patient's metastasis status and tumor stage. Moreover, through longitudinal monitoring, ctDNA was sensitively detected and correlated with either metastasis and recurrence or with CEA levels, a conventional diagnostic marker. An absence of ctDNA mutations at the first clinical follow-up evaluation after treatment and ctDNA clearance were significantly associated with longer progression-free survival (PFS), respectively ($P < 0.0001$, $P = 0.013$).

This study suggests that ctDNA is a useful non-invasive clinical marker for monitoring disease status during follow-up in CRC patients. Serial ctDNA testing was found to improve patient management through monitoring disease progression and the mutational status of the tumor over time.

Keywords: colorectal cancer; liquid biopsy; cell-free DNA; circulating tumor DNA; monitoring; next-generation sequencing

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I. INTRODUCTION

Colorectal cancer (CRC) is the third most common type of malignant tumors and the second most lethal cancer, with an estimated number of 1.8 million new cases and about 881,000 deaths in 2018[1,2]. Incidence and mortality of CRC are ranked the third and the second, respectively, in both sexes worldwide. CRC incidence has been the highest in wealthy Western countries, but it has been drastically increasing with the economic growth in many other parts of the world. Given this trend, CRC is estimated to increase by up to 60 % by 2030[3,4]. According to the 2018 annual report on cancer statistics in Korea, the 5-year survival rate for CRC, which progressively improved from 1993–1995 to 2011–2015, was 21.5 %, but in the case of CRC with metastasis, it falls to 12 %[5]. About 25 % of CRC cases are diagnosed nearly at an advanced stage with metastases. Although overall CRC mortality has declined, CRC is still an aggressive disease with a mortality rate of approximately 40 %[6,7]. CRC patients diagnosed at advanced stages have worse prognoses than patients with other types of cancers diagnosed at earlier or intermediate stages[8,9].

CRC treatment methods include surgery and adjuvant therapy, and, in some cases, chemotherapy or radiation therapy is applied as adjuvant therapy before and after surgery[10]. Recently, various molecular targeting agents for CRC were approved by the United States Food and Drug Administration (FDA), such as anti-EGFR and anti-VEGF/VEGFR agents[11]. Despite the developments in surgery techniques, treatment regimens, and screening implementations for CRC, most patients with adjuvant therapy or surgery have residual disease[12,13]. Also, all patients face the possibility of relapse. Cancer stage itself is not sufficient to predict the effect of adjuvant therapy[9,14]. Determining patients with potential recurrence and accordingly providing an accurate treatment is crucial. Therefore, reliable biomarkers are needed for earlier detection and precise monitoring of treatment outcomes to improve the survival and quality of life in CRC patients[15].

The diagnostic methods for CRC are categorized into three groups: biopsy-based, imaging-based, and blood-based assays. The biopsy-based assay comprises a colonoscopy followed by a histopathological analysis of tumor tissue and serum analysis. Imaging-based assays utilize X-rays, computed tomography, and magnetic resonance imaging[16]. Colonoscopy biopsies are considered as the golden standard for CRC diagnosis but have several limitations. Tissue biopsy is an invasive surgical procedure that is time-consuming, causes discomfort to the patients, and may lead to a failure in detecting critical mutations due to tumor heterogeneity[17,18]. According to a previous study, tumor samples from different regions of the primary site and metastatic sites exhibit heterogeneous characteristics[19]. Since tumors have heterogeneous characteristics, multiple or serial sample collection is required for the monitoring of cancer progression[20]. These factors may negatively affect the treatment progress, underlining the need for the development of new, non-invasive methods with high sensitivity and specificity for the monitoring and early

detection of CRC[21].

As mentioned, the imaging-based assays and serum protein marker analysis can be helpful in monitoring CRC patients[22]. However, imaging-based methods have limitations, including exposure to radiation and failure to detect a potential relapse[23]. Carcinoembryonic antigens (CEAs) are widely used as conventional serum-based CRC markers. CEA detection is less invasive, but it has low sensitivity and specificity[24,25]. Prior studies reported that CEA detection assays have a sensitivity of 35−50 % and a specificity of 62.6–93.3 % for CRC analysis[26-28]. Therefore, a better biomarker with high sensitivity and specificity is needed for monitoring CRC recurrence or treatment response in patients undergoing surgical or systemic therapy.

Liquid biopsy is a feasible and non-invasive method for the analysis of biological fluids collected from cancer patients, including tumor-derived biomarkers such as DNA, RNA, miRNA, and proteins[29,30]. Circulating-tumor DNA (ctDNA) is a liquid biopsy biomarker that refers to tumor-derived fragmented DNA from dead tumor cells through necrosis and apoptosis and can be detected in the plasma as cell-free DNA (cfDNA) in cancer patients[31,32]. It contains specific information on the genetic alterations present in the tumor. Moreover, this biomarker can address the tumor heterogeneity problem by identifying the markers that indicate intratumor or intercellular heterogeneity[33,34]. Therefore, ctDNA has emerged as a useful diagnostic and prognostic biomarker for several cancers with various applications in the diagnosis and monitoring of cancer[35].

Next-generation sequencing (NGS) technologies have facilitated acquiring massive genome data at a reasonable cost in a short time and allows the simultaneous identification of genomic alterations in a single assay[36,37]. Detection of ctDNA requires high sensitivity and specialized assays since ctDNA constitutes less than 1 % of the total cfDNA, and the

cfDNA concentration is usually 5–10 ng/mL in the plasma of healthy individuals[38,39]. Recently, NGS technology has been available for clinical trials and allows sensitive ctDNA analysis for monitoring of tumor-associated genetic aberrations[36,37,40]. Bettegowda *et al.* demonstrated ctDNA level detection in the early stages of CRC[41]. Another study proposed the identification of somatic alterations in ctDNA. Several studies have tried the implementing clinical trials involving ctDNA using the NGS platform[42-44].

In this study, we developed a custom panel targeting CRC-related genes and tested the analytical and clinical performance of the NGS assay for early detection and monitoring of treatment response in CRC patients. We performed a comprehensive analysis of targetable driver and resistance mutations and copy number alterations (CNA) in 263 plasma samples obtained from 105 CRC patients. Furthermore, our analysis showed that ctDNA was detected earlier compared with traditional serum protein biomarkers and could predict progression-free survival (PFS) during CRC progression. We found that ctDNA could indeed serve as a promising biomarker for monitoring clinical outcomes during the management of CRC patients (Figure 1).

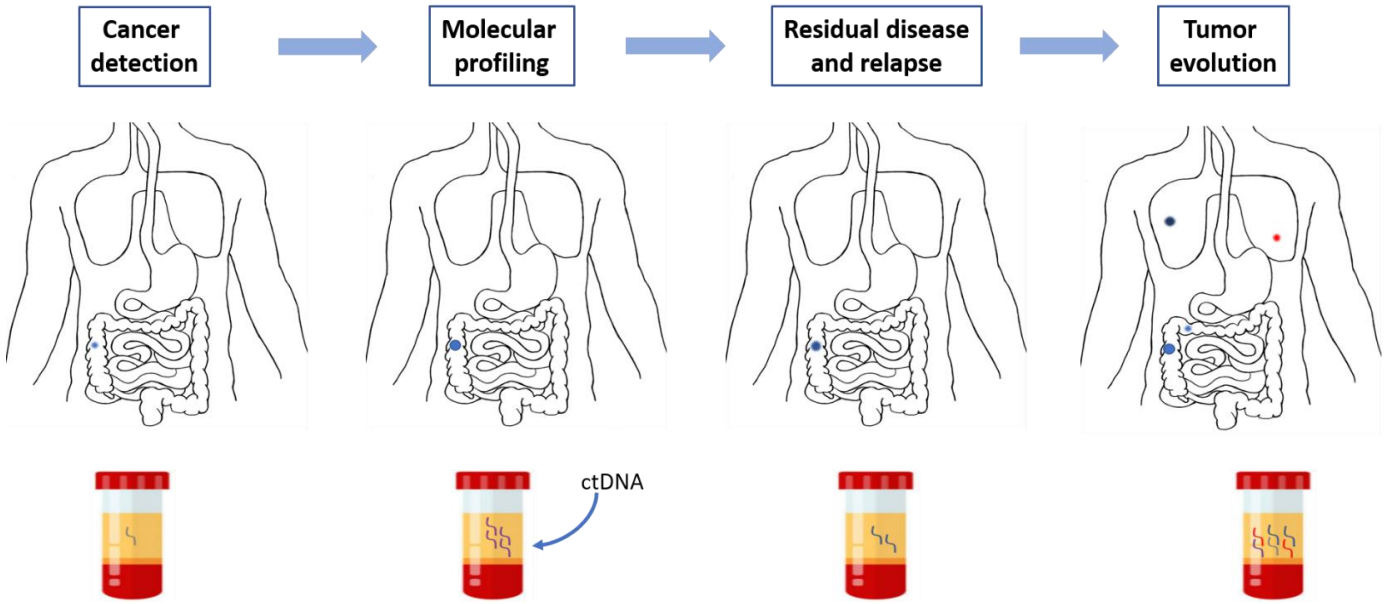

Figure 1. The levels and molecular composition of ctDNA reflect tumor changes.

II. MATERIALS AND METHODS

1. Patient enrollment and stduy design

Patients with CRC at stages I–IV were recruited for this single-institution, prospective, observational study from November 2019 to November 2021 at the Yonsei University Severance Hospital (Seoul, Korea). All patients provided written informed consent. The patients who received surgical resection or other adjuvant chemotherapies after being diagnosed with CRC during follow-up were tested at pre-treatment, post-treatment, and multiple follow-up examinations. Figure 2 shows a schematic of the study workflow. Clinical tumor staging was determined based on the American Joint Committee on Cancer (AJCC)[45] tumor, node, and metastases (TNM) classification, and treatment responses were evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 as partial response (PR), stable disease (SD), or progressive disease (PD)[46]. This study was approved by the Yonsei University Severance Hospital Institutional Review Board (IRB No.4-2019-0811).

