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Utility of *KRAS* mutation detection using circulating cell-free DNA from patients with colorectal cancer

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Key words

Anti-EGFR antibody, circulating cell-free DNA, circulating tumor DNA, EGFR blockade, *KRAS*

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In this study, we evaluated the clinical utility of detecting *KRAS* mutations in circulating cell-free (ccf)DNA of metastatic colorectal cancer patients. We prospectively recruited 94 metastatic colorectal cancer patients. Circulating cell-free DNA was extracted from plasma samples and analyzed for the presence of seven *KRAS* point mutations. Using the Invader Plus assay with peptide nucleic acid clamping method and digital PCR, *KRAS* mutations were detected in the ccfDNA in 35 of 39 patients previously determined to have primary tumors containing *KRAS* mutations using the Luminex method, and in 5 of 55 patients with tumors containing wild-type *KRAS*. Curative resection was undertaken in 7 of 34 patients with primary and ccfDNA *KRAS* mutations, resulting in the disappearance of the mutation from the cell-free DNA in five of seven patients. Three of these patients had tumor recurrence and *KRAS* mutations in their ccfDNA reappeared. Epidermal growth factor receptor blockade was administered to 24 of the *KRAS* tumor wild-type patients. Of the 24 patients with wild-type *KRAS* in their primary tumors, three patients had *KRAS* mutations in their ccfDNA and did not respond to treatment with epidermal growth factor receptor (EGFR) blockade. We also detected a new *KRAS* mutation in five patients during chemotherapy with EGFR blockade, before disease progression was detectable with imaging. The detection of *KRAS* mutations in ccfDNA is an attractive approach for predicting both treatment response and acquired resistance to EGFR blockade, and for detecting disease recurrence.

Epidermal growth factor receptor (EGFR) blockade has improved the outcome of unresectable colorectal cancers (CRC).[1] *KRAS* codon 12 or 13 mutations in exon 2 have been widely reported to be a major predictive biomarker for resistance to EGFR blockade in patients with metastatic CRC (mCRC).[2] Mutations in other members of the *RAS* family may also confer resistance to EGFR blockade in patients without *KRAS* exon 2 mutations.[3] Other oncogenic mutations, such as *BRAF* or *PIK3CA* mutations have also been presented as promising predictors for treatment resistance in these patients, although their predictive value has not yet been established.[4]

Thus, it is important to examine *KRAS* mutation status in patients with CRC. To date, *KRAS* mutation status has been examined using primary tumor samples, even when EGFR blockade is given for the treatment of metastases. However, colorectal tumors are heterogeneous in nature, and tumor heterogeneity and mutational selection are generated by tumor progression. Thus, there are many discordant patients (i.e., patients who show genetic differences between their primary tumors and their metastases)[5,6] and non-responders, with discordance of *KRAS* mutations observed in 8% of mCRC cases.[7]

Epidermal growth factor receptor blockade induces the selection of pre-existing mutant clones and leads to de novo acquisition of *KRAS* mutations.[8] In the past, these two phenomena have not been clinically examined because it is difficult and invasive to collect samples from metastases deep within the body, such as from the lungs or liver. Circulating tumor cells (CTC) and circulating cell-free DNA (ccfDNA) were recently identified in the plasma of patients with malignant disease and are now used for diagnosis, treatment selection, and therapy evaluation.[9] However, CTC cannot always be used to detect *KRAS* mutations because it is difficult to extract sufficiently high CTC yields. Two studies have analyzed *KRAS* mutations using CTC, but both displayed very low sensitivity.[10,11]

Circulating cell-free DNA shows tumor-specific sequence alterations, and advances in sequencing technologies have enabled the rapid identification of somatic genomic alterations.[12] However, both the small number of circulating mutant gene fragments compared with the number of circulating wild-type DNA fragments,[13] and the small amount of ccfDNA able to be extracted in a clinical setting make it difficult to detect mutations, requiring high-sensitivity detection systems. In this study, we evaluated the clinical utility of a

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highly sensitive PCR-based method for detecting *KRAS* mutations in the ccfDNA of mCRC patients.

Materials and Methods

Patients and study design. We prospectively recruited 94 patients with histologically confirmed mCRC with distant metastases. Inclusion criteria for this study were age >20 years and patient performance status of 0 or 1. This study was carried out in accordance with the Declaration of Helsinki, and the study protocol was approved by the Ethics Review Committee of Nippon Medical School (Tokyo, Japan). Written informed consent was obtained from all participants.

Analysis of *KRAS* mutations in primary colorectal tumor tissue. All patients had pathological diagnosis from primary colorectal tumors. DNA was purified from formalin-fixed, paraffin-embedded specimens using a QIAamp DNA Mini kit (Qiagen, Limburg, the Netherlands) according to the manufacturer's recommendations. *KRAS* mutation analysis of the primary tumors was undertaken using the MEBGEN-Luminex method (MBL, Nagoya, Japan) as previously reported.[14]

Blood collection and ccfDNA extraction. Blood samples were collected at the first visit from patients with synchronous metastases. Blood samples were also collected when metastatic sites were detected in patients with metachronous metastases, and every 2–3 months from patients undergoing chemotherapy.

Plasma was prepared by centrifugation of 8 mL EDTA-treated blood at 800 *g* for 10 min at 4°C, followed by transfer to a fresh tube and re-centrifugation at 16 000 *g* for 10 min at 4°C. The supernatants were then either immediately used for DNA extraction or stored at −80°C until use. Circulating cell-free DNA was extracted from 1 mL plasma with the QIAamp Circulating Nucleic Acid kit (Qiagen) according to the manufacturer's plasma and serum fluid protocol. DNA concentrations were determined with the PicoGreen Assay (Quant-iT PicoGreen dsDNA Assay Kit; Thermo Fisher Scientific, Tokyo, Japan).

