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ORIGINAL ARTICLE

Detection of *KRAS* Mutations in Plasma DNA Using a fully Automated Rapid Detection System in Colorectal Cancer Patients

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Abstract *KRAS* mutations have been recognized as predictive markers of primary resistance to anti-EGFR-antibodies in colorectal cancer patients. In addition, newly detected *KRAS* mutations have been reported to be related with acquired resistance to chemotherapy containing anti-EGFR antibody. Considering this evidence, monitoring of *KRAS* mutations is indispensable for making treatment decisions, and the method should be non-invasive allowing repeated examinations. Recently, we established a novel automated sensitive detection system for *KRAS* mutations, named mutation-biased PCR quenching probe system (MBP-QP). The goal of our study was to investigate the potential for monitoring KRAS mutations during treatment with anti-EGFR antibodies. The detection limit of MBP-QP using a control plasmid containing *KRAS* mutations was 1–9 copies, and 0.05–0.3% mutant plasmid was detectable in a mixture of wild type and mutants. One-hundred twenty colorectal cancer patients were genotyped for *KRAS* mutations with MBP-QP as well as polymerase chain reaction reverse sequence-specific oligonucleotide (PCR-rSSO), which has already been applied to cancer tissue samples in the clinical setting. Concordance rates between plasma DNA and cancer tissues were 68% with MBP-QP and 66% with PCR-rSSO, indicating that these systems are equivalent in terms of detecting *KRAS* mutations with plasma DNA. *KRAS* mutations in plasma DNA were frequently observed in systemic metastatic cancer patients, and in three patients *KRAS* mutations appeared after chemotherapy containing anti-EGFR antibody. A prospective study is needed for clarifying whether *KRAS* mutations detected in plasma DNA are predictive markers of treatment efficacy with anti-EGFR antibody.

Keywords Plasma DNA · *KRAS* mutation · Colorectal neoplasms · Molecular targeted therapy

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Introduction

Treatment of metastatic colorectal cancer has progressed rapidly since molecular targeted therapy including anti-EGFR monoclonal antibodies, such as cetuximab and panitumumab, was introduced [1, 2]. Mutations of *KRAS* encoding a GTP-binding protein that contributes to cell proliferation, angiogenesis, and tumor progression were detected in approximately 40% of colorectal cancers, and it has been reported to be related with efficacy of treatment by anti-EGFR antibodies [1–5]. Colorectal cancer patients with *KRAS* wild type

respond better to anti-EGFR antibodies than patients with *KRAS* mutations, indicating that these mutations are predictive markers for primary resistance to anti-EGFR-antibodies. Recently, it has been recommended that *KRAS* and *NRAS* mutations in exons 2, 3 and 4 be examined before treatment with anti-EGFR antibodies, since these mutations are also predictive markers [6, 7].

In addition to the relationship with primary resistance, these mutations were also detected in patients who acquired resistance to anti-EGFR antibodies [8–11]. Mechanisms of acquired resistance to anti-EGFR antibodies include activation of an alternative pathway, such as MET/HGF or EGFR, or activation of RAS/RAF/MAPK mediated through the appearance of *KRAS* mutations [12–14]. Because the emergence of *KRAS* mutations causes resistance to anti-EGFR antibodies, detection of these mutations is indispensable for deciding subsequent treatment. Although re-biopsy has been thought to be the standard method for collection of cancer species, molecular alterations vary depending on the region of biopsy because of tumor heterogeneity [15, 16]. Thus, we have focused on circulating plasma DNA for monitoring *KRAS* mutations during treatment with anti-EGFR antibodies.

We have newly established a fully-automated, sensitive detection system for *KRAS* mutations using the mutation-biased PCR and quenching probe (MBP-QP) method. The method has already been applied to the detection of *EGFR* mutations in lung cancer [17, 18]. Detection limits for T790 M and L858R are 2 and 5 copies, and sensitivities are 0.3 and 0.2%, respectively. T790 M mutation was detected with plasma DNA in 53% of lung adenocarcinoma patients who acquired resistance to EGFR-TKI in our retrospective study, and in 40% of those in a prospective, multi-institutional study [17, 19]. The new detection system for *KRAS* mutations used in this paper contained five types of mutations in codon 12 as well as G13D, which together cover 98% of *KRAS* mutations in colorectal cancer [20, 21]. The goal of our study was to investigate the potential for monitoring *KRAS* mutations during treatment with anti-EGFR antibodies. As the first step, we analyzed the concordance of *KRAS* mutations between cancer tissue and plasma DNA, and compared the result with PCR-rSSO, which has been already applied in the clinic [22].