2. Sample collection and cfDNA extraction from plasma

Whole blood samples (8–10 mL) were collected into DxTubeTM (Dxome, Seongnam, Gyeonggi-do, Korea) to prevent leukocyte lysis and cfDNA degradation. Plasma was separated by centrifugation at 1,900 xg twice. The pure plasma samples were labeled with patient IDs and the dates of sample collection and stored at −80 °C until cfDNA extraction. cfDNA was extracted from 3–4 mL of plasma using the Magnetic Circulating DNA Maxi Reagent (Dxome) according to the manufacturer's instructions. The Cell-free DNA ScreenTape Analysis

(Agilent Technologies, Santa Clara, CA, USA) with the 4150 Tapestation system (Agilent) was performed to measure fragment distribution (140–170 base pairs) and the amount of acquired cfDNA.

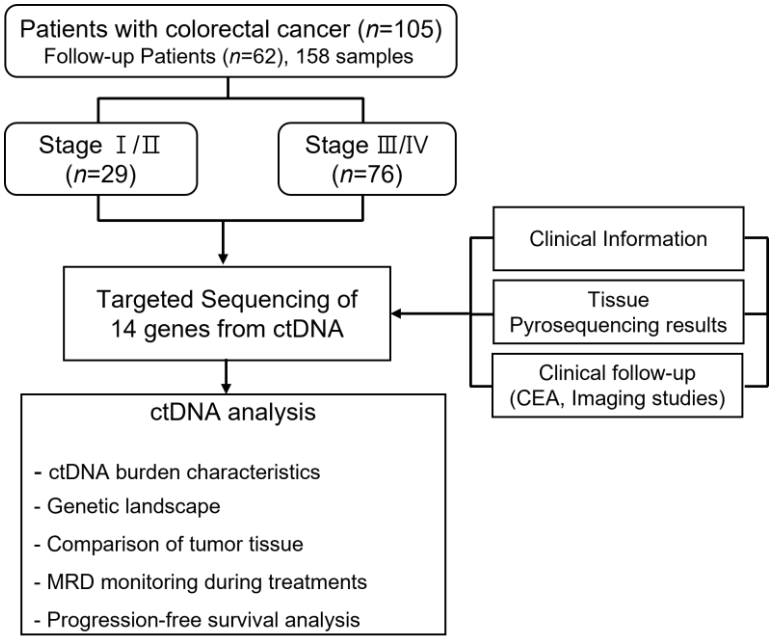

Figure 2. Study workflow for exploring the clinical impact of ctDNA in CRC.

A total of 263 plasma samples were collected from 105 patients diagnosed with CRC. Of the 105 patients, 29 patients were in the early-stage group representing the stages I and II, and 76 patients were in the advanced stage group representing stages III and IV. All patients underwent various chemical and surgical treatments. The specimens were analyzed by targeted sequencing and integrated analysis with clinical features.

3. Customized panel design

A total of 14 genes including those frequently mutated in CRC were selected for the NGS panel. All coding exons were included, and particular intronic regions with previously reported pathogenic mutations were retrieved from the ClinVar database (National Library of Medicine, Bethesda, MD, USA), OncoKB database (Memorial Sloan Kettering Cancer Center, NY, USA), and cBioPortal for Cancer Genomics database (Memorial Sloan Kettering Cancer Center) and were added to the list of target regions. The pathways associated with CRC include WNT/β-catenin signaling, cell cycle control, RAS/MAPK signaling, NOTCH signaling, mTOR signaling, TGF-β signaling, HER2 signaling, cAMP signaling, and AKT signaling pathways. Finally, the following 14 genes were selected: *APC*, *TP53*, *KRAS*, *PIK3CA*, *SMAD4*, *FBXW7*, *BRAF*, *CTNNB1*, *ERBB2*, *NRAS*, *EGFR*, *GNAS*, *AKT1*, and *MAP2K1*.

Double-stranded DNA probes, approximately 120 base pairs (bp) in length, were designed for 2x tiling across target genes and synthesized (Dxome). In total, the size of the capture region was estimated to be 0.043 million bases (Mb) (Figure 3).

Table 1. List of selected genes for customized panel.

Gene	Involved Pathway	Location	Chromosome Position (hg19)
APC	WNT/β-catenin signaling	5q22.2	chr5: 112,041,701-112,183,436
TP53	Cell cycle control	17p13.1	chr17: 75,702,197,592,368
KRAS	RAS/MAPK signaling	12p12.1	chr12: 25,356,222-25,405,365
PIK3CA	PIK3K/mTOR signaling	3q26.3	chr3: 178,864,810-178,959,381
SMAD4	TGF-β/SMAD4 signaling	18p21.1	chr18: 48,555,082-486,129,11
FBXW7	NOTCH signaling	4q31.3	chr4: 153,240,909-153,457,893
BRAF	MAPK signaling	7q34	chr7: 140,432,312-140,626,064
CTNNB1	WNT/β-catenin signaling	3p22.1	chr3: 41,239,441-41,283,439
ERBB2	HER2 signaling	17q12	chr17: 37,842,836-37,886,415
NRAS	RAS/MAPK signaling	1p13.2	chr1: 115,245,584-115,261,015
EGFR	EGFR/MAPK signaling pathway	7p11	chr7: 55,085,224-55,276,531
GNAS	cAMP signaling	20q13	chr20: 57,413,272-57,487,751
AKT1	PI3K/AKT signaling pathway	14q32.33	chr14: 105,234,186-105,263,580
MAP2K1	MAPK signaling	15q22.31	chr15: 66,677,710-66,785,382

4. Assay performance evaluation with standard materials

Limit of detection (LOD) was evaluated using Seraseq® ctDNA Mutation Mix v2 (SeraCare Life Sciences, Milford, MA, USA) with known SNVs and indels. To evaluate assay sensitivity and specificity, five levels of reference material (RM) with an allele frequency (AF) of 0.05–1.00 % including 22 markers (SNVs and indels) were used with three replications per single run.

5. NGS library construction and sequencing

The library of cfDNA was constructed using the Library Prep Reagent for Illumina (Dxome) from approximately 5–20 ng of cfDNA. The cfDNA library was processed for the end-repair, dA-tailing, and adapter ligation. Then, adapter-ligated products were amplified with Illumina UDI primers from the High-Fidelity PCR Master Mix and purified.

Target enrichment was performed with a custom design enrichment panel following the manufacturer's instructions (Dxome). The target-enriched DNA libraries were quantified using Qubit dsDNA HS Assay Kit (Invitrogen) and Agilent D1000 ScreenTape Assay Kit (Agilent). Finally, the libraries were pooled, and sequenced on a NovaSeq 6000 instrument (Illumina, San Diego, CA, USA), achieving approximately 150 million reads per sample. Sequencing with a 151 bp, dual-indexed, paired-end sequencing configuration was performed.

6. Data processing and variant calling (PiSeq)

Raw sequence data were demultiplexed into Fastq format using bcl2fastq conversion software (Illumina). The Fastq data were trimmed for adaptor

sequences and then aligned to hg19 using the Burrows-Wheeler Alignment tool (BWA; version 0.7.12; Wellcome Trust Sanger Institute, Cambridge, UK)[47]. and ExomeDepth[48] and a customized algorithm were used for copy number alteration (CNA) analysis. Furthermore, the PiSeq algorithm (Dxome), which improves the accuracy of molecular barcoding by calculating genome positions of the mapped read groups, was conducted to call the variants (Figure 2). The software automatically predicts preliminary classifications of somatic variants according to the Association of Molecular Pathology (AMP), American Society of Clinical Oncology (ASCO), and College of American Pathologists (CAP) Standards and Guidelines[49]. All true somatic variants were visually filtered by Integrative Genomics Viewer (IGV) to exclude false positives. Germline variants were filtered when the variant allele frequency (VAF) was maintained at 50 % from the serial sampling data.

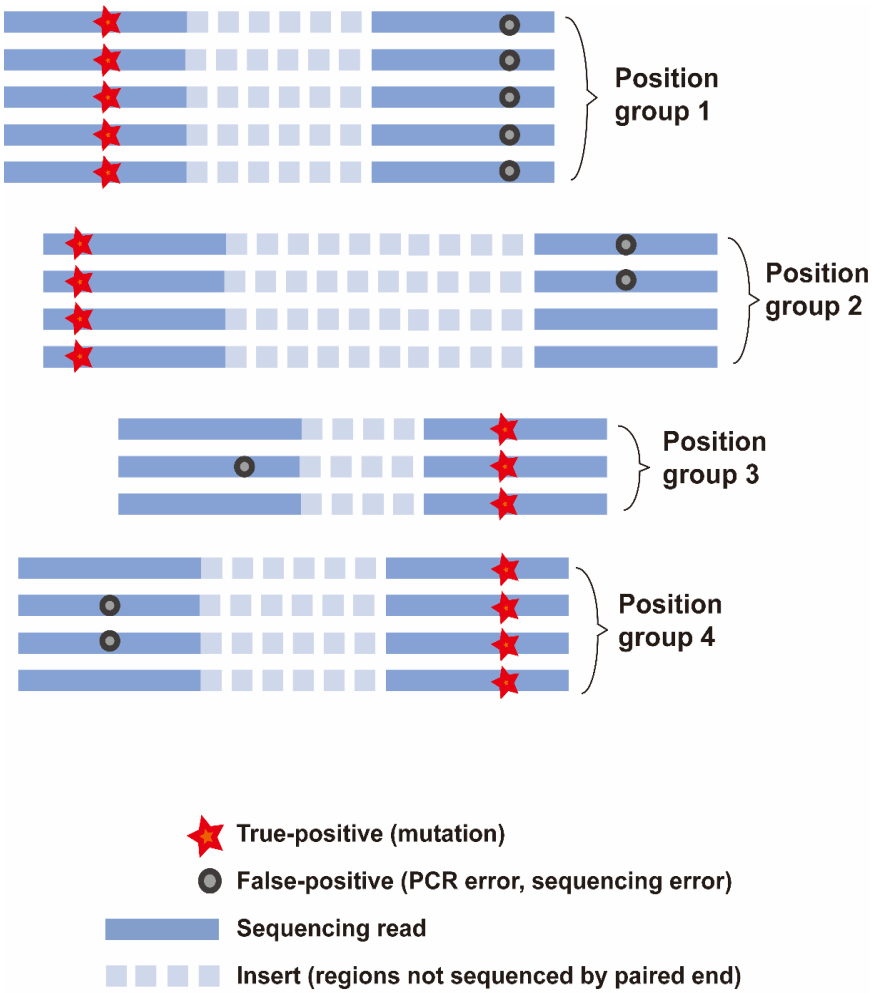

Figure 3. Schematic of analysis pipeline.

