Original

The clinical validity of digital PCR based circulating tumor DNA monitoring in patients with colorectal cancer who received adjuvant chemotherapy

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Abstract

To improve survival rate, postoperative adjuvant chemotherapy (ACT) and surveillance are performed for patients with colorectal cancer (CRC). A recent study demonstrated that a circulating tumor DNA (ctDNA)-guided approach to the treatment of stage II CRC reduced ACT use without compromising recurrence-free survival. We examined whether longitudinal ctDNA assays by digital PCR (dPCR) can provide early relapse prediction in CRC patients who received ACT. Variant allele frequency (VAF) of ctDNA was informative in all 52 patients with dPCR using 87 selected mutations. Among 51 patients who underwent curative resection, patients who had relapse (n = 9) showed higher ctDNA VAF than those without relapse at the first postoperative (post-op) timepoint (n = 42, p < 0.0001). Among 14 patients who received ACT, 4 out of 6 (66.7%) patients who had relapse were ctDNA-negative at post-op. However, all 6 patients with relapse showed ctDNA elevation before relapse was confirmed by computed tomography scan during the postoperative period. Patients with at least one ctDNA-positive timepoint during the postoperative period (n = 6) showed a higher risk of relapse than those who had sustained ctDNA-negative status (n = 8) (HR 39.6, 95%CI 6.4-243.9, p < 0.0001). Longitudinal dPCR-ctDNA assay can complement the postoperative ctDNA-guided approach for both ACT intervention and relapse detection based on conventional surveillance.

I. Introduction

Colorectal cancer (CRC) is the third most common cancer type worldwide [1]. Approximately two-thirds of patients with stage II or III CRC undergo resection with curative intent [2]. To increase the cure rate, postoperative adjuvant chemotherapy (ACT) is followed by surgery to eradicate micro metastases. The benefit of ACT has been clearly demonstrated in stage III disease, whereas that in stage II disease remains controversial. To improve

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Table 1. Characteristics of patients with CRC in the study cohort

Characteristic	Preoperative-ctDNA	
	positive (n = 31)	negative (n = 21)
Sex - % (no.)		
Male	61.3 (19)	42.9 (9)
Female	38.7 (12)	57.1 (12)
Age group - % (no.)		
≦ 70	51.6 (16)	52.4 (11)
＞ 70	48.4 (15)	47.6 (10)
Tumor site - % (no.)		
Left	67.7 (21)	52.4 (11)
Right	32.3 (10)	47.6 (10)
Tumor stage* - % (no.)		
T1	22.6 (7)	9.5 (2)
T2	67.7 (21)	71.4 (15)
T3	9.7 (3)	9.5 (2)
T4	0 (0)	9.5 (2)
pStage# - % (no.)		
I	22.6 (7)	9.5 (2)
II	67.7 (21)	71.4 (15)
III	9.7 (3)	4.8 (1)
IV	0 (0)	14.3 (3)

* TNM classification, 8th edition
\# Pathological stage
CRC, colorectal cancer; pre, pre-operation; ctDNA circulating tumor DNA

disease-specific and overall survival (OS) by allowing detection of relapse and subsequent treatment intervention, postoperative surveillance is performed for patients with stage II or III CRC using computed tomography (CT) scans and serum carcinoembryonic antigen (CEA) testing during the 5 years after surgery[3-6].

Recently, circulating tumor DNA (ctDNA) has emerged as a promising noninvasive biomarker for the molecular diagnosis and monitoring of several cancer types[7-13]. Clinical validities[14] of the ctDNA assay have been demonstrated for early prediction of therapeutic efficacy in patients with metastatic CRC[15-18] and relapse in those with localized CRC[9,12,19,20]. Several clinical trials are currently testing the clinical utility of ctDNA testing in CRC patients[21]. The most recent randomized controlled trial demonstrated that a ctDNA-guided approach to the treatment of stage II CRC reduced ACT use without compromising recurrence free survival[22]. In these studies, next-generation sequencing (NGS) of plasma DNA has been used for ctDNA detection. However, due to the cost and labor, NGS-based ctDNA monitoring has not been adopted for use in daily practice in which many cancer patients are frequently and longitudinally tested.