Materials and Methods

Cell Lines and Human Samples

Human cancer cell lines A549, H226B, Calu-1, SK-Lu-1, HCT116, and RPMI8226 were purchased from the American Type Culture Collection (Manassas, VA), and RERF-LC-MT was kindly provided by Dr. Tomonori Hayashi of the Radiation Effects Research Foundation. These cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum at 37°C in 5% CO_2. Tissue and patient-matched plasma samples were obtained from 120 colorectal cancer patients who underwent treatment at Saga Medical School Hospital, the Cancer Institute Hospital of the Japanese Foundation for Cancer Research, Saitama Medical Center, Jichi Medical University, or Saga Prefectural Hospital between 2013 and 2015. The examination of *KRAS* mutation status in primary lesions was performed using the TheraScreen®: K-RAS Mutation Kit in the laboratories of SRL, Inc. (Tokyo, Japan), and using the MEBGEN™ KRAS kit in the Cancer Institute Hospital. The examination using TheraScreen® contained seven different kinds of *KRAS* mutations, G12S, G12C, G12R, G12D, G12 V, G12A, G13D, and that using the MEBGEN™ KRAS kit contained twelve kinds of *KRAS* mutations, G12S, G12C, G12R, G12D, G12 V, G12A, G13D, G13S, G13C, G13R, G13 V, and G13A. Examination of *KRAS* mutations in cancer tissue was mostly performed before the start of treatment. Pathological stage was determined according to criteria of the 8th edition of the Japanese Classification of Colorectal Carcinoma. The study protocol was approved by each institute, and all patients gave informed consent for obtaining surgical specimens according to the Declaration of Helsinki.

Preparation of Control Plasmid and Analysis of *KRAS* Mutations

The control plasmid was prepared as follows by GenScript USA, Inc. A 320 bp DNA fragment (Accession No. NG_007524, 10,380–10,699) was obtained by PCR, purified, and subcloned into the pUC57 vector. Genomic DNA was isolated from cancer cell lines using the QIAamp® DNA mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Peripheral blood samples from colorectal cancer patients were collected into tubes containing 3.8% citric acid, and plasma was immediately separated by 3000 rpm centrifugation at 4 °C for 20 min. DNA was isolated from 200 μl of patient plasma using a QIAamp® DNA mini kit, as described previously [17–19]. *KRAS* mutations of codon 12/13 including G12A, G12C, G12D, G12S, G12 V, and G13D were determined by the mutation-biased PCR and quenching probe system (MBP-QP) using the fully automated genotyping system i-densy™ IS-5320 (ARKRAY Inc., Kyoto, Japan) (Fig. 1). The MBP-QP method was already established for detecting *EGFR* mutations T790 M and L858R, as described previously [17–19]. In brief, the system includes an MBP step, modified PCR, and a QP system for detection using a TAMRA-conjugated, guanine-specific quenching fluorophore probe (QProbe, J-Bio21, Tokyo, Japan). The MBP-QP method covered 6 different *KRAS* mutations: G12A, G12C, G12D, G12S, G12 V, and G13D.