The PiSeq tool (Dxome) is an algorithm that detects mutations in ctDNA with high sensitivity that can replace the molecular barcoding system. This tool was adopted in this study.

7. Pyrosequencing of tumor tissue samples

Genomic DNA (gDNA) from FFPE tissue blocks was extracted using a Maxwell CSC DNA FFPE Kit (Promega Corporation, Madison, WI, USA) and quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Fremont, CA, USA). Therascreen *KRAS* Pyro Kit (Qiagen, Valencia, CA, USA) was used to screen for *KRAS* mutations. Pyrosequencing was performed on a T100 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) and PyroMark Q24 System (Qiagen). Pyrosequencing results were analyzed using PyroMark Q24 Software version 2.0 (Qiagen) according to the manufacturer's instructions.

8. Statistical analysis and visualization

Pearson correlation was used to analyze the correlation of VAFs between different groups. The comparison of categorical variables was performed by using Chi-square test or Fisher's exact test, whereas continuous variables were evaluated through the Wilcoxon signed rank test. PFS was calculated from the start of treatments to the date of disease progression or death from any cause. Kaplan-Meier method with the log-rank test was used for the statistical analysis of PFS data. All statistical analyses were performed on R (https://www.R-project.org/) version 4.0.3. The 'ComplexHeatmp' and 'maftools' package in R was used to analyze and visualize the mutation data, and differences with *P* values less than 0.05 were considered statistically significant.

III. RESULTS

1. Patients characteristics

Overall, 263 peripheral blood samples were obtained from 105 patients with stage I–IV CRC. Patient characteristics are summarized in Table 3. The median age of the patients were 64.0 years (range, 39–88 years), and 67.6 % were male. Of the 105 patients, 45 patients who planned to receive surgery or chemotherapy enrolled in the study at the beginning of the treatments or after a maximum of 2 weeks, whereas 60 patients received no surgical procedures or neoadjuvant therapy, including cytotoxic chemotherapies. Seventy-six patients (72.4 %) had a primary tumor site in the colon, and 29 patients (27.6 %) had one in the rectum. Also, 77 (73.3 %) patients had CRC with metastasis, and 28 (26.7 %) patients did not show metastasis.

Table 2. Clinical characteristics of CRC patients included in this study

		Total Patients (*n = 105*)
Age (y)		
	Median [range]	64.0 [39-88]
Gender (%)		
	Female	34 (32.4 %)
	Male	71 (67.6 %)
Location (%)		
	Colon	76 (72.4 %)
	Rectum	29 (27.6 %)
Differentiation (%)		
	Well/Moderately	83 (79.0 %)
	Poorly/Undifferentiated	22 (21.0 %)
Stage (%)		
	I	8 (7.6 %)
	II	21 (20.0 %)
	III	21 (20.0 %)
	IV	55 (52.4 %)
MSI status (%)		
	MSS	91 (86.7 %)

	MSI-High	6 (5.7 %)
	Unknown	8 (7.6 %)
Distant metastasis (%)		
	Yes	77 (73.3 %)
	No	28 (26.7 %)
Treatment (%)		
	Chemotherapy & Surgery	58 (55.2 %)
	Chemotherapy	26 (24.8 %)
	Surgery	20 (19.0 %)
	No treatment	1 (1.0 %)

MSS, Microsatellite stable; MSI-High, Microsatellite Instability-High

2. Sequencing metrics

To evaluate monitoring treatment response in clinical samples, we performed target sequencing of cfDNA using a customized panel. The target region size of the panels was 0.043 Mb. 4.3 Giga base pairs (Gbp) were allocated to each sample to achieve the targeted coverage of 30,000 x. The cfDNA analysis of the plasma samples provided deep sequencing with an average depth of 67,735 x (range, 4,373–396,284). To exclude false positive variants, we filtered out variants with duplex-matched molecular barcodes of 0–1. On average, 85 % of bases had more than 3 duplicates per molecular barcode. The sequencing run metrics are summarized in Table 4.

Table 3. NGS run statistics for plasma samples

	Average	SD
Total reads (bam)	35,886,360	22,658,187
Average depth (x)	59,109	41,103
Duplicates	75.2 %	13.9 %
On target	48.4 %	10.3 %
% Covered (>100× pi)	90.8	27.8
% Covered (>200× pi)	89.3	29.4
% Covered (>300× pi)	87.4	30.4
% Covered (>500× pi)	80.7	34.7
% Covered (>1000× pi)	55.8	41.6
Total number of molecular barcodes	445,579	276,962
Duplex matched molecular barcodes (%)	383,379 (82.8 %)	230,608 (15.7 %)
Median number of duplicates per molecular barcode	17	19
Percentage of duplicates per molecular barcode ≥3	83.9 %	17.8 %

3. Analytical performance validation of the customized panel

We designed a customized CRC panel to target 14 genes related to CRC. The performance of the customized CRC panel was evaluated by an average ultra-deep sequencing depth of above 30,000 x using commercial reference standard cfDNA samples with known allele frequencies (AF; 0.005–1.000 %). Overall, single nucleotide variants (SNVs) and small insertion/deletions (indels) with 0.25–1.00 % allele frequency (AF) were detected in 20 ng input cfDNA (Table 4). The five reference samples were analyzed with a mean sensitivity of 97.8 % and a specificity of 97.0 % (Table 5). Moreover, this panel's LOD was 0.26 % with 95 % CI (Figure 4).

Table 4. Performance evaluation of the customized CRC panel

Variant Type	RM type	AF of RM, (%)	Detection rate			
			1st	2nd	3rd	Average
SNVs	Seraseq® ctDNA Reference Material v2	1	100 % (14/14)	100 % (14/14)	100 % (14/14)	100.0 %
		0.5	100 % (14/14)	100 % (14/14)	100 % (14/14)	100.0 %
		0.25	100 % (14/14)	100 % (14/14)	100 % (14/14)	100.0 %
		0.125	71.4 % (10/14)	92.9 % (13/14)	85.7 % (12/14)	83.3 %
	Seraseq® ctDNA MRD Panel Mix	0.5	100 % (9/9)	100 % (9/9)	100 % (9/9)	100.0 %
		0.05	11.1 % (1/9)	0 % (0/9)	0 % (0/9)	3.7 %
Indels	Seraseq® ctDNA Reference Material v2	1	100 % (8/8)	100 % (8/8)	100 % (8/8)	100.0 %
		0.5	100 % (8/8)	100 % (8/8)	100 % (8/8)	100.0 %
		0.25	100 % (8/8)	100 % (8/8)	100 % (8/8)	100.0 %
		0.125	62.5 % (5/8)	87.5 % (7/8)	87.5 % (7/8)	79.2 %
	Seraseq® ctDNA MRD Panel Mix	0.5	80 % (4/5)	100 % (5/5)	80 % (4/5)	86.70 %
		0.05	0 % (0/5)	0 % (0/5)	0 % (0/5)	0 %

RM, Reference Material; AF, Allele Frequency; LOD, Limit of Detection; SNVs, Single Nucleotide Variants; Indels, Insertion and deletions; ctDNA, Circulating DNA

Table 5. Analytical sensitivity and specificity assessment of the customized CRC panel

AF of RM, %	Sensitivity, %	Specificity, %	PPV, %	NPV, %
1	100.0	97.0	94.3	100.0
0.5	100.0	97.0	94.3	100.0
0.25	100.0	97.0	94.3	100.0
0.125	93.1	97.0	93.1	91.4

AF, allele frequency; RM, reference material; PPV, positive predictive value; NPV, negative predictive value