We previously demonstrated that frequent ctDNA monitoring by digital PCR (dPCR) enabled early relapse prediction, treatment efficacy evaluation, and disease-free corroboration in the management of gastro-
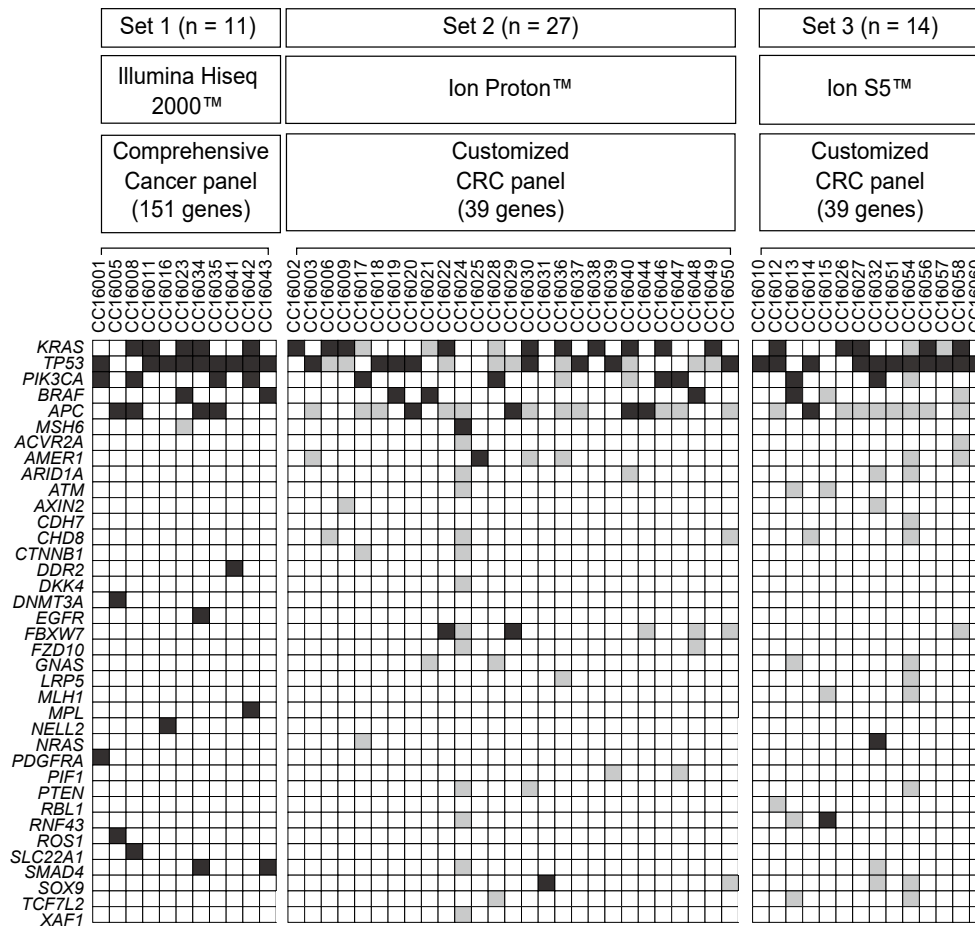
Fig. 1. Mutation profile of primary CRC tumors from 52 patients.
　　　Mutation profile of 52 CRC tumors. Sequencing platforms and gene panels using in Set 1, 2, and 3 are shown in the top panel. Mutated genes are shown in the bottom panel. Color boxes indicate the mutated genes. Black boxes indicate mutations used for dPCR analysis.

intestinal cancers[23-26]. Especially in CRC, our recent study by dPCR-based ctDNA assay highlighted the possibility of reducing the frequency of CT scan during postoperative surveillance for CRC patients[27].

In this study, we examined the results of longitudinal ctDNA assays during the postoperative period and found that ctDNA monitoring by dPCR can provide early relapse detection at various time points in CRC patients who received ACT.