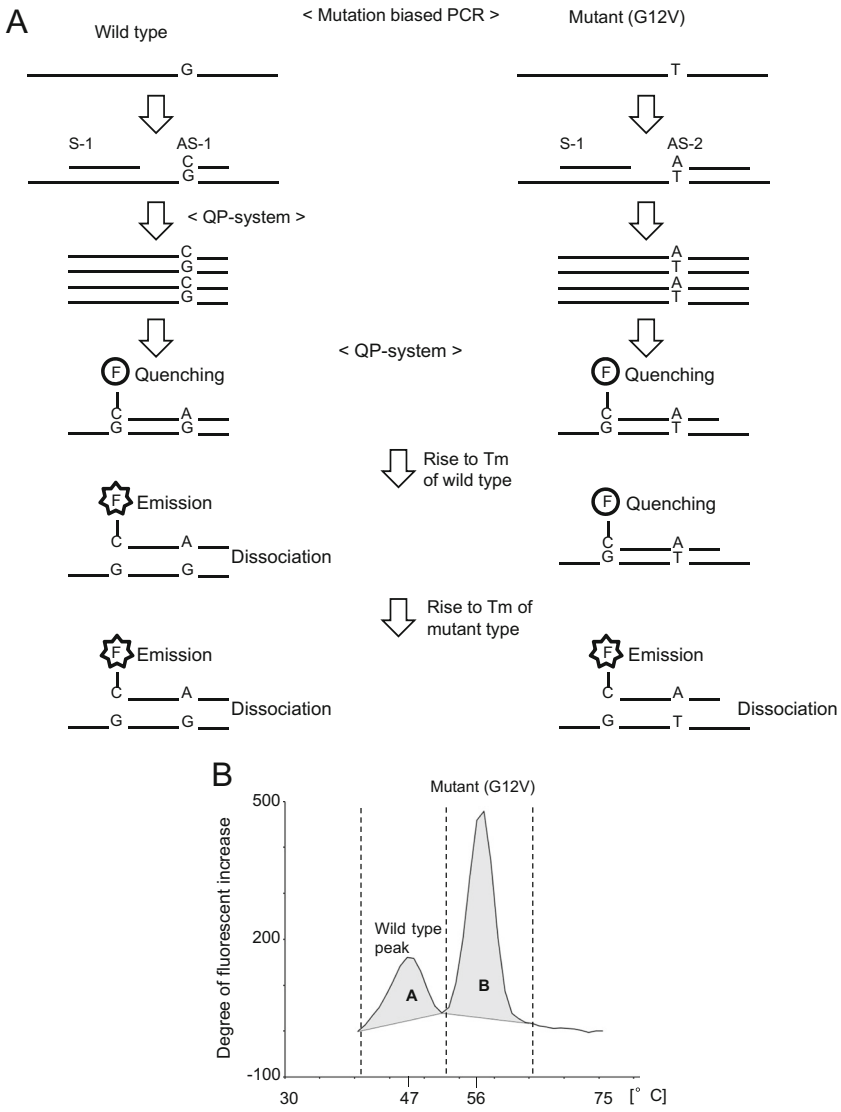

Fig. 1 **a** Principles of the MBP-QP method. Figure 1a illustrates the method in the case of *KRAS* mutation G12 V. The MBP-QP method consists of mutation-biased PCR (MBP) and a quenching probe (QP)-system. In MBP, the difference in primer lengths for wild type and mutant sequences leads to a higher efficiency of amplification of the mutant sequence relative to that of the wild type. Presence of *KRAS* mutant amplicons is determined by the QP-system using specific guanine quench fluorophore probe. Because the probe for wild type is mismatched, melting temperatures (Tm) differ, resulting in the ability to distinguish between wild-type and mutant amplicons. **b** The result with MBP-QP method using genomic DNA isolated from RERF-LC-MT (p. G12 V). Areas under the curve of wild type (A) and mutant (B) are shown

Criteria for declaring a sample mutation-positive were defined by the areas under each mutation peak divided by that of the wild type peak. Area under the peak was determined by the "idensy AreaAna®" software (ARKRAY Incorporated, Japan). *KRAS* mutations with plasma DNA were also analyzed by polymerase chain reaction reverse sequence-specific oligonucleotide (PCR-rSSO), GENOSEARCH™ HS KRAS kit (MEDICAL & BIOLOGICAL LABORATORIES CO., LTD., Nagano, Japan) using the Luminex® 100/200™ system (Luminex Japan Corporation Ltd., Tokyo, Japan), which contains a system for detecting thirteen different *KRAS* mutations: G12S, G12C, G12R, G12D, G12 V, G12A, G13D, G13S, G13C, G13R, G13 V, G13A and Q61H. Because the two systems for detecting *KRAS* mutations in primary lesions, the TheraScreen®: K-RAS Mutation Kit and the MEBGEN™ KRAS kit, did not contain Q61H, data on Q61H with plasma DNA were excluded in the analysis comparing tissue and plasma DNA.

Statistical Analysis

Association between *KRAS* mutations in plasma DNA and clinicopathological characteristics was analyzed using the chi-square test for contingency tables. Statistical analyses were conducted using IBM SPSS Statistics 22 (SPSS Inc., IBM Company).