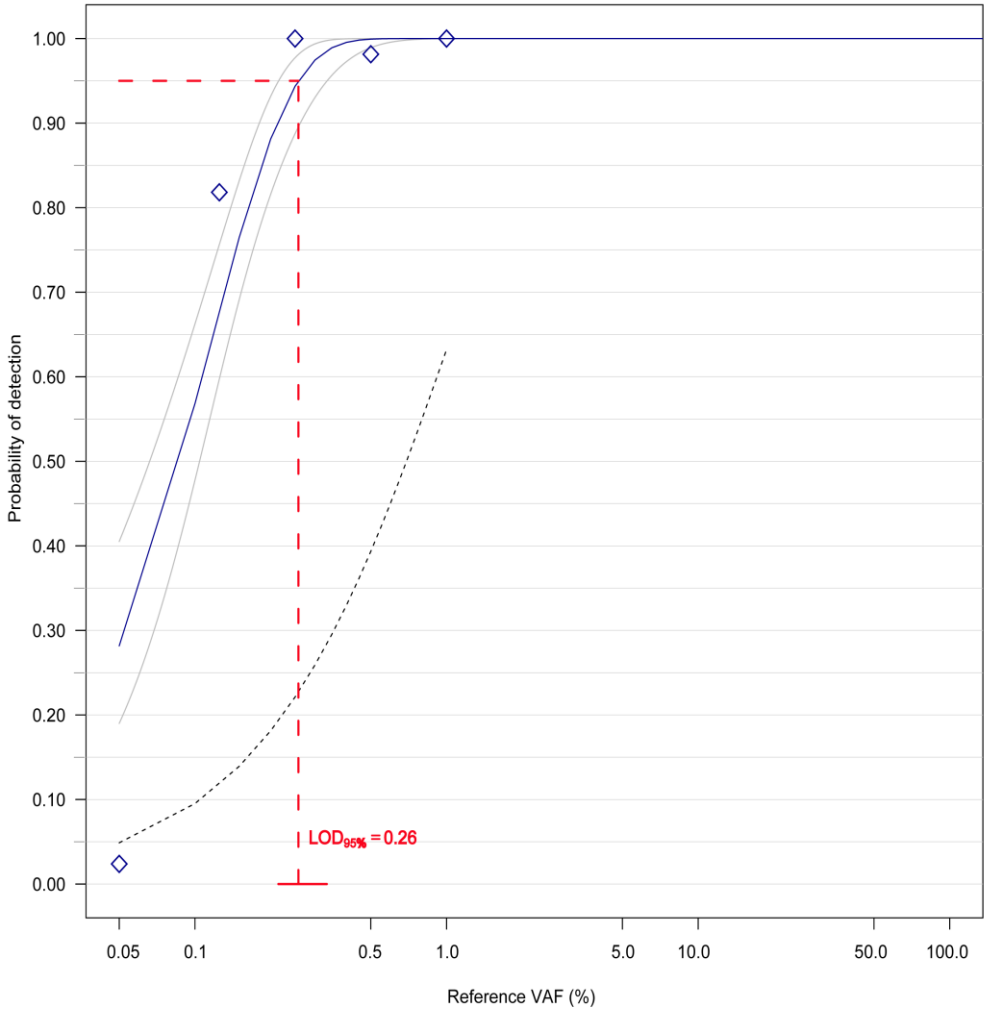

Figure 4. LOD of customized CRC panel.

Validation with standard reference samples. Six standard reference samples from Seracare, including five mutated samples and one wild-type sample, were used for assay validation. Each mutated sample had 22 known mutations covered in the panel, with AF from 0.05 % to 1 %. VAF, Variant allele variant allele frequency; AF, Allele frequency; LOD, Limit of detection

4. Association of ctDNA mutation burden with tumor characteristics

The ctDNA mutation burden was calculated using the average VAF. The average VAF was defined as the average value of the VAF of tier 1/2 mutations. The average VAF value obtained for each patient was compared with the patient's tumor characteristics including the TNM stage, tumor stage, and serum CEA level. The results showed that the average VAF increased with the TNM stage ($P = 0.11$, 0.0015, 0.00013), and the average VAF of stage III/IV was higher than that of stage I/II ($P = 0.00079$). Furthermore, the average VAF of the "> 5 ng/mL" group was significantly higher than that of the "< 5 ng/mL" CEA level group (Figure 4E; $P = 0.000053$). These results indicated that the average VAF reflected the ctDNA mutation burden.

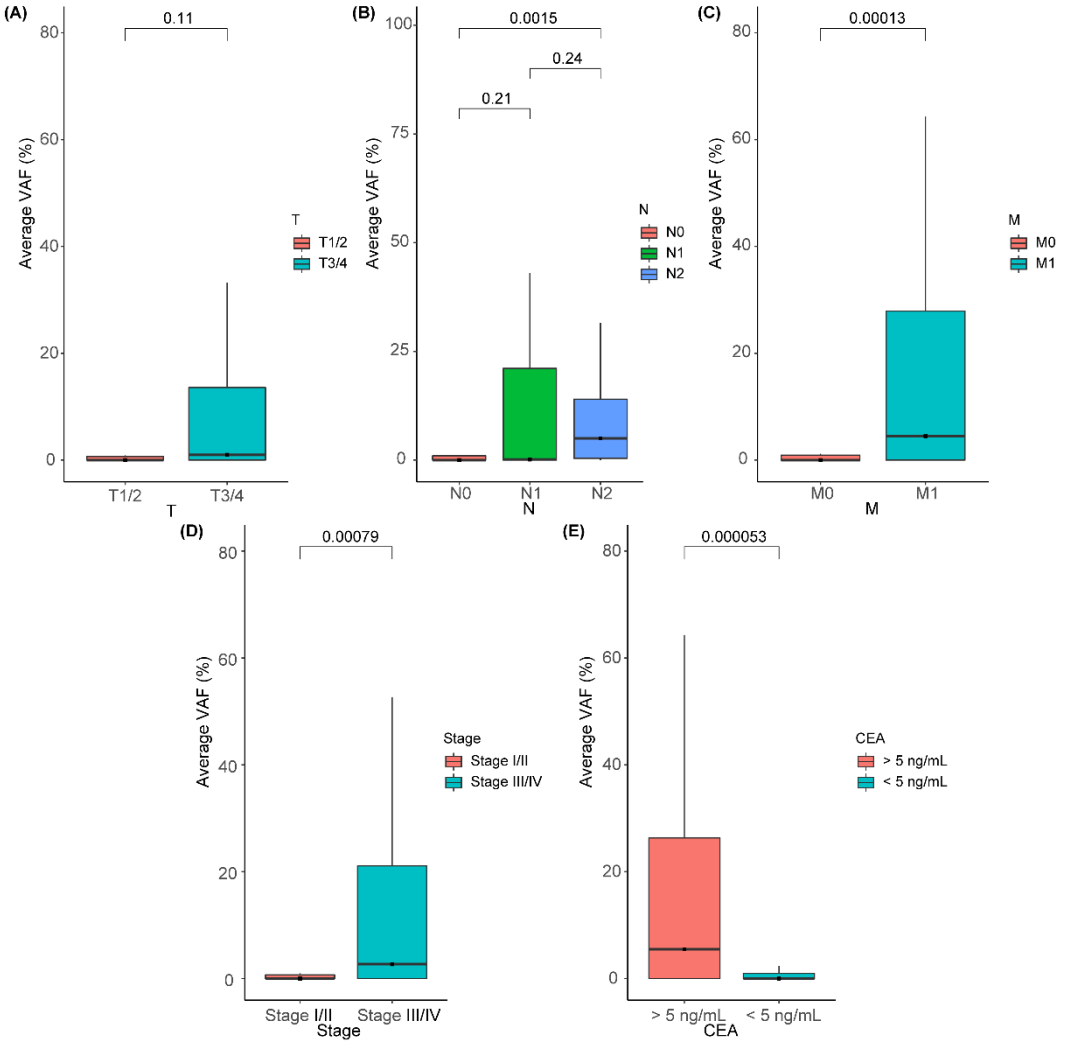

Figure 5. ctDNA burden and clinical feature analysis.

Analysis of various clinical features according to molecular tumor burden. **(A–C)** Relationship between the TNM staging and ctDNA mutation burden. **(D)** Tumor stage in relation to ctDNA mutation burden. **(E)** Association of CEA values with ctDNA mutation burden. T, Tumor; N, Node; M, Metastasis; CEA, Carcinoembryonic antigen; VAF, Variant allele frequency

5. Identification of somatic variants in ctDNA

To identify somatic mutations in CRC patients, we analyzed genetic distribution. Plasma samples were obtained from 105 patients. The overall detection rate of significant tier 1/2 variants showed 56.2 % (59/105). The variants classification of ctDNA was mostly founded SNV class. Also, variants of C>T commonly appeared, and patients with pathogenic variants have an average of 1.35 variants (Figure 5). A total of 140 variants were observed in 105 samples. The detection rate was 75.0 % (42/56) in patients at stage IV, and 67.5 % (52/77) in patients with metastasis. Significant *APC* variants (36.0 %) were the most commonly observed, followed by *TP53* (35.0 %), *KRAS* (23.0 %), *SMAD4* (6.0 %), *FBXW7* (6.0 %), *CTNNB1* (3.0 %), *ERBB2* (3.0 %), *NRAS* (2.0 %), *PIK3CA* (2.0 %), *BRAF* (2.0 %), *GNAS* (2.0 %), and *EGFR* (1.0 %) (Figure 6). All *APC* variants were truncation variants. Also, *TP53* mutations were the second most common ones after *APC*. Most of *TP53* variants were found in the P53 DNA-binding domain. The most common combination of mutated genes was *TP53* and *APC*. Mutations in *TP53* and *APC* were found in 12 patients, 7 of whom were stage III/IV with metastases. In addition, *KRAS* variants were detected alone or with *APC* or *TP53* variants. They were found much more frequently in tumor samples at stage III/IV compared with those at stage I/II. Statistical analysis confirmed that the mutations in *TP53–APC*, and *KRAS–APC* exhibited significant co-occurrences ($P < 0.05$). The somatic alterations had a variable variant allele frequency (VAF; median, 8.6 %; range, 0.2–90.0 %). Especially, genetic distribution of mutations showed characteristic patterns so that the sequential accumulation of mutations in specific genes was associated with the tumor stage.