II. Materials and Methods

1. Patients and sample collection

This study was approved by the Institutional Review Board of Iwate Medical University (IRB #HGH 28-15 and #MH 2021-073). Written informed consent was obtained from all patients. 52 patients who had undergone complete resection of the tumor were enrolled in this study between March 11, 2016, and June 20, 2018. A summary of patient characteristics is provided in Table 1. Surgically acquired primary tumor tissue samples and corresponding serial blood samples were
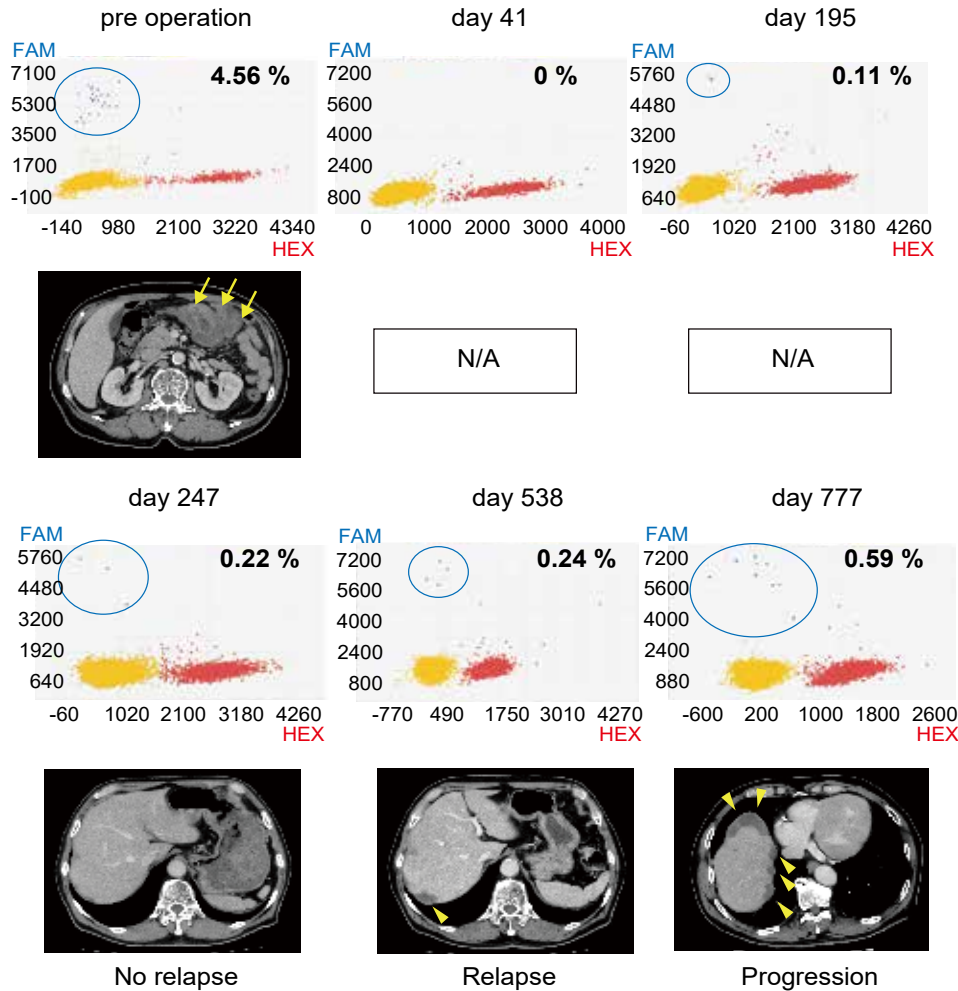
Fig. 2. Representative results of ctDNA analyses and corresponding CT images.
Results of ctDNA analyses by dPCR at postoperative timepoints of Patient CC16019 are shown in the upper panels. Red and blue dots indicate wild-type and mutant reactions for a specifically designed primer/probe set for the tumor-specific mutation (TP53 c.G818A). CT images corresponding to the timing of dPCR analysis are shown in the lower panels. From days 195 to 777, ctDNA VAF levels showed an increasing trend (blue circles). Arrows and arrow heads in the CT images indicate primary tumor and dissemination relapse surrounding liver. Variant allele frequencies (%) by dPCR were show in scattergrams. ctDNA, circulating tumor DNA; N/A, not applicable.

obtained for the ctDNA assay.

2. Primary tumors sequence using NGS

NGS-based tumor sequencing analyses were performed using three different platforms; 1) the ClearSeq Comprehensive Cancer Panel (Agilent Technologies, Inc., Santa Clara, CA, USA) targeting 151 genes on an Illumina Hiseq 2000 (Illumina, Inc., San Diego, CA, USA)[24] ; 2) Ion Proton™; and 3) the Ion S5™ system (Thermo Fisher Scientific, Waltham, MA, USA), using a customized CRC panel targeting 39 genes[28](Fig. 1).