Results

Detection Limits for *KRAS* Mutation

The detection limit of the MBP-QP method using control plasmids containing six types of *KRAS* mutations at codon 12/13 was examined. The detection limits were 1–9 copies, and 0.05–0.3% mutant plasmid was detectable (Table 1). We also analyzed genomic DNA isolated

Table 1 Detection of *KRAS* mutations using the MBP-QP method

	Threshold	
	Quantity	Ratio (%)
Plasmid		
G12A	9 copy	0.05*
G12C	1 copy	0.05*
G12D	3 copy	0.10*
G12S	1 copy	0.10*
G12 V	8 copy	0.30*
G13D	2 copy	0.30*
Genomic DNA		
G12A (RPMI8226)	0.02 ng	0.10†
G12C (Calu-1)	0.04 ng	0.03†
G12D (SK-Lu-1)	0.02 ng	0.30†
G12S (A549)	0.02 ng	0.03†
G12 V (RERF-LC-MT)	0.04 ng	0.30†
G13D (HCT116)	0.02 ng	0.30†

* Percentage of control plasmids carrying *KRAS* mutations relative to the amount of wild type plasmid in the mixture

† Percentage of genomic DNA isolated from RPMI8226, Calu-1, SK-Lu-1, A549, RERF-LC-MT, or HCT116 in the mixture with that from H226B (wild type)

MBP-QP, mutation biased PCR-quenching probe system

from six cancer cell lines in which the *KRAS* mutation status was known. The detection limit was 0.02–0.04 ng DNA (Table 1). When genomic DNA isolated from H226B, which carries *KRAS* wild type, was combined with those from the six cell lines having *KRAS* mutations, 0.03–0.3% of mutants could be detected.

KRAS Mutations Were Detected with Plasma DNA Isolated from Colorectal Cancer Patients

One hundred and twenty colorectal cancer patients were examined for *KRAS* mutation status with plasma DNA. Characteristics of the patients are shown in Supplementary Table S1. They comprised 56% with colon cancer and 44% with rectal cancer. Most of the patients' tumors were at stage IV (60%) at the time of peripheral blood collection, and sample collection was conducted during chemotherapy in 82% of the cases. Chemotherapy containing anti-EGFR antibody was administered in 29 patients. *KRAS* mutations at codon 12/13 in cancer tissues were detected in 41% of the patients and frequency of mutation types was similar to that reported in the Catalogue of Somatic Mutations in Cancer (COSMIC) database (20, 21). With plasma DNA, among patients in whom *KRAS* mutations were positive in cancer tissue, *KRAS* mutations were detected in 33% using MBP-QP and 33% using PCR-rSSO (Table 2). Frequency of mutation type G12D was higher, and G12 V was lower with MBP-QP than with PCR-rSSO, which corresponds with results obtained using control plasmid and genomic DNA isolated from cancer cell lines. Among the patients with *KRAS* wild type in primary lesions, *KRAS* mutations were observed with plasma DNA in two patients using MBP-QP and in one patient using PCR-rSSO (Table 3). Concordance rates were 68% in MBP-QP and 66% in PCR-rSSO, indicating that these systems are equivalent in terms of detecting *KRAS* mutations in plasma DNA. The detection rate did not differ according to stage, mutation type, or history of chemotherapy (Table 4). However, detection rate was significantly higher in the patients with metastatic colorectal cancer involving three or more organs, although the detection rate was 32% among entire patients with metastasis. *KRAS* mutations tend to be less frequent in patients during chemotherapy than in patients not treated with chemotherapy, although the difference was not statistically significant. In addition, *KRAS* mutations were more frequently detected in plasma DNA among colon cancer patients

Table 2 Mutation analysis with plasma DNA

	Plasma DNA	
KRAS mutations in tissue	MBP-QP	PCR-rSSO
Total (n = 49)	16 (33%)	16 (33%)
Mutation type, no. (%)		
G12A (n = 1)	0	0
G12C (n = 5)	2 (40%)	2 (40%)
G12D (n = 13)	5 (38%)	3 (23%)
G12R (n = 5)	-	0 (0.0)
G12S (n = 1)	1 (100%)	1 (100%)
G12 V (n = 11)	3 (27%)	5 (45%)
G13D (n = 13)	5 (38%)	5 (38%)

MBP-QP, mutation biased PCR-quenching probe system;
PCR-rSSO, PCR-reverse sequence-specific oligonucleotide

Table 3 Concordance between results with cancer tissue and those with plasma DNA

		Plasma DNA				
		MBP-QP		PCR-rSSO		
	KRAS	Mutant	WT	Mutant	WT	NE
Tissue	Mutant (n = 49)	14	35	15	34	0
	Wild type (n = 67)	2	65	1	64	2
	Total (n = 116)	16	100	16	98	2

MBP-QP, mutation biased PCR-quenching probe system;
PCR-rSSO, PCR-reverse sequence-specific oligonucleotide; WT, wild type, NE, not evaluated

Table 4 Characteristics of patients with *KRAS* mutations detected in plasma DNA analysis using MBP-QP among the patient with *KRAS* mutations in tumor tissue