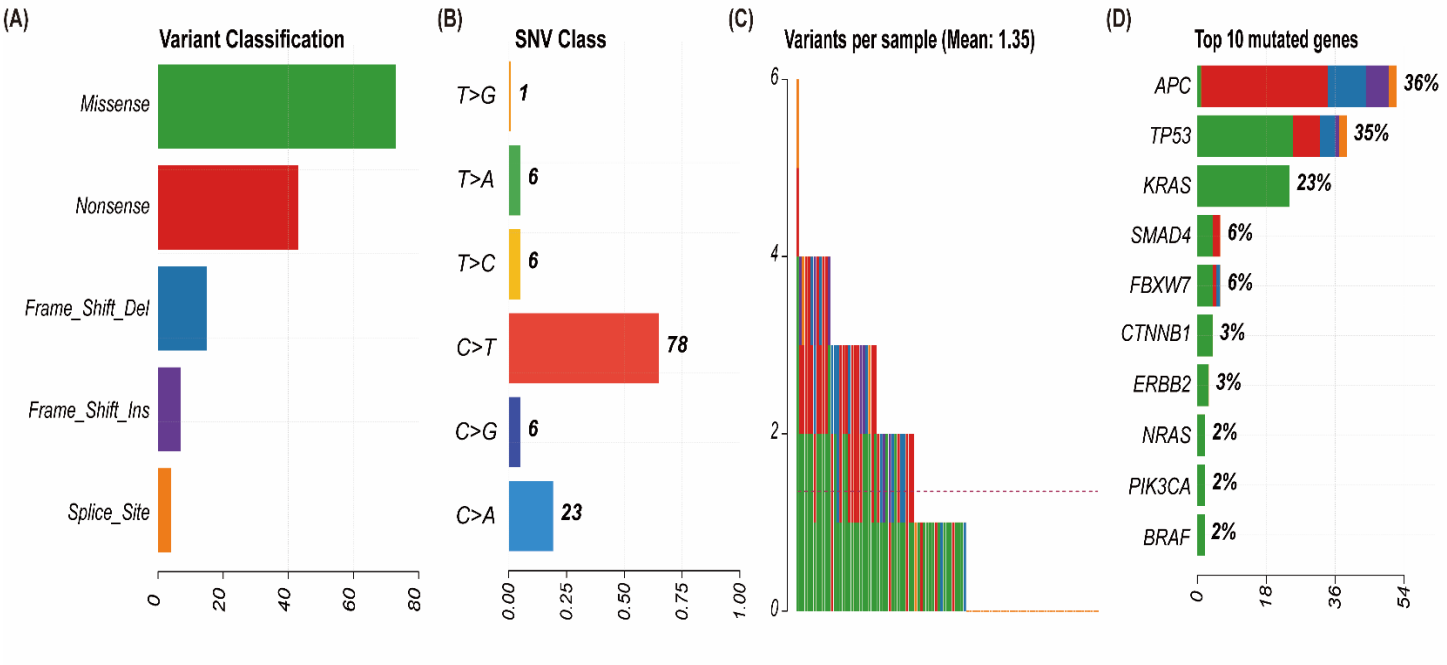

Figure 6. Variants summary.

Variants summary plot showing variants classification **(A)** SNV class distribution. **(B)** SNV type distribution. **(C)** Tier 1/2 mutations of the top 10 most mutated genes. **(D)** Variant characteristics. Del, Deletion; Ins, Insertion; SNV, Single nucleotide variant

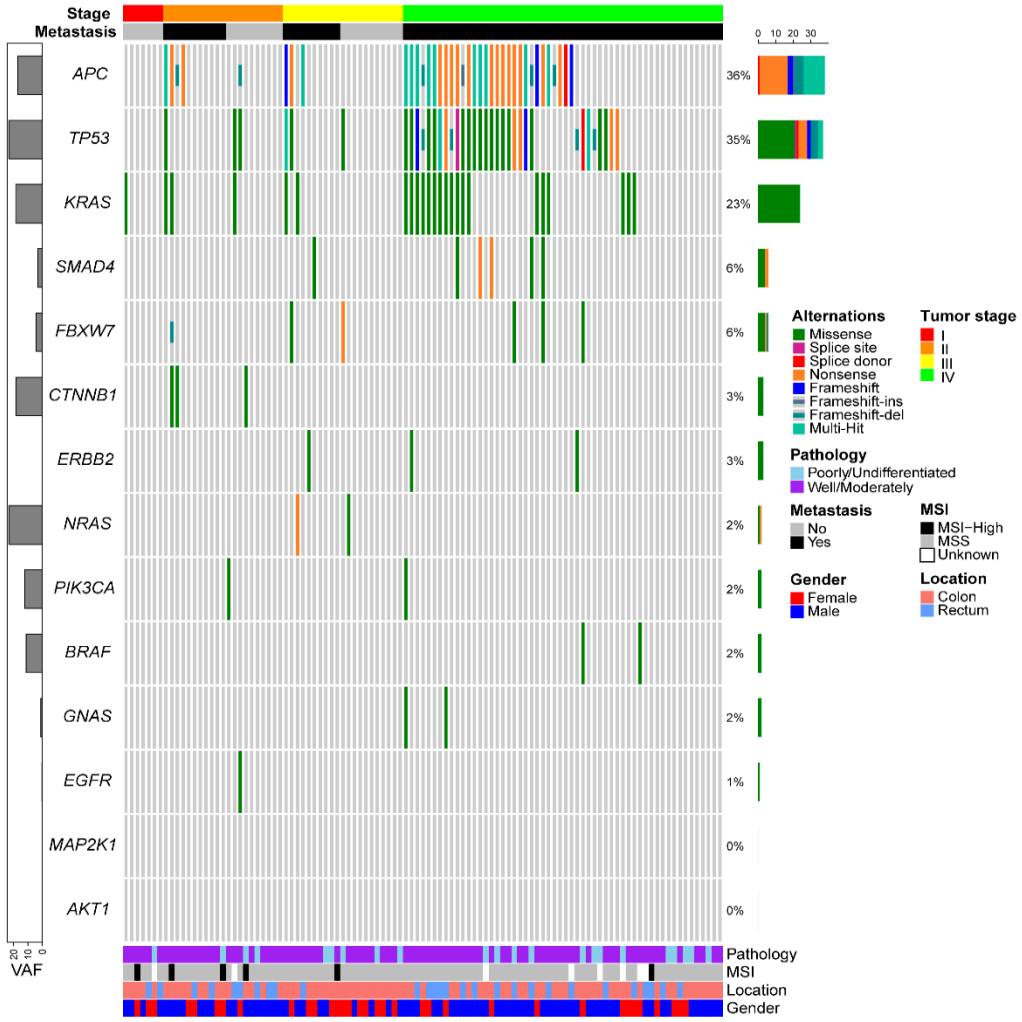

Figure 7. Spectrum of variants in ctDNA of CRC patients according to the cancer-associated clinical significance.

The landscape of variants from 105 patients with CRC. The oncoplot shows the frequency and distribution data of the tier 1/2 variants detected in ctDNA of CRC patients. The central plot shows the types of mutations in each patient sample. The upper bar graph shows the tumor stage and metastasis status of each patient, and the numbers on the right indicate the overall frequency. The lower part of the figure shows the clinical features of each patient, and the percentage number on the left indicate the VAF. MSI, Microsatellite instability; MSS, Microsatellite stable; MSI-High, Microsatellite Instability-High; VAF, Variant allele frequency

6. The comparison of *KRAS* mutation status between ctDNA and matched tumor tissues

Of the 105 patients enrolled in this study, tissue biopsy samples from 39 patients were collected before the treatment and subjected to pyrosequencing. The presence of gene hotspot variants was compared in cfDNA samples and tumor tissue samples. Since pyrosequencing of tissue samples was conducted for *KRAS* codon 12,13, and 61 regions, the comparison of genetic variation presence was performed only for *KRAS* mutations. The concordance rate, positive predictive value (PPV), and negative predictive value (NPV) were 77.8 %, 88.9 %, and 74.1 %, respectively. In one case, ctDNA from a patient with a stage II tumor of size less than 4 cm exhibited a *KRAS* mutation, but the tissue biopsy sample did not. In contrast, *KRAS* mutations were detected in tissue samples but not found in ctDNA samples collected from the same patients in 9 cases (Table 6). Nine cases were stage I/II patients with no metastasis Other than that, the specific variation detection was perfectly consistent. Especially, *KRAS* gene mutations showed significant differences between tumor tissue and ctDNA ($P = 0.002$).

Table 6. Comparison of *KRAS* mutation between tumor tissue and plasma samples

***Patients* (*n* = 39)**			**Tissue**			
			Meta		Non-meta	
			Mutant	Wild-type	Mutant	Wild-type
ctDNA	Meta	Mutant	6	0	–	
		Wild-type	1	18		
	Non-meta	Mutant	–		0	0
		Wild-type			8	6

Meta, Metastasis; Non-meta, Non-metastasis

7. Correlation with serum protein biomarkers for monitoring CRC patients

To compare the changes in serially detected ctDNA variants with CEA levels, the longitudinal ctDNA analysis was conducted by monitoring ctDNA samples from 32 patients, which were collected at more than 3 time points (range, 116–585 days). Among the 32 patients, 21 patients exhibited ctDNA variants. In four patients who underwent surgery after chemotherapy, ctDNA variants were not detected immediately after the surgery, but two of them who had metastasis showed the variants during the follow-up. To examine the clinical utility of ctDNA for the early detection of clinical outcomes during follow-up, the average VAFs in 8 patients with ctDNA variants were compared to CEA levels. There was a significant correlation between the decreases in the average VAFs in ctDNA (range, 0.0–41.0 %) and CEA levels ($P < 0.001$). Especially, the maximum change in the average VAF observed before (range, 0.0–41.0 %) and after treatment (range, 0.0–1.7 %) was found to be more significant than the corresponding change in CEA value ($P < 0.001$), confirming that the ctDNA analysis had a high sensitivity in detecting dynamic tumor changes. The changes in ctDNA VAF were reflected in the CEA levels and CT scan results (Figure 8). Seven of 18 patients (38.9 %) without baseline did not exhibit ctDNA somatic variants. Especially, among the 18 patients, 11 of 14 patients who previously received surgery were free of ctDNA variants during the follow-up. Of the 11 patients who did not exhibit ctDNA variants after the surgery, 9 patients had a sustained absence of ctDNA variants at all 3 time points. On the other hand, the two patients who eventually tested positive for ctDNA variants showed clinical characteristics of metastasis or increased tumor size (Figure 9). To establish a useful biomarker of

ctDNA in patients with any clinical characteristics during treatments, maximal change in the average VAF (range: 0.0–77.0 %) was compared with that in the CEA levels (range, 1.52–5,592 ng/mL). There were no significant differences between the changes in the average VAF and CEA levels.