3. Monitoring ctDNA levels using dPCR

The dPCR assay for quantitative monitoring of ctDNA levels was performed as described previously[23-26]. Briefly, specific primers and probes labeled for wild-type and mutant alleles were specifically designed for each mutation
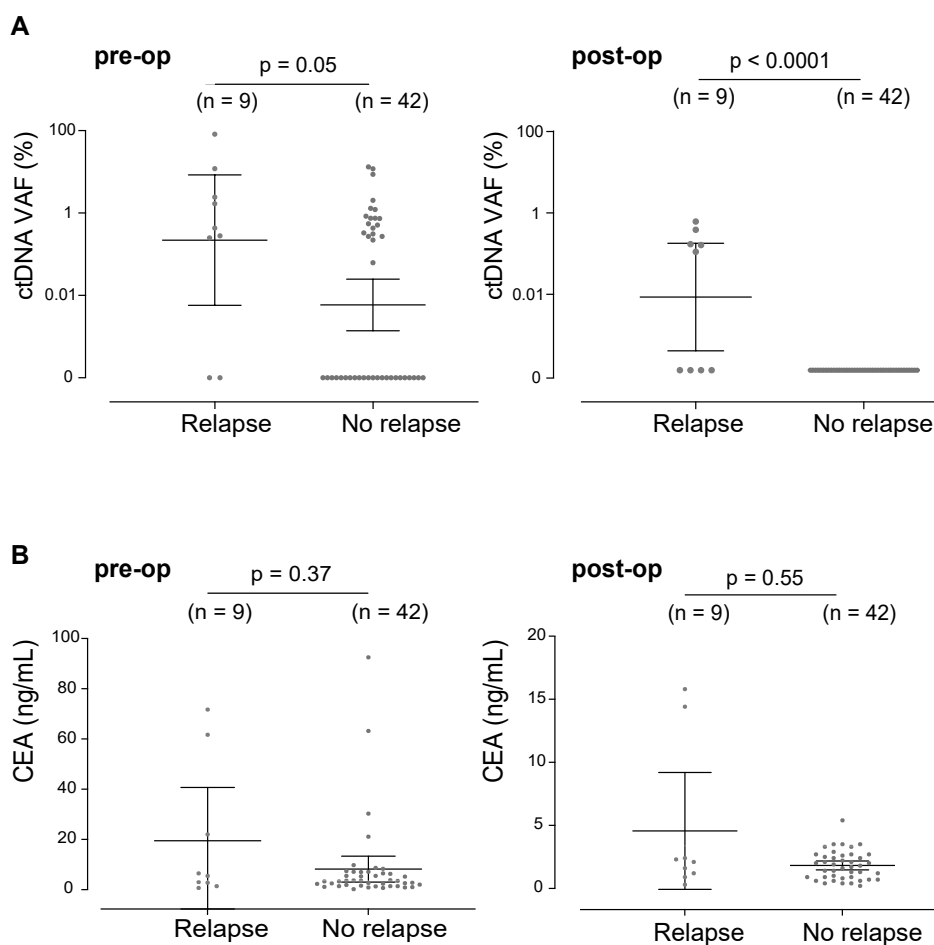
Fig. 3. Association between status of blood biomarkers and relapse.
Preoperative and postoperative levels of ctDNA (A) and CEA (B) in CRC patients with or no relapse. Pre-op and post-op indicate preoperative and first postoperative timepoint from the initial surgery. ctDNA, circulating tumor DNA; VAF, variant allele frequency.

identified in a primary tumor, using Hypercool Primer & Probe™ technology (Nihon Gene Research Laboratories, Sendai, Japan). For frequently recurring missense mutations, commercially available primer/probe sets were used (Thermo Fisher Scientific, Waltham, MA, USA and Quantdetect, Inc., Tokyo, Japan). One to five mutations per tumor that had a variant allele frequency (VAF) higher than 10% in primary tumors were prioritized for dPCR analysis. ctDNA data for VAFs were plotted on a time course along with therapy type and clinical information of 14 CRC patients who

received ACT.