Invader Plus assay with peptide nucleic acid clamping for *KRAS* mutations. We detected *KRAS* mutations in ccfDNA using the Invader Plus assay with peptide nucleic acid clamping (Inv-Clamp assay; Hologic, Inc. (Marlborough, MA, USA) Figs S1–S3).[15] *KRAS* mutation status was blinded to investigators. To evaluate the assay's sensitivity for mutation detection, a titration study of plasmid DNA was initially carried out using plasmid DNA (0.15 fg) containing seven *KRAS* mutations (G12V, G12A, G12D, G12S, G12C, G12R, and G13D) mixed with 150 fg wild-type DNA. Oligonucleotide sequences used in this study are shown in Table S1. These seven somatic *KRAS* mutations were detected by the Inv-Clamp assay with a positive threshold ratio for all mutations defined as a 0.1% mutant to 99.9% wild-type mixture of plasmid DNA as per previous reports.[16] These mutant and wild-type DNAs were PCR-amplified and cloned into plasmid pCR2.1 (Thermo Fisher Scientific); the synthesized mutant and wild-type templates were verified with sequencing.

Validation with digital PCR. We also detected *KRAS* mutations in ccfDNA using digital PCR (dPCR). Digital PCR was carried out using a QuantStudio 3D Digital PCR System platform comprising a Gene Amp 9700 PCR machine including a chip adapter kit, an automatic chip loader, and the QuantStudio 3D Instrument (Thermo Fisher Scientific). The primers were synthesized by Thermo Fisher Scientific and the assay identification numbers of the primers were: G12C, AH0JEUD; G12S, AHI14FA; G12R, AHMSYXY; G12V, AHX1IHY; G12D, AH6R5PI; G12A, AHPAVDP; and G13D, AHD2BW0. We prepared 18 reaction mixtures containing 9 μL of 2× QuantStudio 3D Digital PCR Master Mix (Thermo Fisher Scientific), 0.45 μL of 40× TaqMan Assay by Design primer-probe mix, and 8.55 μL diluted sample genomic DNA (110–40 300 ng/mL). We loaded 14.5 μL of each reaction mixture onto a QuantStudio 3D Digital PCR 20K Chip (Thermo Fisher Scientific) using the automatic chip loader according to the manufacturer's instructions. Loaded chips underwent amplification in the Gene Amp 9700 PCR System under the following conditions: 96°C for 10 min, 39 cycles at 56°C for 2 min and at 98°C for 30 s, followed by a final extension step at 60°C for 2 min. After amplification, the chips were imaged on the QuantStudio 3D Instrument, which assesses raw data and calculates the estimated concentration of the nucleic acid sequence targeted by the FAM and VIC dye-labeled probes according to the Poisson distribution. The resulting data were reported in copies/μL together with the results of the data quality assessment metrics. For deeper analysis of the chip data, QuantStudio 3D Analysis Suite Cloud Software (Thermo Fisher Scientific) was used for relative and quantitative data analysis. An illustration of *KRAS* rare allele quantification by digital PCR is shown in Figure S4.

Treatment plan. Twenty-four patients with *KRAS* wild-type primary tumors were treated with chemotherapy including anti-EGFR antibody therapy. Ten patients were treated with mFOLFOX6 (day 1, oxaliplatin 85 mg/m², folinic acid 400 mg/m², and fluorouracil 400 mg/m² i.v. bolus, followed by 2400 mg/m^2 over 46 h of continuous infusion) and cetuximab (400 mg/m^2 on day 1). Eight patients were treated with mFOLFOX6 and panitumumab (6 mg/kg on day 1). Three patients were treated with FOLFIRI (day 1, irinotecan 150 mg/m^2, folinic acid 400 mg/m^2, and fluorouracil 400 mg/m^2 i.v. bolus, followed by 2400 mg/m^2 over 46 h of continuous infusion) and panitumumab (6 mg/kg on day 1). Two patients were treated with irinotecan (150 mg/m^2 on day 1) and panitumumab (6 mg/kg on day 1) and one patient was treated with the De Gramont regimen (day 1, folinic acid 400 mg/m^2 and fluorouracil 400 mg/m^2 i.v. bolus, followed by 2400 mg/m^2 over 46 h of continuous infusion) and panitumumab (6 mg/kg on day 1).

Evaluation of clinical response. Tumor response was assessed with computed tomography (CT) according to the Response Evaluation Criteria in Solid Tumors version 1.1. We also measured carcinoembryonic antigen (CEA) and CA19-9 levels every month throughout chemotherapy. A "normal" level of CEA falls under 5.0 ng/mL, while that of CA19-9 falls under 37 U/mL.

Results

***KRAS* status and ccfDNA yield.** Using the MEBGEN-Luminex method previously reported,[14] 39 of the 94 patients recruited were determined to have colon cancers with mutated *KRAS* in their primary tumors (primary *KRAS* mutations) and 55 patients had colon cancers with wild-type *KRAS* in their primary tumors (primary *KRAS* wild-type). We obtained ccfDNA from all 94 patients with a median yield of 790 ng/mL (Fig. 1a). No significant difference was observed between the yield from patients with or without *KRAS* mutations (Fig. 1b; $P = 0.26$). In addition, ccfDNA yield did not correlate with CEA ($P = 0.24$) or CA19-9 ($P = 0.50$) levels (data not shown). No significant difference was observed in the yield between the *KRAS*-detectable patients and the non-detectable patients (Fig. 1c; $P = 0.47$).