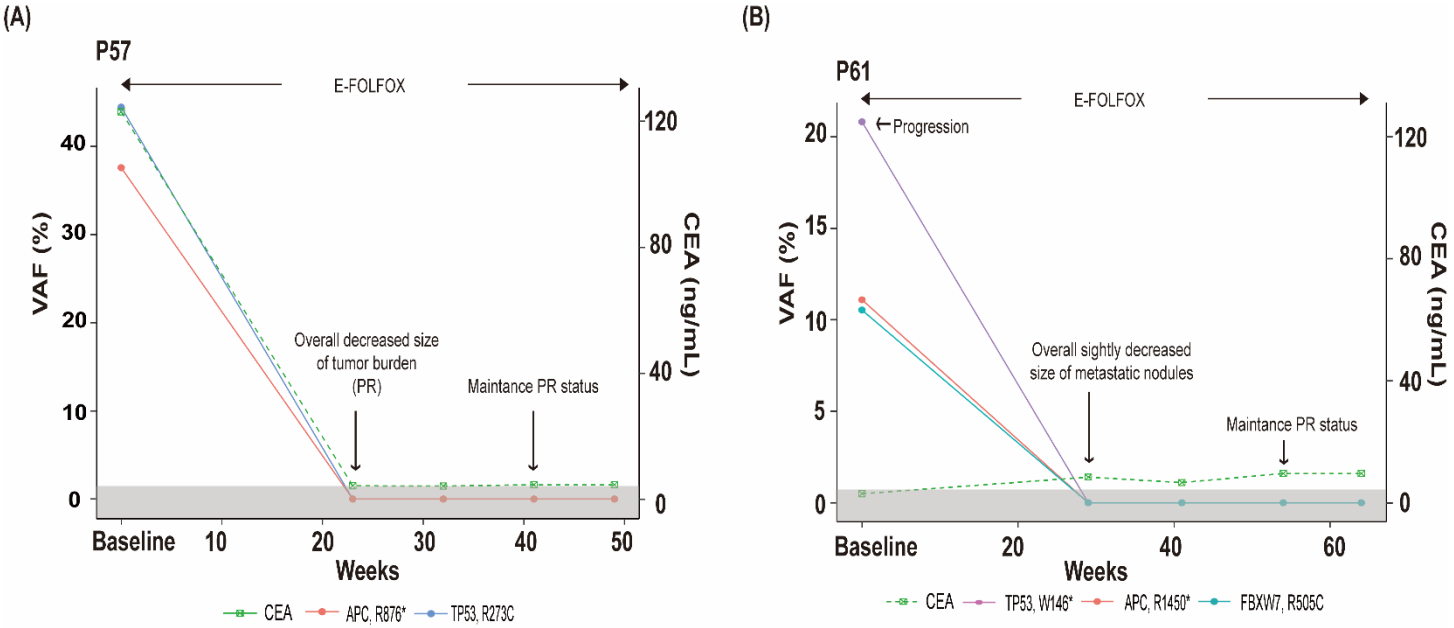

Figure 8. Correlation of ctDNA variants with serum protein biomarkers for monitoring in CRC patients relative to the baseline levels (representative cases).

Comparison of ctDNA VAFs and CEA levels. The arrows indicate changes in patients' clinical phenotypes. The grey zone indicates the region of normal CEA levels (< 5 ng/mL). VAF, Variant allele variant allele frequency; CEA, Carcinoembryonic antigen; PR, Partial response

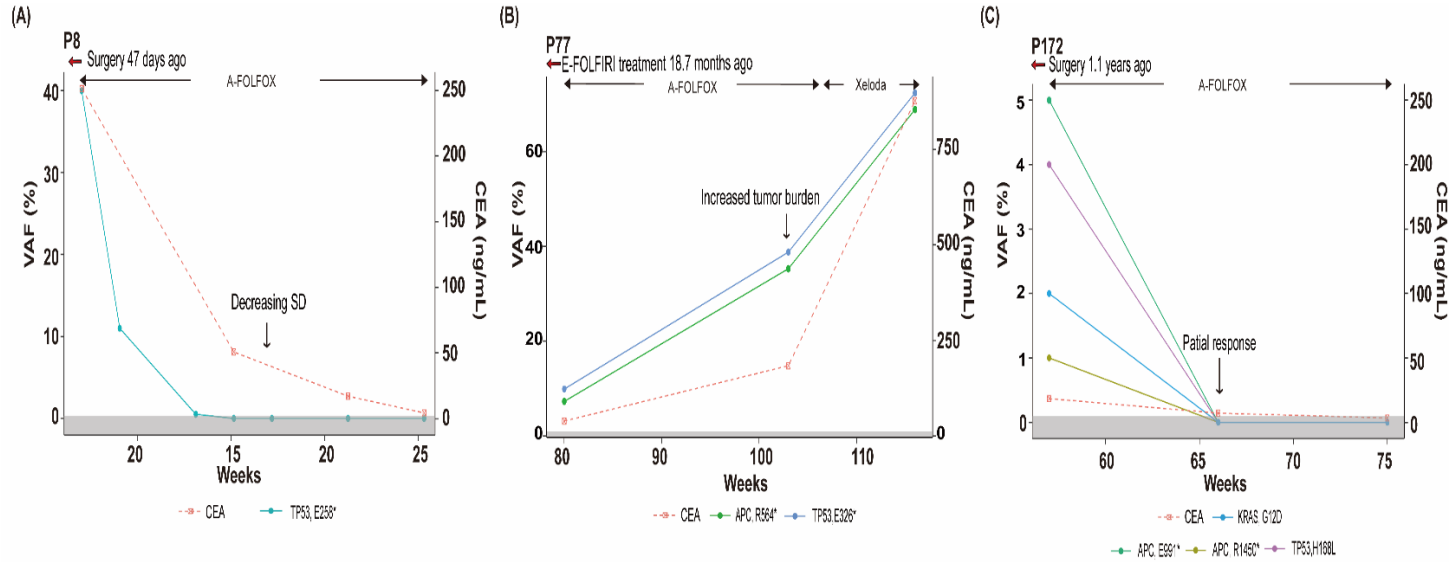

Figure 9. Correlation with serum protein biomarkers for monitoring in CRC patients without baseline data (representative cases).

Comparison of ctDNA VAFs and CEA levels. The arrows indicate changes in patients' clinical phenotypes. The grey zone indicates the region of normal CEA levels (< 5 ng/mL). VAF, Variant allele variant allele frequency; CEA, Carcinoembryonic antigen; SD, Stable disease

8. Somatic variants status and risk of recurrence

To assess the changes in ctDNA VAF for monitoring treatment response, we performed longitudinal ctDNA analysis in a total of 32 patients with and without baseline data who had various clinical features and received treatments, including surgery and cytotoxic chemotherapy. The variants with a stable VAF of 50 % were considered to represent germline mutations and filtered out. We found clinical impact in eight representative cases. In five cases with baseline data, CEA levels were found to be in the normal ranges or decreased, but ctDNA VAF increased to levels above 5 %. At this time, the patients exhibited higher levels of metastasis or increased tumor burden (Figure 10). In three patients with no pre-surgery or pre-medication baseline data, the CEA levels showed dynamic changes or remained in the normal range in 2 cases. However, the patient showed tumor progression or metastasis at the time of detection of VAF at 4 % (Figure 11A, C). In addition, at the time point when the CEA level was high but no ctDNA variant was detected, the patient was stable, or the tumor burden was reduced (Figure 11B). These results showed that tumor characteristics were reflected in changes in the average ctDNA VAF and CEA levels through the CT scan result, and the changes in VAF reflected the patients' condition with a higher sensitivity compared with the CEA levels.