4. Statistical analysis

For group comparisons, the Mann-Whitney U test and Fisher's exact test were used. Relapse-free survival (RFS) was estimated using Kaplan-Meier method. RFS based on ctDNA status (i.e., positive or negative) at the first postoperative timepoint from the initial surgery and throughout the postoperative surveillance period were compared with log-rank test. The Cox proportional hazards model was used to estimate risks, based on RFS. We considered $p < 0.05$ to be statistically signifi-
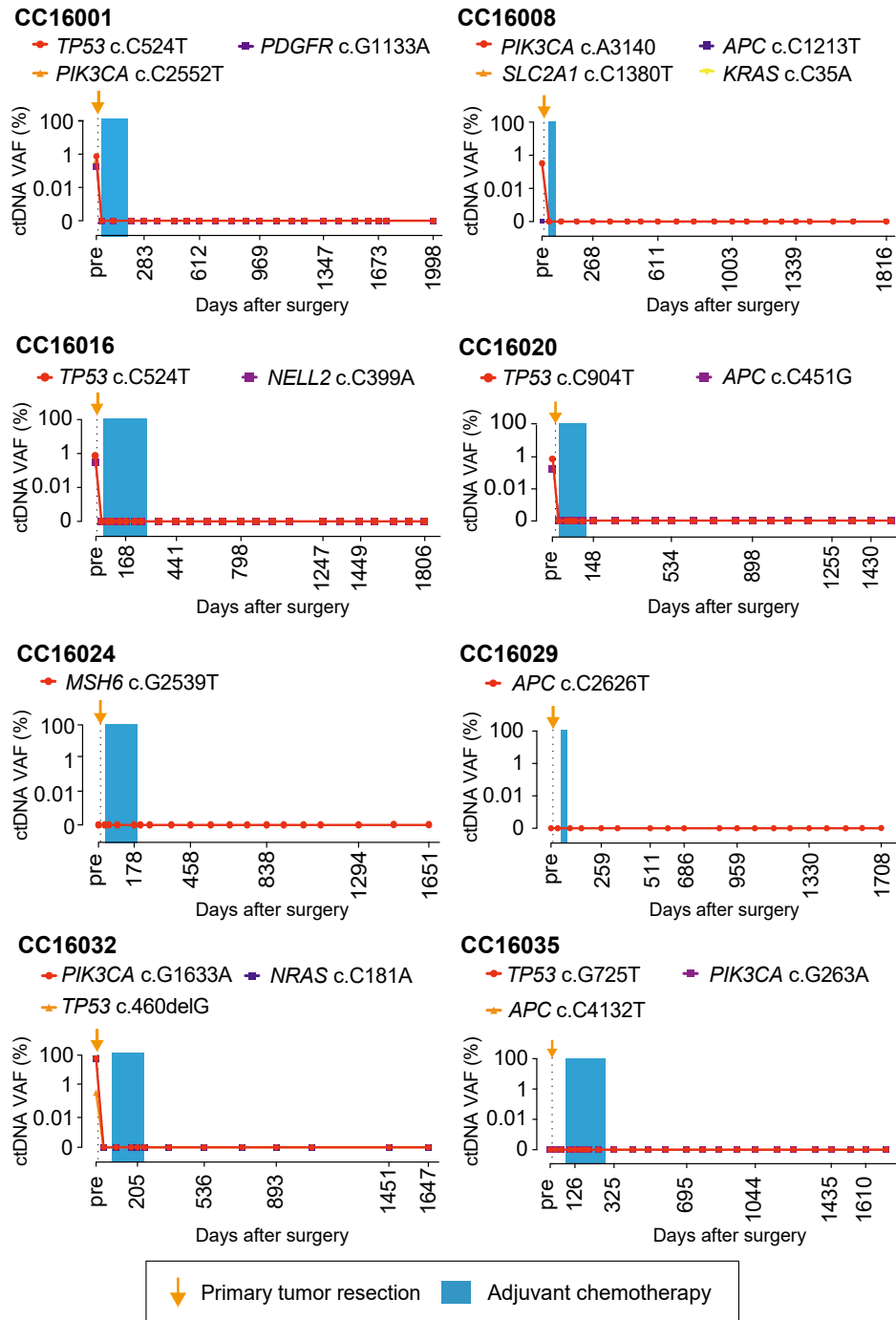
Fig. 4. Dynamics of ctDNA in 8 patients with CRC who received adjuvant chemotherapy without relapse.
Patient specific mutations using for ctDNA monitoring were arranged above the graph   of each case. These mutations were detected by primary tumor sequencing. ctDNA, circulating tumor DNA; VAF, variant allele frequency; Pre, preoperative timepoint.

cant for all analyses. All analyses were performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA).