		$n = 44^{*}$
Characteristics	No.	$P^{\dagger}$
Detection with plasma DNA, no. (%)	14/44 (32)	
Clinical stage, no. (%)		
I	0/0 (0)	0.58
II	2/5 (40)	
III	3/16 (19)	
IV	9/23 (39)	
Mutation type, no. (%)		
G12A	0/1 (0)	0.73
G12C	2/5 (40)	
G12D	4/13 (31)	
G12S	1/1 (100)	
G12 V	3/11 (27)	
G13D	4/13 (31)	
Chemotherapy, no. (%)		
Naive	4/8 (50)	0.22
During treatment	10/36 (28)	
No. of organs with cancer involvement, no. (%)		
< 3	4/25 (16)	0.01
≥ 3	10/19 (53)	

* The cases with G12R detected in cancer tissues were excluded

† Exact *p* value based on the chi-squared test

MBP-QP, mutation biased PCR-quenching probe system

than in rectal cancer patients, and the difference was statistically significant.

KRAS Mutations in Plasma DNA Appeared after Treatment with Anti-EGFR Antibody

When *KRAS* mutation was not detected in cancer tissues, the results in plasma DNA were consistent with those in cancer tissues except for three patients (Table 3). The clinical courses of these patients are shown in Fig. 2. *KRAS* was wild type in cancer tissue before treatment in all three cases. After treatment with chemotherapy including anti-EGFR antibody, *KRAS* mutations G12D, G13D, and G12 V were detected in plasma DNA of these three patients. Peripheral blood was obtained after acquired resistance to chemotherapy containing anti-EGFR antibody in all three patients.

Discussion

In this paper, we demonstrated that *KRAS* mutations are detectable in plasma DNA using the MBP-QP method. Detection rate and concordance with cancer tissues using MBP-QP were equivalent to those obtained with PCR-rSSO, which has been already applied to cancer tissue in the clinical setting. *KRAS* mutations were frequently detected in systemic metastatic cancers, and these were newly detected after acquired resistance to anti-EGFR antibody.

Recently, mutation analysis using peripheral blood—so-called liquid biopsy—has been applied to colorectal cancer [9, 10, 23–25]. In most published reports, quantitative PCR and BEAMing were used for detection with plasma DNA. Among colorectal cancer patients with *KRAS* mutation detected in cancer tissue, the same mutations were observed with

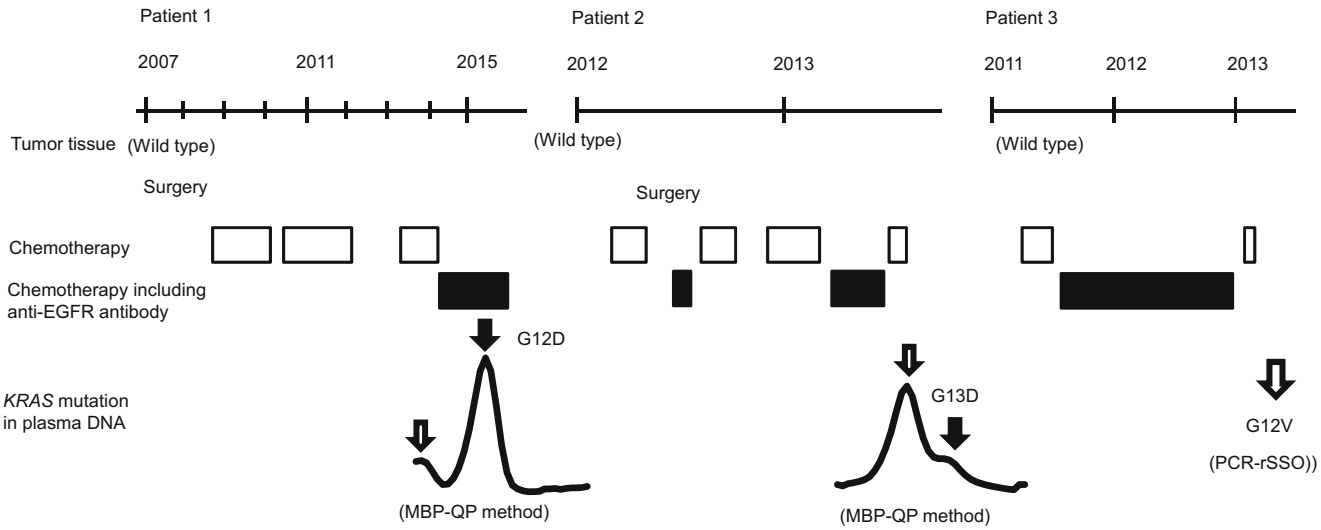

Fig. 2 Clinical courses of three colorectal cancer patients, all of whom had wild type *KRAS* in cancer tissues at the time of diagnosis and were treated with chemotherapy containing anti-EGFR antibody. Mutation status of *KRAS* with plasma DNA was analyzed after the start of chemotherapy using the MBP-QP and PCR-rSSO methods. White and black arrows in "*KRAS* mutation in plasma DNA" indicate the peaks for *KRAS* wild type and mutant type, respectively