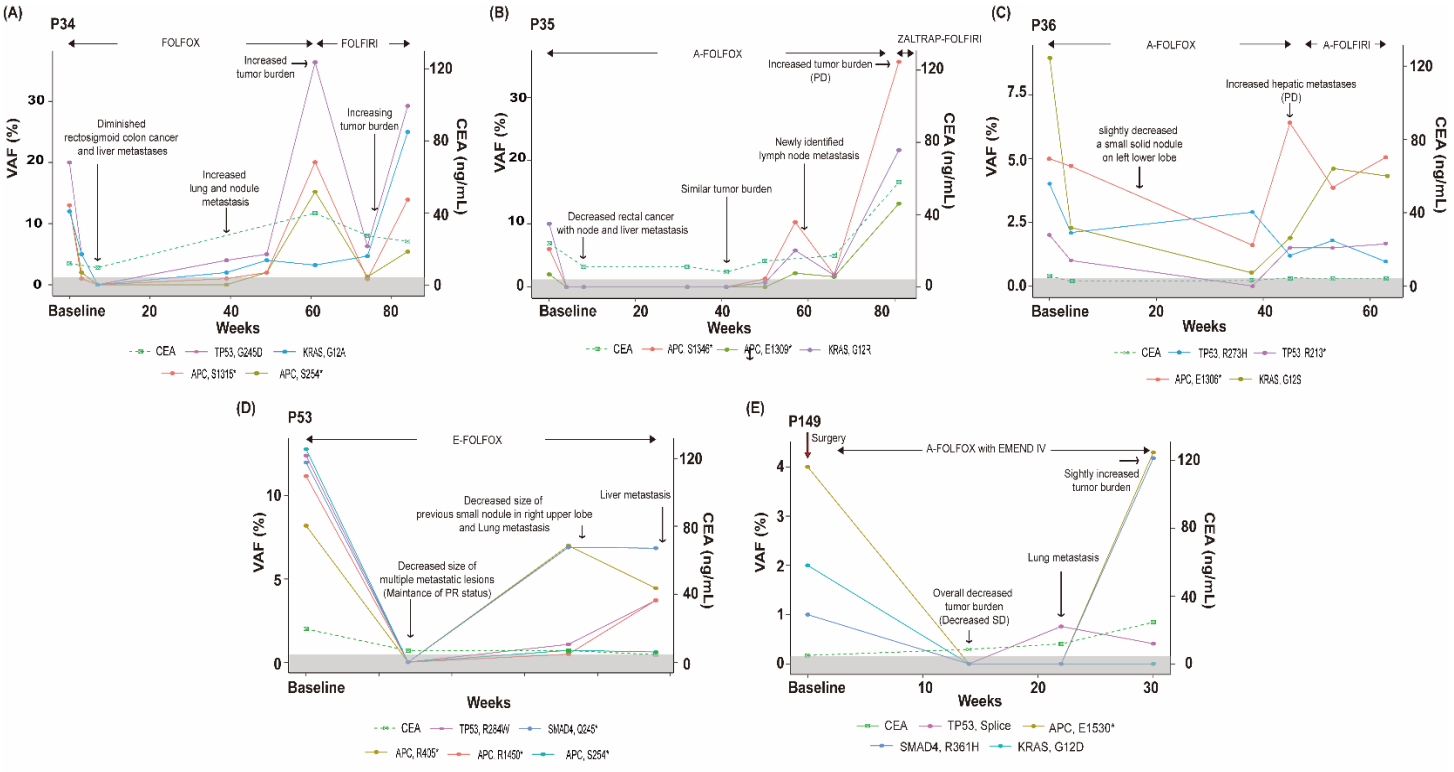

Figure 10. Residual lesion and recurrence monitoring in CRC patients with baseline data (representative cases).

Comparison of changes in ctDNA variant frequency and CEA levels. **(A)** P34. **(B)** P35. **(C)** P36. **(D)** P53. **(E)** P149. The arrows mark changes in the patients' clinical phenotype. The grey zone indicates the region of normal CEA levels (< 5 ng/mL). VAF, Variant allele variant allele frequency; CEA, Carcinoembryonic antigen; PD, Progression disease

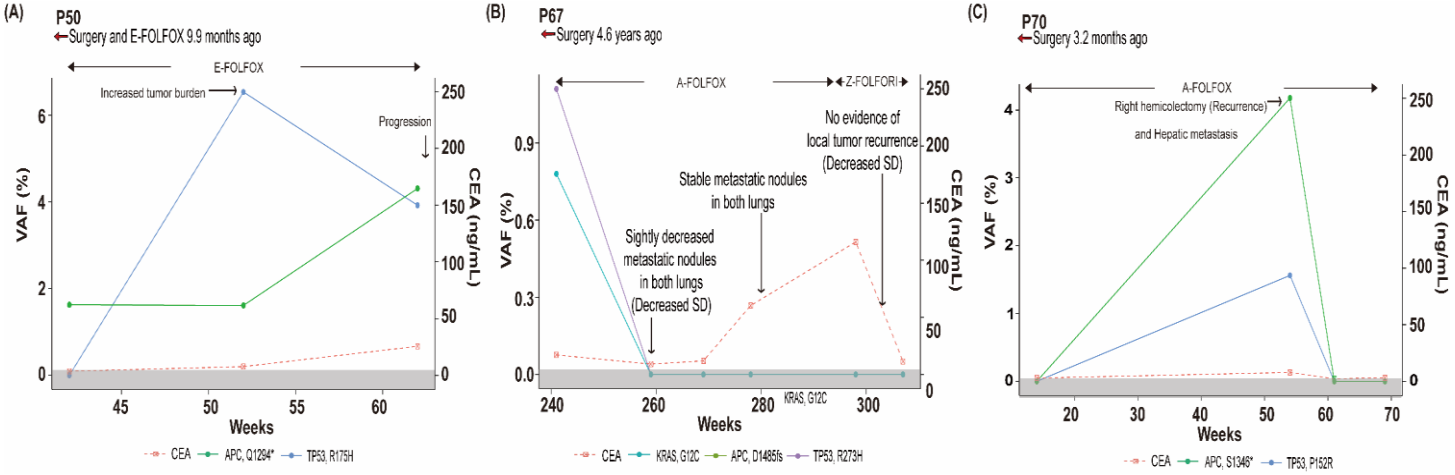

Figure 11. Residual lesion and recurrence monitoring in CRC patients without baseline data (representative cases).

Comparison of changes in ctDNA variant frequencies and CEA levels. **(A)** P50 **(B)** P67 **(C)** P70. The arrows indicate changes in the patient's clinical phenotype. The grey zone indicates the region of normal CEA levels (< 5 ng/mL). VAF, Variant allele variant allele frequency; CEA, Carcinoembryonic antigen; SD, Stable disease

9. Survival analysis of treatment outcome by serial ctDNA monitoring

We analyzed the treatment outcomes concerning the mutation detection or the average VAF in ctDNA containing only all tier 1/2 variants in 25 patients. The average ctDNA VAF was measured at each time point. The mutation evaluation was performed during the first clinical follow-up after the treatment. Also, ctDNA clearance was defined as the complete absence of all tier 1/2 variants in ctDNA that were detectable in at least two consecutive samples collected during the treatment period. The patterns of ctDNA mutations during the treatment period were grouped into four categories: (1) the mutation found in baseline samples was persistently detected or (2) vanished; (3) the mutation was not detected in the baseline samples and was also absent in later time points, or (4) was newly acquired. Patients with persistent mutations (n = 6) had significantly worse outcomes, whereas the median PFS of patients with no mutations was the longest ($P < 0.0001$) (Figure 12A). Furthermore, 18 (72.0 %) patients exhibited decreases in the average VAF, and a complete ctDNA clearance was observed in 15 (60.0 %) patients during the follow-up period (range 33–800 days). Patients who exhibited ctDNA clearance (+) had a longer PFS compared to patients with no ctDNA clearance ($P = 0.013$). The median PFS was 489 days in the ctDNA clearance (+) group and 317 days in the ctDNA clearance (-) group (Figure 12B).

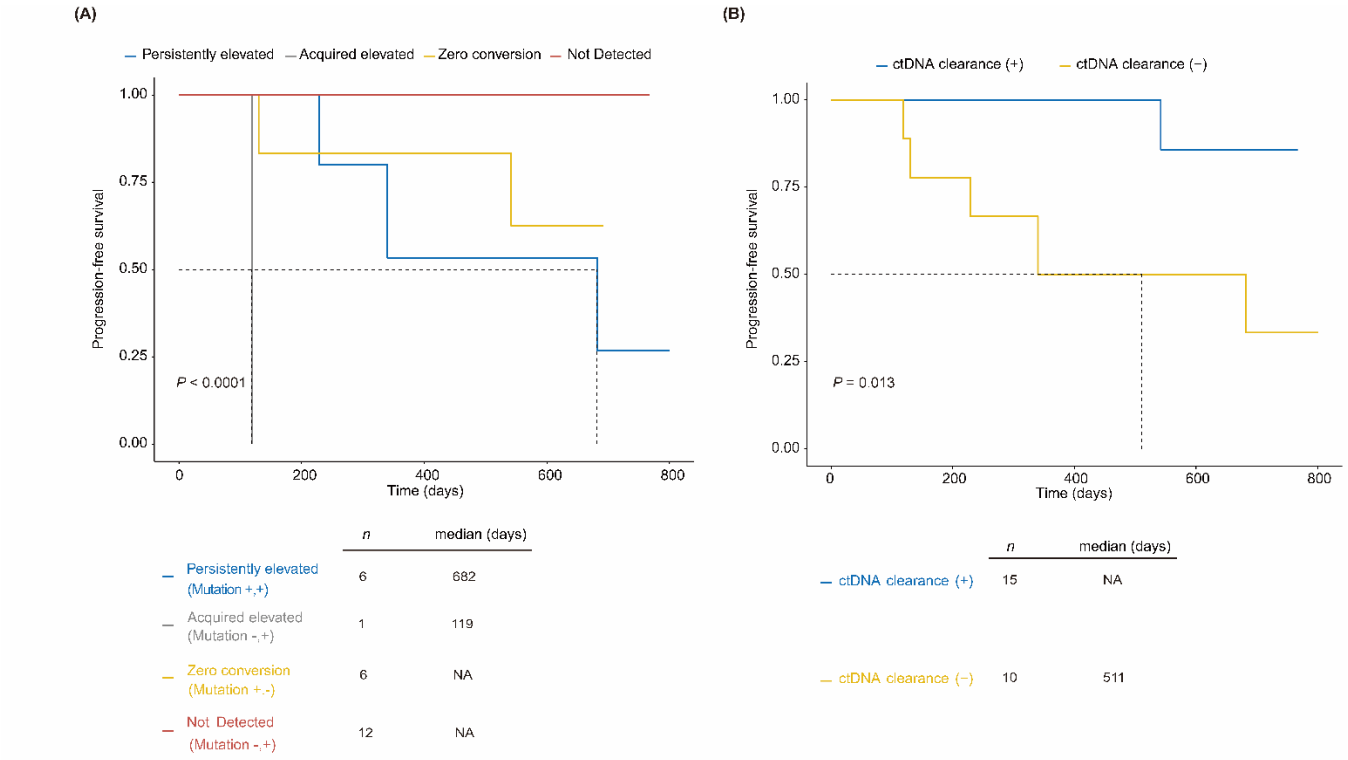

Figure 12. Treatments response outcomes by ctDNA variants.