Mutation detection in ccfDNA of patients with primary *KRAS* mutations. Using the Inv-Clamp assay and dPCR, we were

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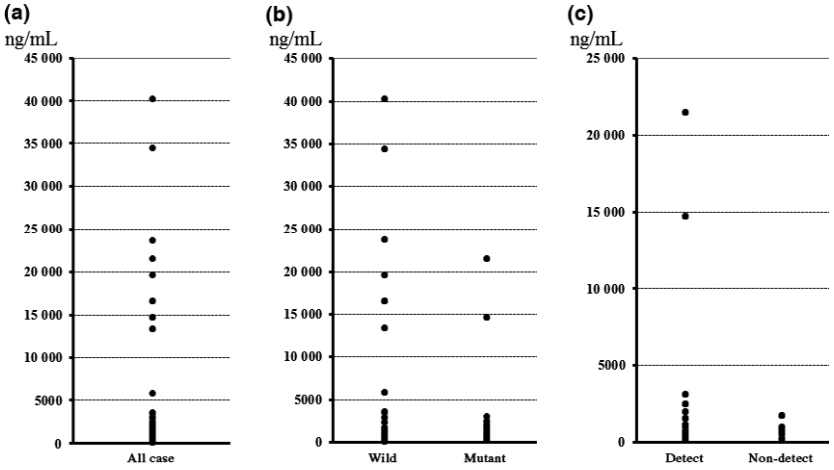

Fig. 1. Circulating cell-free (ccf)DNA yield for *KRAS* mutation analysis. (a) Median yield of ccfDNA from all 94 patients with metastatic colorectal cancer was 790 (110–40 300) ng/mL; the mean yield was 3343 ± 7398 ng/mL. (b) Median yield of ccfDNA from 39 patients with primary *KRAS* mutations was 720 (230–40 300) ng/mL; the median yield from the 55 patients without primary *KRAS* mutations was 885 (110–21 500) ng/mL ($P = 0.26$). (c) *KRAS* mutations were detected with the Inv-Clamp assay in the ccfDNA of 34 of the 39 patients with primary tumor *KRAS* mutations. Median yield of ccfDNA in *KRAS*-detectable cases (Detect) was 706 ng/mL, whereas that for the non-detectable (Non-detect) cases was 830 ng/mL ($P = 0.47$).

able to detect mutant *KRAS* in ccfDNA from 35 of the 39 patients with primary *KRAS* mutations (87%; Table 1). Patient characteristics of all individuals with primary *KRAS* mutations are shown in Table 2. The genotype of the primary tumor was coincident with the genotype of the ccfDNA in all cases except for five non-detectable patients (cases 4, 8, 16, 33, and 34). All discordant patients had small metastases (Fig. 2). Their CEA (<5 ng/mL) and CA19-9 (<37 U/mL) levels were normal (Table 2). Four of the five patients were treated with FOLFOX/bevacizumab and all patients obtained an objective clinical response. Three of these four patients (cases 4, 16, and 33) had a complete response (CR), whereas the other patient (case 8) showed an extended partial response (PR) over 18 months. For 6 of the 11 out of 39 patients with primary *KRAS* mutations but normal CEA and CA19-9 levels, *KRAS* mutations were also detected in the ccfDNA (Table 2).

Using the Inv-Clamp assay, a detection rate of 85% (34/39) was obtained, and dPCR yielded a detection rate of 80% (31/39). Case 26 was the only case that was detected by dPCR but not the Inv-Clamp assay. Cases 3, 9, and 12 were all detected by the Inv-Clamp assay but not by dPCR. Digital PCR analysis was also used to determine the number of mutations present in the ccfDNA (compared with wild-type DNA); this varied from 0.00% to 48.10%, with a median of 0.67%. For 15 patients, <1.00% of ccfDNA was determined to contain *KRAS* mutations (Table 2).

Analysis of ccfDNA *KRAS* mutations following curative resection. Curative resection of primary and metastatic lesions was carried out for 7 of the 34 patients (cases 2, 6, 7, 27, 35, 36, and 39) with primary *KRAS* mutations and ccfDNA *KRAS* mutations. Resection in five of the seven patients (cases 2, 6, 7, 27, and 36) resulted in the disappearance of the *KRAS* mutations from the ccfDNA within the first month following resection.

Three of these patients had tumor recurrence (cases 2, 6 and 7) and *KRAS* mutations in their ccfDNA reappeared before detection with any other methods (i.e., CT imaging or CEA and CA19-9 biomarker levels). Two of these patients (cases 27 and 36) had no tumor recurrence and no detectable *KRAS* mutations in their ccfDNA after resection.

For the remaining two patients (cases 35 and 39), the ccfDNA *KRAS* mutations had not disappeared 1 month subsequent to resection, but a reduction in the mutation rate was observed (case 35, 3.98% to 0.85%; case 59, 0.97% to 0.64%); tumor recurrence was still not detected 3 months after resection.

Case 6 had rectal cancer with large lymphatic metastases including the para-aortic lymph nodes. The clinical course and CT imaging for this patient is shown in Figure 3(a). Two months after the curative surgery, no *KRAS* mutations were detected in the ccfDNA. However, 8 months after the operation, a *KRAS* mutation was detected in the ccfDNA. Two months after detecting the *KRAS* mutation in the ccfDNA, a CT scan revealed a liver metastasis (Fig. 3b), and ccfDNA yield and the percentage of ccfDNA with *KRAS* mutations continued to increase. Lung metastases subsequently developed (Fig. 3c). Carcinoembryonic antigen was within normal limits until identification of the lung metastases, whereas CA19-9 (data not shown) remained within normal limits after identification of the lung metastases.