plasma DNA in from 76% to 98% of cases using these detection methods [21, 23, 24]. According to results using digital PCR, tumor derived DNA was detected in more than 75% of colorectal cancer patients, and the detection rate was equivalent to advanced pancreas, ovarian, breast, and gastroesophageal cancers [24]. *KRAS* mutations with plasma DNA are sometimes detected even if *KRAS* mutations were not found in cancer tissues [21]. Technology for mutation detection has progressed recently, and the sensitivity has been reported to be less than 0.01% with some methods. However, the relationship between mutation detection and treatment efficacy has not yet been elucidated. According to a recent report, quantity of *KRAS* mutations in plasma was significantly correlated with efficacy of chemotherapy using cetuximab and irinotecan [26]. A higher level of mutated *KRAS* in plasma detected by quantitative PCR corresponded with a lower disease control rate (DCR); patients with greater than the upper quartile (75%) of *KRAS* mutation level had a DCR of 0%, whereas those with less than the upper quartile had a DCR of 42%. These data indicate that the anti-cancer effect of chemotherapy containing anti-EGFR antibody is correlated with the mutant load.

The detection rate using MBP-QP was relatively low compared to other detection systems previously reported [21, 23, 24]. It seems to be related to the fact that patients in our study included non-metastatic cancer patients, and sample collection was conducted during chemotherapy in 2/3 of those patients. Due to the inclusion of patients with a variety of clinical features, some clinical characteristics related with detection of *KRAS* mutations in plasma DNA could be observed. First, it was frequently detected in systemic metastatic patients, especially those with involvement of three or more organs. Second, the frequency was higher before treatment than during chemotherapy. These results suggest that appearance of tumor derived DNA in peripheral blood is related with tumor burden and tumor progression. According to our previous paper, detection of tumor derived DNA in plasma was associated with tumor burden in an animal model with systemic metastasis [27]. The detection rate was also significantly associated with metastasis. It seems reasonable to assume that shrinking tumor volume, including metastatic lesions after chemotherapy, caused the disappearance of tumor-derived DNA in peripheral blood. The rate of detection of *KRAS* mutations in plasma among colon cancer patients was significantly higher than among rectal cancers. Considering that it has been reported that the outcome of colon cancer is worse than that of rectal cancer, the result would also seem to be associated with tumor progression [28]. In addition, we confirmed that *KRAS* mutations were newly detected after acquired resistance to anti-EGFR antibody contained in chemotherapy, as other papers have reported. Since the first report of a patient with acquired resistance to cetuximab having newly detected *KRAS* mutation in cancer tissue, several papers have demonstrated acquired *KRAS* mutations, including mutations detected in plasma [8, 10, 11]. The mechanism of appearance of *KRAS* mutations after acquired resistance to anti-EGFR antibody has been assumed to be clonal selection from a minor population of pre-existing mutations or novel spontaneous mutations, but this mechanism has not been clarified.

The goal of our study was to investigate the potential for monitoring *KRAS* mutations during treatment with anti-EGFR antibodies. This system has a possibility to become an alternative method when biopsy is difficult to perform, as with recurrent cases demonstrating distant metastases. Investigation of mechanisms of acquired resistance to molecular targeted therapy would be the most promising application of our system. As the first step, we examined the sensitivity of our system and the clinical characteristics of patients with tumor-derived DNA. After improving the sensitivity of our system and extending it to allow examination of other kinds of mutations, including all three *RAS* genes, the next step will be to investigate whether mutation detection can facilitate making decisions regarding subsequent treatment strategy. To solve that question, it is urgently needed to proceed to conducting a prospective study investigating the relationship between the anti-cancer effect of chemotherapy containing anti-EGFR antibody and the detection of *KRAS* mutations with plasma DNA.

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Compliance with Ethical Standards

Conflict of Interest Statement Toshiya Hosomi and Mitsuharu Hirai are employees of ARKRAY Inc.

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