(A) Kaplan-Meier analysis of PFS according to the change of in the average VAF at each time point during the treatment period. **(B)** Kaplan-Meier analysis of PFS according to ctDNA clearance detection.

IV. DISCUSSION

In this study, we have analyzed serially collected ctDNA samples from CRC patients with targeted deep sequencing during the treatment period, and comprehensively examined the mutation profiles of the ctDNA samples to assess the clinical relevance of longitudinal ctDNA monitoring in CRC patients.

The two strategies for minimal residual disease (MRD) monitoring are "fixed-panel" and "bespoke." Since MRD detection requires high sensitivity and specificity, many studies adopted an efficient strategy to monitor patients by performing ultra-deep sequencing using a small gene panel. Lee *et al.* evaluated the Oncomine™ Colon cfDNA Assay including 14 genes relevant to tumor and determined the LOD as 0.5 %[50]. Verma *et al.* reported that AVENIO ctDNA assay Kit had a sensitivity of 100 % at 1 % AF, 100 % at 0.5 % AF, and 50 % at 0.1 % AF with 40 ng sample input[51]. The sensitivity of the "fixed-panel (0.043 Mbp)" used in our study was confirmed to be 100.0 %, and the LOD was 0.26 % AF. Thus, these data suggest that our approach is amply inclusive and clinically relevant as it allows mutational profiling in a cost-effective way for MRD tracking during follow-up.

The average VAFs in ctDNA samples showed statistically significant differences between patients with tumors at different stages ($P < 0.01$). In recent years, several studies have demonstrated that ctDNA levels correlate with tumor burden[52,53]. TNM stage and tumor stage are tumor-associated parameters that can be used to assist in prognostic cancer staging in solid cancers. The average VAF in ctDNA at each TNM stage and tumor stage is different (lower in early stages and higher in later stages). Since the average ctDNA VAF can represent the molecular tumor burden, the average VAF in ctDNA present in the plasma has an independent prognostic value in CRC[54]. The ctDNA analysis could provide accurate data about the primary and molecular characteristics of tumor tissues without requiring

invasive biopsies, particularly in patients with advanced tumor stage. This data is consistent with the Lee *et al*. reported a 45 % detection rate in CRC patients and a high detection of ctDNA mutations at higher TNM stages and CEA levels[50].

The ctDNA variants were detected in 59 of 105 patients (56.2 %), and *APC*, *TP53*, and *KRAS* were the most frequently mutated genes. A total of 140 alterations were found in 105 patients; the average number of tier 1/2 variants per patient was 1.35. Also, the detection rate was high in stage 4 tumors with metastasis (75 %), and 67.5 % in patients with metastasis. *APC* tumor-suppressor gene plays a vital role in the early stage of CRC, are found in about 80 % of sporadic CRC[55]. *APC* gene mutation activated Wnt signaling; it transmits a signal from the receptor on the cell surface to the nucleus, changing the expression of genes related to tumor formation of growth[56,57]. *KRAS* is another crucial oncogene associated with VEGF and EGFR pathways. *KRAS* mutations occur in approximately 40–50 % of sporadic colorectal cancer or adenomas over 1 cm but are rarely found in small adenomas and are known to be involved in the growth stage of adenoma and correlate with metastasis and vascular formation[58-60]. P53 is the first known and most studied tumor suppressor protein that regulates various downstream genes in different signaling pathways. The *TP53* mutation is founded in up to 70 % of colorectal cancer and can observe the loss of heterozygosity of 17p locus[61]. Furthermore, Somatic variant interactions were identified in *TP53*-*APC* and *APC*-*KRAS* ($P < 0.05$). These three genes were shown to significantly interact in CRC patients, and this result is consistent with the previous report[62]. This data demonstrated the use of a customized panel including 14 genes for detecting somatic variants in patients with CRC, and found good detection rates in progressive tumor with metastasis, as reported in several previous studies[63,64]. Moreover, this result strongly highlights that some hypermutated cases in our cohort could have

metastasis.

The *KRAS* mutations are known to be important biomarkers to determine target therapy, and were detected in 23 % of the samples in our study, similar to a previous study[65]. However, the comparison of *KRAS* gene mutations between ctDNA samples and tumor tissues revealed nine inconsistent cases. Of these, 9 cases were tumors at early stages without metastasis. Ye *et al.* reported a meta-analysis indicating that the sensitivity of *KRAS* mutation detection is higher in metastatic CRC (0.79; 95 % CI, 0.76–0.82)[66]. These results show that the presence of the *KRAS* mutation in ctDNA correlates with the metastasis and tumor stage of the patient, and was reflected in patients' tumor progression state. Integrated analysis along with tumor tissue results can help in accurate selection of the target therapy.

The dynamic changes in ctDNA levels during the therapy significantly correlated with tumor shrinkage and regrowth reflecting treatment responses, and similar imaging results were obtained. The dynamic changes in VAF were indicative of higher sensitivity to patients' clinical outcomes compared to CEA in several cases. In seven patients with normal or stable CEA levels, changes in VAF discriminated between tumor progression and metastasis. These results suggest that linear monitoring of ctDNA can provide reliable information on the patient's tumor progression throughout the treatment process and could potentially constitute a better predictor of treatment response.

In our study, PFS analysis indicated an improved progression-free survival rate when mutations in ctDNA were not detected during the follow-up examination or more than two consecutive times after the first treatment. The analysis of ctDNA at multiple time points after the treatment confirmed that the detection of mutations and dynamic changes in ctDNA reflected the patients' clinical treatment responses and allowed to clonal tracking. While there are several arguments for a single ctDNA test to predict patients' outcomes,

accumulating data support the idea that molecular changes occurring in ctDNA through a time period can serve as a better biomarker in CRC. In addition, integrated analysis with CT scan results, rather than just an independent evaluation of ctDNA, allows accurate monitoring of patients' treatment responses.

However, our study lacks evidence to guide the time point during treatment or surveillance, and the overall survival analysis could not be calculated with a short collection period. Further studies are needed to conduct prospective randomized interventional clinical trials.

V. CONCLUSION

In conclusion, ctDNA reflected the underlying stepwise genetic events in colorectal cancer development and might be a potential predictive marker of mutation burden. Serial ctDNA testing can improve patient management by monitoring disease progression and clonal evolution. Moreover, the dynamic genomic changes in ctDNA can be a clinically significant prognostic marker for monitoring treatment outcomes. The present study suggests that the use of ctDNA in CRC patients can constitute a valuable non-invasive tool for monitoring disease states during or after treatment.

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ABSTRACT (IN KOREAN)

대장암 환자의 치료반응 모니터링을 위한 순환종양 DNA 분석의 임상적 중요성

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이 현 아

대장암은 발병률과 발생률이 세계적으로 3위를 차지한다. 치료 방법의 발달로 사망률은 개선이 되었지만, 여전히 전이와 높은 재발률을 보이기 때문에 치료 후 모니터링을 하는 것이 중요하다. 현재 가장 널리 사용되고 있는 비침습성 바이오 마커는 암배아항원 (CEA) 마커로 모니터링에 사용하기에는 낮은 민감도와 특이성의 한계를 가지고 있다. 높은 민감도와 특이성을 가지는 비침습적 바이오마커로 순환 종양 DNA (ctDNA)는 대장암 환자를 모니터링하는 데 유용한 바이오 마커가 될 수 있다. 본 연구에서는 대장암을 진단받은 105명의 환자를 등록하고 263개의혈장 샘플을 수집했다. 105명의 대장암 환자의 치료 반응을 모니터링하기 위해 관련 유전자 14개를 대상으로 패널을 설계해 NGS를 실시했으며, 염기서열 분석 결과는 병리학적 특성 및 임상 결과와의 상관관계에 대해 평가되었다. ctDNA 분석은 환자의 56.2 %에서 총 140개의 변이를 식별했다 (n = 105). 그 결과 그 결과 *APC* (36.0 %), *TP53*

(35.0 %), *KRAS* (23.0 %)이 가장 많이 변이된 유전자로 나타났다. 선형 분석은 최대 변이 대립유전자 주파수와 종양 단계 사이의 양의 상관관계($P < 0.01$)를 보여주었다. 조직 생검 결과(*KRAS*)가 있는 39명의 환자의 ctDNA와 종양조직의 *KRAS* 변이 검출은 30명은 일치하였으며, 일치되지 않는 9명은 전이되지 않거나 종양 2기에 해당되었다. 이는 전이의 여부가 돌연변이 검출에 영향을 미치는 것으로 시사되었다. 또한 연속적 ctDNA의 분석을 통해서 질병 진행을 예측하는 데 있어 치료 후 첫 임상 추적 평가의 ctDNA 돌연변이가 검출되지 않거나 두 번 연속적으로 변이가 나타나지 않는 환자의 경우, 무진행생존율 (PFS)가 그렇지 않은 환자보다 더 길었으며 영상 이미지 결과와 복합적으로 분석 시 통계적으로 유의하게 긴 무진행생존율을 나타냈다 ($P < 0.0001$, $P = 0.013$).

이러한 데이터는 ctDNA의 연속적 분석이 시간의 경과에 따른 종양의 질병 진행 및 돌연변이 상태 모니터링을 통해 환자 관리를 개선할 수 있음을 의미한다. 본 연구는 ctDNA가 다양한 임상 조건의 대장암 환자의 추적 관찰 중 질병 상태를 모니터링하는 데 유용한 비침습적 바이오 마커임을 시사한다.

핵심되는 말: 대장암, 액체생검, 세포 유리 DNA, 순환 종양 DNA, 모니터링, 차세대 염기서열 분석