Case 7 had rectal cancer with liver and lung metastases. Low anterior resection was carried out. Treatment with FOLFOX and bevacizumab was given and the lung metastases disappeared (data not shown). Liver resection was subsequently carried out, and the *KRAS* mutation in the ccfDNA disappeared within 1 month after surgery. A *KRAS* mutation was detected in the ccfDNA 3 months after resection, but CEA and CA19-9 levels were within normal limits at that time. Recurrence of liver metastases was later detected with imaging 2 months after detecting the *KRAS* mutation in the ccfDNA.

Detection of *KRAS* mutations in ccfDNA of patients with wild-type primary tumors. Using Inv-Clamp assay and dPCR, *KRAS* mutations in the ccfDNA were detected in 5 of the 55 (9%) patients determined to have wild-type *KRAS* in their primary tumors (Table 1).

Chemotherapy with EGFR blockade was given to 24 of the 55 patients with wild-type primary tumors; these patient characteristics are shown in Table 3. Cases 47, 56, and 63, who had a *KRAS* mutation detected in their ccfDNA, did not show treatment response. Case 59 was the only patient who had no

Table 1. Detection rate of *KRAS* mutations in circulating cell-free (ccf)DNA of patients with stage IV colorectal cancer using the Inv-Clamp Assay and digital PCR (dPCR)

	ccfDNA *KRAS* wild-type (%)	ccfDNA *KRAS* mutants (%)
Primary *KRAS* wild-type ($n = 55$)	91	9
Primary *KRAS* mutants ($n = 39$)	13	87

Using the Inv-Clamp assay and dPCR, we were able to detect mutant *KRAS* in ccfDNA from 34 of the 39 patients (87%) with primary *KRAS* mutations. Using the Inv-Clamp assay, a detection rate of 85% (33/39) was obtained, whereas dPCR yielded a detection rate of 80% (31/39). *KRAS* mutations in the ccfDNA were detected in 5 of the 55 (9%) patients determined to have wild-type *KRAS* in their primary tumors by both methods (Inv-Clamp assay and dPCR).

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Table 2. Characteristics of patients with stage IV colorectal cancer with primary *KRAS* mutations

Case	Sex	Age, years	Primary site	Metastatic site	*KRAS*, primary	*KRAS*, ccfDNA	Mutation rate, %	CEA, <5.0 ng/mL	CA19-9, <37 U/mL
1	M	67	A-colon	Liver	G12V	G12V	16.30	7.8	8.6
2	M	70	A-colon	Liver	G12D	G12D	0.33	26.5	3726.0
3	M	67	Rectum	Liver, lung	G12D	G12D	0.00	231.0	1991.2
4	M	69	Rectum	Lung	G12V	Wild	0.00	3.5	9.2
5	F	68	T-colon	Peritoneum bone	G13D	G13D	0.39	3.5	3.9
6	F	66	Rectum	PA-LN	G12A	G12A	0.29	2.0	10.3
7	M	78	S-colon	Liver, lung	G12D	G12D	4.63	61.5	44.8
8	M	59	Rectum	Lung	G12V	Wild	0.00	2.4	16.5
9	F	69	Rectum	Peritoneum	G12V	G12V	0.00	55.2	38.7
10	M	82	Rectum	Lung	G12V	G12V	0.67	467.0	636.6
11	F	88	Cecum	Liver	G12V	G12V	48.10	433.7	8.1
12	F	67	Rectum	Liver, lung	G12A	G12A	0.00	2.3	15.4
13	F	65	A-colon	Lung	G13D	G13D	0.36	7.3	7.9
14	F	80	Cecum	Liver	G12V	G12V	24.70	34.7	153.6
15	F	81	A-colon	Peritoneum	G12D	G12D	4.21	34.3	44.6
16	M	74	A-colon	Lung	G12D	Wild	0.00	3.3	9.1
17	M	76	A-colon	Liver, lung	G12V	G12V	0.49	90.0	645.3
18	M	62	Rectum	Lung	G12V	G12V	3.49	14.2	25.9
19	M	70	A-colon	Peritoneum	G12D	G12D	0.78	3.5	35.5
20	F	62	A-colon	Liver, lung	G13D	G13D	18.22	965.6	4277.2
21	M	57	S-colon	Liver	G12D	G12D	2.25	3.9	203.4
22	M	69	S-colon	PA-LN	G12D	G12D	0.05	87.5	85.1
23	M	68	Rectum	Peritoneum	G13D	G13D	0.03	39.9	70.9
24	M	78	A-colon	Liver, peritoneum	G12V	G12V	0.46	142.5	474.0
25	F	74	S-colon	Liver, lung, bone	G12A	G12A	28.57	39.9	288.6
26	M	64	S-colon	Liver, lung	G12V	G12V	0.05	58.8	230.5
27	F	53	S-colon	Liver	G12D	G12D	0.68	54.2	2.0
28	F	82	Cecum	Liver	G12D	G12D	3.06	43.3	245.8
29	M	72	A-colon	Liver	G12D	G12D	2.18	8.0	23.4
30	M	56	Rectum	Liver	G12D	G12D	40.03	298.5	2.0
31	M	69	S-colon	Lung, peritoneum	G12D	G12D	0.05	87.5	85.1
32	M	67	Rectum	Lung, bone	G13D	G13D	3.95	98.0	1368.4
33	F	72	Cecum	Lung	G12V	Wild	0.00	4.9	2.0
34	M	84	S-colon	Liver	G13D	Wild	0.00	2.6	4.0
35	M	84	Rectum	Liver	G12S	G12S	3.98	2.6	2.0
36	M	82	Rectum	Liver	G12V	G12V	0.26	11.0	44.3
37	M	83	S–colon	Liver	G12V	G12V	35.52	6.5	7.1
38	M	70	S-colon	Liver, bone	G12V	G12V	41.72	2500.2	4219.0
39	M	73	S-colon	Liver	G12D	G12D	0.97	4.9	5.3