III. Results

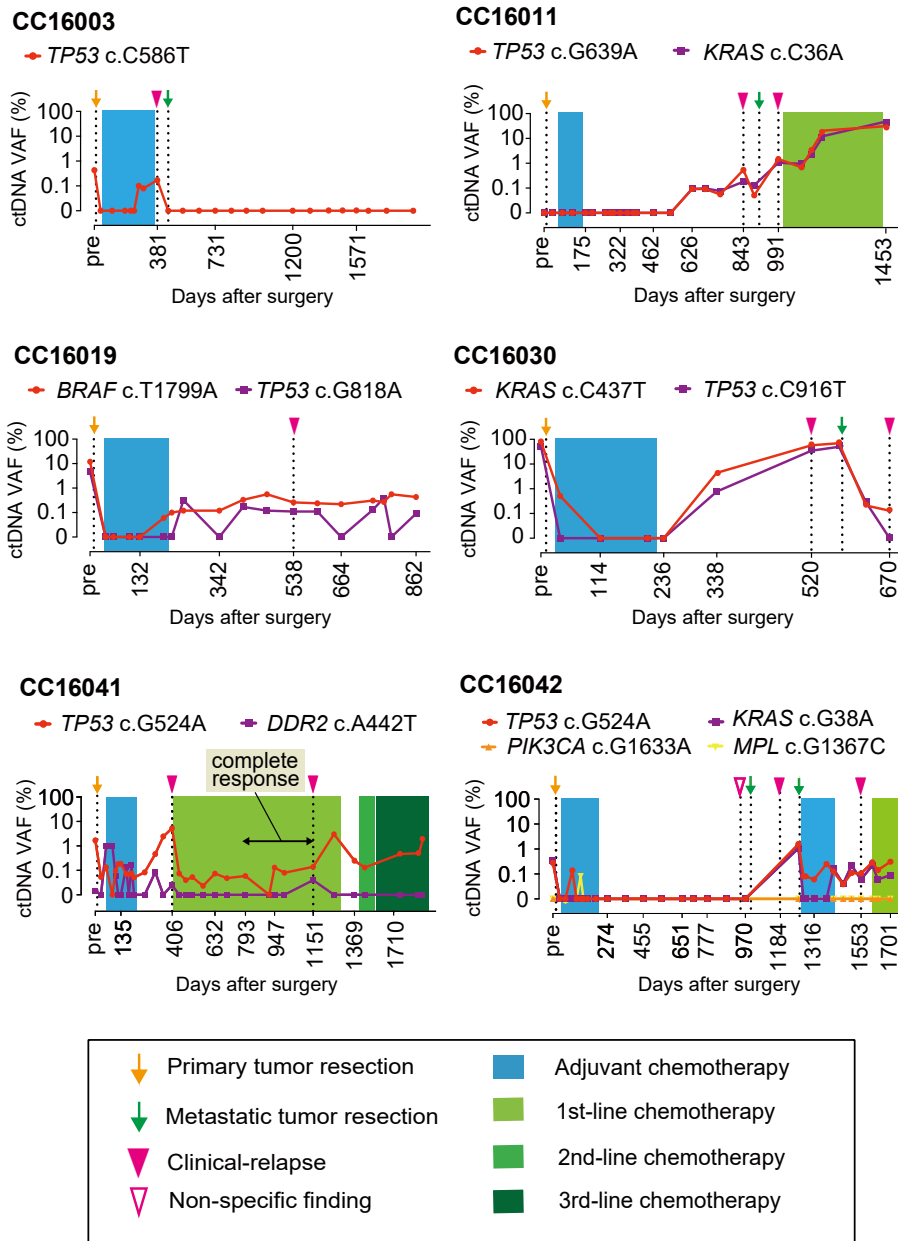1. Mutations selected for ctDNA detection in CRC patients

Fig. 5. Dynamics of ctDNA in 6 patients with CRC who received adjuvant chemotherapy with relapse.
Patient specific using for ctDNA monitoring were arranged above the graph of each case. These mutations were detected by primary tumor sequencing. ctDNA, circulating tumor DNA; VAF, variant allele frequency; Pre, preoperative timepoint.