Using the Inv-Clamp assay and digital PCR, we were able to detect mutant *KRAS* in circulating cell-free (ccf)DNA from 35 of the 39 patients with primary *KRAS* mutations (87%). The genotype of the primary tumor was coincident with the genotype of the ccfDNA in all cases except five non-detectable patients. A-colon, ascending colon; CEA, carcinoembryonic antigen; F, female; M, male; PA-LN, para-aortic lymph node; S-colon, sigmoid colon; T-colon, transverse colon.

KRAS mutation in the ccfDNA and did not show treatment response. All other patients presented an objective response to therapy (1 CR, 19 PR).

Case 40 represents an instance wherein our assay was able to predict continued response to EGFR blockade. For this patient, a complete response lasted for over 18 months (data not shown). After 18 months of chemotherapy, treatment was stopped; 6 months later, a metastatic liver tumor was detected. Wild-type *KRAS* in the ccfDNA continued to be present; therefore, treatment with FOLFIRI and panitumumab was restarted. Three months after re-initiation of chemotherapy, the metastatic liver tumor had markedly decreased in size. The presence of wild-type *KRAS* in the ccfDNA continued throughout treatment (Fig. S5).

Eight of the 24 patients acquired resistance to EGFR blockade, and *KRAS* mutations in the ccfDNA were detected in five of these eight patients 2–3 months before detection of disease progression was possible with CT. The clinical course and CT imaging of case 41 is shown in Figure 4. In this case, PR to chemotherapy was observed for 12 months (Fig. 4a–c). The G12C *KRAS* mutation was then detected in the ccfDNA, but no tumor size increase was noted and both CEA and CA19-9 levels remained within normal limits. Two months after detecting the mutation, the liver tumor increased in size (Fig. 4d). Treatment with FOLFIRI and bevacizumab was given, but no response occurred. The patients died 9 months after the initial detection of the KRAS mutation.

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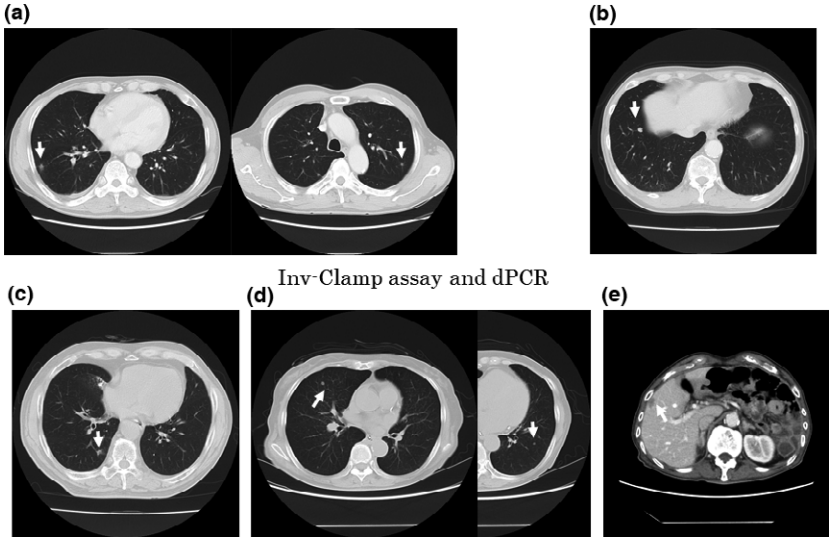

Fig. 2. Computed tomography of five patients with stage IV colorectal cancer in whom *KRAS* was not detectable. White arrows indicate the metastatic tumors. In all five cases, the size and the number of metastatic tumors were very small (<1.5 cm) (arrows). (a) Case 4. (b) Case 8. (c) Case 16. (d) Case 33. (e) Case 34.