At least one somatic mutation was identified in the primary tumor of all 52 patients. Fig. 1 summarize the mutation profile; the detailed mutation profile is available in our previous reports[24,27]. The most frequently mutated genes were *TP53* (37 / 52, 71.2%), *APC* (28 / 52, 53.8%), and *KRAS* (24 / 52, 46.2%). Probe/ primer sets for the 87 selected mutations from 52 patients were validated by dPCR using corresponding primary tumor DNA (Fig. 1). Preoperative plasma from 31 out of the 52 (59.6%) patients was positive for ctDNA
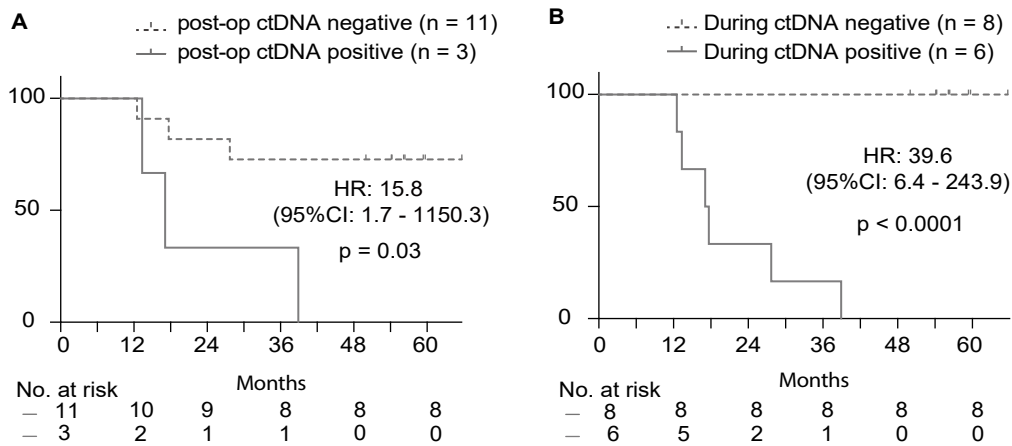
Fig. 6. Relapse-free survival according to ctDNA status in patients with CRC who received
adjuvant chemotherapy.
Relapse-free survival according to ctDNA status at first postoperative timepoint (A) and
during postoperative period (B). p values were derived from the Kaplan–Meier log-rank
test. HR and 95% CI were estimated by the Cox proportional hazards model. post-op,
postoperative; ctDNA, circulating tumor DNA; HR, hazard ratio; CI, confidence interval.

(Table 1). As shown in Fig. 2, results of ctDNA analyses by dPCR were compared to radiographic finding.

2. Association between ctDNA status and relapse in patients with CRC

The median observation period for our cohort was 1,503 (range, 322-1,951) days. Radiographic relapse was observed for 10 out of the 52 (19.2%) patients with CRC. Among the 10 patients with radiographic relapse, one patient who underwent two-stage resection of the primary tumor and liver metastasis with curative intent was excluded in the subsequent analysis. Among the 51 patients who underwent resection with curative-intent as the initial surgery, no significant differences in the preoperative ctDNA levels were observed between patients with (n = 9) and without relapse (n = 42, p = 0.05; Mann–Whitney U test). However, patients who did have relapse (n = 9) showed significantly higher ctDNA VAF than those without relapse at post-op (n = 42)

(p < 0.0001, Mann–Whitney U test; Fig. 3A). In terms of CEA status, no significant difference was observed between relapse and non-relapse patients (Fig. 3B).

3. Longitudinal ctDNA analysis of patients with CRC received ACT

Plasma samples at the first postoperative timepoint from the initial surgery were collected an average of 34.0 days (range, 20-58) after resection. Fig. 4 and 5 show the ctDNA dynamics for 14 patients with CRC who received ACT. For 8 patients who had no relapse, ctDNA-negative results were obtained throughout the postoperative period (Fig. 4). Of these 8 patients, 5 were pretreatment ctDNA-positive. All exhibited a decrease in ctDNA levels below the detection limit at the post-op timepoint and continued to be ctDNA-negative. Meanwhile, all patients (6/6, 100%) with relapse showed an increase in ctDNA prior to radiographical relapse. In the 6 patients with relapse, ctDNA elevation was observed during ACT

in 4 patients (CC 16003, CC 16019, CC 16041 and CC 16042) and after ACT in 2 patients (CC 16011 and CC 16030). Four out of the 6 (66.7%) patients with relapse showed ctDNA-negative at the first post-op timepoint (Fig. 5).

4. Plasma ctDNA status and risk of recurrence in CRC patients who received ACT

We next evaluated the RFS rate stratified with ctDNA status for 14 patients who received ACT. Patients who were ctDNA-positive at the first postoperative timepoint from the initial surgery (n = 3) showed a significantly higher risk of relapse than those who were ctDNA-negative (n = 11) (HR 15.8, 95%CI 1.7-1150.3, p = 0.03, log-rank test; Fig. 6A). Furthermore, patients with at least one ctDNA-positive timepoint during the post-operative period (n = 6) showed a significantly higher risk of relapse than those who had sustained ctDNA-negative results (n = 8) (HR 39.6, 95%CI 6.4-243.9, p < 0.0001, log-rank test; Fig. 6B).