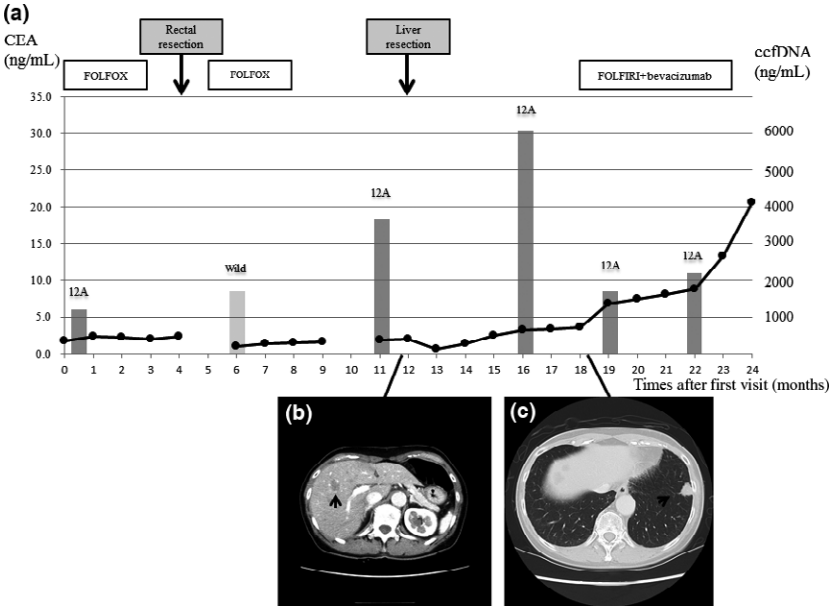

Fig. 3. Clinical course of a patient with metastatic colorectal cancer with a *KRAS* mutation that disappeared from the circulating cell-free (ccf)DNA following curative resection but subsequently re-appeared with tumor recurrence. (a) Carcino-embryonic antigen (CEA) (black circles), *KRAS* status, and ccfDNA yield (gray columns: light gray, wild-type; dark gray, mutated). Curative resection was carried out after six courses of mFOLFOX6 (oxaliplatin, folinic acid, and fluorouracil). (b) A computed tomography scan shows liver metastasis (arrow) 8 months later, leading to partial liver resection. (c) Lung metastasis (arrow) was detected 6 months after the resection, and mFOLFOX6 with bevacizumab was then administered. A *KRAS* mutation in the ccfDNA was detectable at the primary visit; this disappeared after curative resection but reappeared after recurrence of the liver metastasis. The ccfDNA yield (1050–6110 ng/mL) and mutation rate (0.29% to 2.64%) increased after the second resection. CEA was negative until detection of the lung metastasis.

Discussion

In this study, we showed the utility of *KRAS* mutation detection in the ccfDNA of patients with tumors containing *KRAS* mutations or wild-type *KRAS*. Many studies have reported the utility of detecting *KRAS* mutations in the ccfDNA of patients with *KRAS*-mutated tumors. However, few studies have reported its utility in patients with *KRAS*-wild-type tumors, and no studies have reported its utility as a biomarker for monitoring disease progression in patients with *KRAS*-mutated tumors. Based on the above results, it is apparent that *KRAS* mutations in ccfDNA could be used as a biomarker for the early detection of recurrence in mCRC patients with primary *KRAS* mutations. Moreover, this approach can provide real-time assessment of patients' mutation status and enable both prediction of the efficacy of EGFR blockade and analysis of acquired resistance to this blockade before clinical resistance is observed.

Using the Inv-Clamp assay and dPCR, we detected *KRAS* mutations in the ccfDNA of mCRC patients with primary *KRAS* mutations at a high rate (87%). Previous studies have reported detection rates for mutant *KRAS* in ccfDNA of 36–92%.[17–22] Our study was blinded but many of these previous studies were non-blinded (paired formalin-fixed, paraffin-embedded tumors and plasma). Detection rates can be affected by the detection method, tumor extent, and patient selection. Our patients included only stage IV patients, and our detection rate is second only to that reported by Thierry *et al.* (92%).[21] In that study, researchers recruited 106 patients but excluded 11 patients (10.4%) with insufficient sample quality. In our study, all recruited patients were included in the analysis, which may account for the slight difference in detection rates.

The reason for lack of detection in certain patients is unclear; however, it does not appear to be related to the yield of ccfDNA. In general, 1000 ng ccfDNA includes approximately 150 000 copies of the genome. In early studies, the ccfDNA concentrations obtained varied from 0 to >1000 ng/mL blood, with an average of 180 ng/mL.[23,24] However, advances in technology have allowed increased ccfDNA

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Table 3. Characteristics of patients with *KRAS*-wild-type primary colorectal tumors treated with epidermal growth factor receptor blockade

Case	Sex	Age	Primary site	Metastatic site	ccfDNA	Regimen	Best response	CEA	CA19-9
40	M	54	S-colon	Liver	Wild	FOLFIRI/Pmab	CR	2.3	11.9
41	F	70	S-colon	Liver	Wild	FOLFOX/Cmab	PR	19.7	85.2
42	F	79	Rectum	Liver	Wild	FOLFOX/Pmab	PR	2.3	4.4
43	M	66	A-colon	Liver, lung	Wild	FOLFIRI/Pmab	PR	22.3	169.8
44	M	68	Rectum	Liver	Wild	FOLFOX/Pmab	PR	24.4	24.2
45	F	66	T-colon	Liver	Wild	FOLFOX/Cmab	PR	15 000.0	693.2
46	M	60	Rectum	Liver	Wild	FOLFOX/Cmab	PR	9.6	45.1
47	M	71	Rectum	Liver	G13D	FOLFOX/Cmab	SD	9.7	25.9
48	M	73	Rectum	Lung	Wild	Irinotecan/Pmab	PR	39.4	53.1
49	M	79	Rectum	Liver	Wild	DeGramont/Pmab	PR	18.6	13.0
50	F	60	A-colon	Lung	Wild	FOLFOX/Cmab	PR	2.4	16.7
51	M	72	S-colon	Liver	Wild	FOLFOX/Cmab	PR	32.4	11.6
52	M	56	S-colon	Liver	Wild	FOLFOX/Pmab	PR	1223.0	1878.5
53	M	73	Rectum	Liver	Wild	FOLFOX/Cmab	PR	19.1	22.0
54	M	68	Rectum	Liver	Wild	FOLFOX/Pmab	PR	2722.0	11 405.8
55	M	69	Rectum	Liver	Wild	FOLFOX/Pmab	PR	403.6	110.8
56	M	51	A-colon	Peritoneum	G12A	FOLFOX/Cmab	SD	6.0	10.3
57	F	66	S-colon	Liver	Wild	FOLFOX/Pmab	PR	44.6	174.1
58	F	79	S-colon	Liver	Wild	FOLFOX/Cmab	PR	15 000.0	609.4
59	M	74	S-colon	Liver	Wild	FOLFOX/Pmab	PD	467.7	155.4
60	F	60	S-colon	Lung	Wild	DeGramont/Cmab	PR	13.1	22.7
61	M	60	Rectum	Lung	Wild	Irinotecan/Pmab	PR	17.4	35.8
62	F	37	Rectum	Liver	Wild	FOLFIRI/Pmab	PR	127.4	401.8
63	F	64	Rectum	Liver, lung	G12R	FOLFOX/Cmab	SD	38.5	21.5