IV. Discussion

Early detection of recurrence and treatment intervention can improve the prognosis and survival of patients with cancer. Although intensive follow-up with CT scan and CEA testing is now still recommended after CRC resection with curative intent, recent randomized trials showed that intensive follow-up for patients with CRC provided no significant benefit[29,30]. In terms of cost and invasiveness of diagnostic modalities, CEA testing is a quite reasonable method and has been widely used for predicting relapse. A previous study showed that CRC patients with elevated postoperative CEA had increased risk of relapse[31]. In our study, however, no significant difference was observed in CEA level at the first postoperative timepoint from the initial surgery between patients with and without relapse (Fig. 3B). Meanwhile, our dPCR-based ctDNA testing demonstrated that patients who were ctDNA-positive at post-op showed a significantly higher risk for relapse than those who were ctDNA-negative (Fig. 3B), as in previous studies[9,12,20]. Therefore, post-op ctDNA status is a promising biomarker for relapse prediction for patients with CRC after resection with curative intent.

ACT for minimal residual disease (MRD) after surgical resection for cancer patients is the earliest intervention for recurrent disease and its benefit has been demonstrated in various cancer types, including CRC. However, because surgery alone can cure more than 80% of stage II and 50% of stage III CRC patients, many patients are exposed to unnecessary chemotherapy[32,33]. Analysis of ctDNA directly evaluates evidence of MRD that could ultimately signal clinical recurrence and is a promising alternative strategy for relapse detection. Indeed, clinical validities of the ctDNA assay have been demonstrated for early prediction of therapeutic efficacy in patients with metastatic CRC[15-18] and relapse in those with localized CRC[9,12,19,20]. Recently, it has been demonstrated that a ctDNA-guided approach could reduce ACT use for stage II CRC patients, most of whom do not require additional treatment after surgery[22]. Kotani et al. also reported that patients with high-risk stage II or III and ctDNA-positive status at 4 weeks after surgery deriverd significant benefit from ACT[34]. In these randomized trial, ctDNA status was evaluated at only

or two points (4 or 7 weeks) after surgical resection and about 10% of patients with ctDNA-negative status at those timepoints had relapse within 3 years. Longitudinal ctDNA-negative just after surgery during the postoperative period. In our present study, all 42 patients without relapse were ctDNA-negative at the post-op timepoint. Among the 42 patients without relapse, 8 (19%) patients who had ctDNA-negative results at post-op received ACT. It is possible that these 8 ctDNA-negative patients could have avoided ACT in the ctDNA-guided approach. Meanwhile, 4 out of 9 (44.4%) patients with relapse were ctDNA-negative at post-op (Fig. 3A). These 4 ctDNA-negative patients could not receive ACT in the ctDNA-guided approach and this may result in early relapse. Therefore, some patients may suffer the disadvantage of ACT omission as a result of evaluation of ctDNA status at only one or two postoperative time-points. Among 14 patients who received ACT, 4 out of 6 (66.7%) patients with relapse were ctDNA-negative at the first postoperative timepoint (Fig. 5). However, all 6 patients with relapse showed ctDNA elevation before relapse was confirmed by CT scan during the postoperative period. The average day of first ctDNA-detection after surgical resection in the 6 relapse patients was day 209 (range, 30-626 days). The average leading time from ctDNA-elevation to radiographic relapse was 253 (range, 112-376 days) . Our results indicate that longitudinal dPCR-based ctDNA assay may enable early treatment intervention even in relapsed patients without ctDNA-detection at the post-op timepoint.

In conclusion, our results indicate that longitudinal ctDNA monitoring by dPCR can

complement the postoperative ctDNA-guided approach for both ACT intervention and relapse detection based on conventional surveillance using CT scans and CEA testing.

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術後化学療法を受けた大腸癌患者における
デジタル PCR 法を用いた
ctDNA モニタリングの臨床的妥当性

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要旨

　大腸癌術後症例では生存率向上を目的とし，補助化学療法とサーベイランスが行われている．Stage II 大腸癌では術直後の circulating tumor DNA（ctDNA）情報により無再発生存率を低下させることなく補助療法を削減可能であることが示されている．本研究では，根治手術を受けた大腸癌 52 症例における digital PCR による ctDNA の長期モニタリングと再発の有無について評価した．再発例の術後初回採血での ctDNA 値は無再発例に比し有意に高かった．補助療法施行 14 例のうち，再発例 6 例中 4 例（66.7 ％）は術直後 ctDNA 陰性であったが，6 例全例で経過中の CT での再発確認前に ctDNA の上昇が見られた．術後経過中の ctDNA 陽性症例は陰性維持例に比し有意に再発リスクが高かった．経時的な ctDNA 解析は補助療法施行の判断と早期再発発見の両方に妥当性を有することを示した．