Chemotherapy with epidermal growth factor receptor blockade was given to 24 of 55 patients with wild-type primary tumors. Cases 47, 56, and 63, who had a *KRAS* mutation detected in their circulating cell-free (ccf)DNA, did not show any treatment response. Case 59 was the only patient who had no *KRAS* mutation in the ccfDNA and did not show treatment response. All other patients presented an objective response to therapy (1 complete response [CR], 19 partial response [PR]). A-colon, ascending colon; CEA, carcinoembryonic antigen; Cmab, cetuximab; F, female; FOLFIRI, irinotecan, folinic acid, and fluorouracil; FOLFOX, oxaliplatin, folinic acid, and fluorouracil; M, male; PD, progressive disease; Pmab, panitumumab; S-colon, sigmoid colon; SD, stable disease; T-colon, transverse colon.

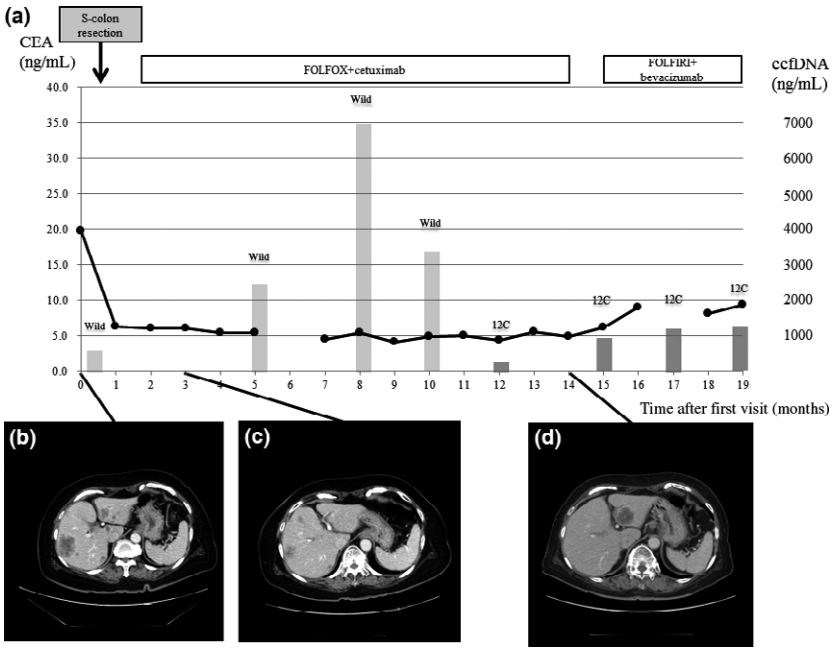

Fig. 4. Clinical course of a patient with stage IV colorectal cancer with a detected *KRAS* mutation in circulating cell-free (ccf)DNA before acquired resistance to epidermal growth factor receptor blockade. (a) Carcinoembryonic antigen (CEA) (black circles), *KRAS* status, and ccfDNA yield (gray columns: light gray, wild-type; dark gray, mutated). (b) Sigmoidectomy and treatment with mFOLFOX6 (oxaliplatin, folinic acid, and fluorouracil) and cetuximab were used for treatment of the liver metastasis. (c) The metastatic tumor decreased in size 3 months after starting chemotherapy. (d) Progression of the liver tumor was detected 14 months after initiation of chemotherapy, leading to treatment with FOLFIRI (irinotecan, folinic acid, and fluorouracil) and bevacizumab. No *KRAS* mutations in the ccfDNA were detected at the first visit or at 8 months after starting chemotherapy. We detected the G12C *KRAS* mutation in the ccfDNA 12 months after starting chemotherapy, followed by progression of the metastatic tumor 2 months after detecting the *KRAS* mutation in the ccfDNA.

extraction yields.[25] In our study, the median concentration of ccfDNA extracted was 790 ng/mL. In the five cases we observed with non-detectable primary *KRAS* mutations (i.e., when using the Inv-Clamp assay and dPCR), extraction yielded 275–1760 ng/mL ccfDNA. Of note, however, is that patients who had lower amounts of extracted ccfDNA (230, 234, 266,

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and 270 ng/mL) still had detectable *KRAS* mutations. Indeed, Taly *et al.*[22] reported that *KRAS* mutations can be detected in ccfDNA with a concentration as low as 100 ng/mL.

Another possibility for the lack of detection is that these patients had mutation rates lower than the detection limit. Using dPCR, we were able to detect *KRAS* mutations in the ccfDNA of 15 of 32 patients (47%) who had mutation rates <1.00%. Taly *et al.*[22] also reported that mutation rates observable with dPCR-based detection were <1.00% in 6 of 17 patients (35%). For case 8, we detected the *KRAS* mutation in the primary tumor but not in the ccfDNA. The *KRAS* mutation was detected in the lung metastasis obtained by CT-guided biopsy after disease progression, but the *KRAS* mutation was again not detected in the ccfDNA. Particularly with case 4 in mind, another possibility may be that these patients have discordant primary and metastatic tumors; we did not obtain samples specifically from the metastatic site to confirm or exclude this hypothesis. Discordance of *KRAS* mutations has previously been observed in 8% of mCRC patients.[7]

We can use *KRAS* mutations in ccfDNA as a tumor marker for the prediction of poor prognosis or for early detection of recurrence in patients with *KRAS* mutations. Importantly, we were able to detect *KRAS* mutations in the ccfDNA of patients who were both CEA- and CA19-9-negative. This result is similar to that of another study that also reported detection of *KRAS* mutations in the serum of patients with normal CEA levels.[18] Although CEA is a useful prognostic marker, its sensitivity is only 65% in stage IV CRC.[26] Using CEA to identify recurrent or metastatic disease in asymptomatic patients is valuable to identify candidates for survival-prolonging therapy;[27] however, the sensitivity for recurrence is 77% with a specificity of 98%,[28] indicating that the development of more sensitive biomarkers or supportive biomarkers is expected. *KRAS* mutations in ccfDNA may be just such a novel marker for patients with *KRAS* mutations because it can detect CEA-negative recurrence.

Additionally, CRCs that show *KRAS* mutations are associated with poor prognosis, and *KRAS* status is an independent prognostic factor indicative of overall and progression-free survival.[29] *KRAS* mutations in ccfDNA are also reported to be predictors of poor prognosis for CRC with distant metastases.[17] Thus, we believe that *KRAS* mutations in ccfDNA could be used as not only novel, sensitive biomarkers but also as prognostic indicators.

We can now determine the risk of recurrence in patients with primary *KRAS* mutations using ccfDNA. *KRAS* mutations in ccfDNA may disappear after removal of tumors with *KRAS* mutations within the short term, because ccfDNA is cleared from the blood and has a variable half-life in the circulation ranging from 15 min to several hours.[30] Thus, *KRAS* mutations detected in ccfDNA after surgery can indicate residual cancer in patients with *KRAS* mutation-containing tumors. In this study, *KRAS* mutations in ccfDNA disappeared following curative resection of the primary and metastatic tumors in five of the seven patients who underwent curative resection. However, three of these five patients had tumor recurrence and *KRAS* mutations in the ccfDNA reappeared before detection of recurrence was possible with CT or by analyzing the CEA or CA19-9 levels. Although this study included only three patients who had recurrence, the clinical course of these patients indicated that in addition to CEA and CA19-9 levels, the presence of *KRAS* mutations in the ccfDNA may be useful for early detection of recurrence. Two patients with *KRAS* mutations in their ccfDNA after curative resection did not have tumor recurrence within 3 months after operation; these patients should be monitored closely.

We can also now predict EGFR blockade efficacy in patients with wild-type *KRAS* primary tumors. We detected *KRAS* mutations in the ccfDNA of 5 of the 55 patients (9%) with wild-type primary tumors, one of whom was later found to have a metastatic liver tumor with a G13D *KRAS* mutation. Another study has also reported the detection of a ccfDNA *KRAS* mutation in one of 37 (4%) primary *KRAS* wild-type patients.[18] In our study cohort, 24 patients with primary wild-type tumors underwent EGFR blockade. Objective responses were observed in 20 of the 21 patients with wild-type *KRAS* in the primary tumor and in their ccfDNA. Conversely, the remaining three patients with a wild-type primary tumor but the presence of a *KRAS* mutation in their ccfDNA showed no treatment response. Although this was observed for only three cases it is highly suggestive, leading us to propose that the detection of a discordant patient by ccfDNA analysis should influence the choice of treatment.

We can predict acquired resistance to EGFR blockade by using ccfDNA before clinical resistance occurs. Diaz *et al.*[31] reported in a retrospective study that new *KRAS* mutations in ccfDNA were detected in 9 of 24 (38%) patients with mCRC refractory to EGFR blockade, before radiographic evidence of disease progression was observed. Morelli *et al.*[32] also showed in a retrospective study that new *KRAS* mutations in ccfDNA were detected in 27 of 62 (44%) patients with mCRC refractory to EGFR blockade. In this study, we prospectively showed that new *KRAS* mutations in ccfDNA were detected in five of eight patients before disease progression was detected with CT imaging. Although other reasons for acquired resistance were considered, no detection of any *KRAS* mutations during chemotherapy, which included EGFR blockade, occurred during 1 year of treatment. Furthermore, we did not detect *KRAS* mutations in the ccfDNA of another patient with wild-type *KRAS* who underwent similar treatment and maintained an optimal response. These data highlight the importance of monitoring *KRAS* mutation status during treatment, as new mutations can lead to acquired resistance.

Our study has several limitations. First, although it is a prospective study, it is a small study. Second, it included only metastatic patients. Future studies with larger numbers of patients will be required to validate the utility of *KRAS* mutation detection in ccfDNA, as well as to evaluate its utility in non-metastatic patients. Studies to determine the detection limit when using ccfDNA for metastatic cancer *KRAS* mutation detection for patients with small (<1 cm) metastases are also required.

In conclusion, we showed the utility of molecular diagnosis in the detection of *KRAS* mutations in ccfDNA, which is an attractive method for both monitoring and predicting response to EGFR blockade, as well as for the detection of recurrence or the prediction of prognosis.

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Disclosure Statement

The authors have no potential conflicts of interest to declare.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1–S3. Invader Plus Method and the results for *KRAS* point mutation detection in serum and plasma.

Figure S4. The results for *KRAS* point mutation detection in serum and plasma by using digital PCR.

Figure S5. A patient with primary and ccfDNA KRAS wild who respond well to EGFR blockade.

Table S1. Primers to detect *KRAS* mutations.

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