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Clinical Study

Phase II Trial of Cetuximab plus Irinotecan for Oxaliplatin- and Irinotecan-Based Chemotherapy-Refractory Patients with Advanced and/or Metastatic Colorectal Cancer: Evaluation of Efficacy and Safety Based on *KRAS* Mutation Status (T-CORE0801)

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Key Words
Cetuximab · Colorectal carcinoma · Epidermal growth factor receptor · *KRAS*

Abstract
Background: Mutations in the *KRAS* gene have been identified as negative predictors of response to anti-epidermal growth factor receptor (EGFR) monoclonal antibody therapy by patients with metastatic colorectal cancer (mCRC). However, it has been based on the study of mainly Caucasian mCRC patients. This prospective study investigated the relationship between the mutation status of EGFR-related genes including *KRAS* and the response rate (RR) to cetuximab plus irinotecan therapy in Japanese mCRC patients. ***Methods:*** Samples taken from 43 chemotherapy-refractory mCRC patients who had undergone cetuximab plus irino-

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tecan therapy at 11 medical centers in Japan were subjected to direct DNA sequencing to determine the *KRAS*, *BRAF*, *PIK3CA*, *NRAS*, and *AKT1* mutation status. The clinical outcome after the treatment was evaluated for each mutation status. ***Results:*** *KRAS* mutations were detected in 31.7% of 41 eligible patients. The RR to cetuximab plus irinotecan therapy was found to be 17.9 and 0% in the *KRAS* wild-type and mutant subgroups, respectively. ***Conclusion:*** Despite the identification of a lower-than-expected RR to treatment by the *KRAS* wild-type subgroup, *KRAS* mutation status appears to be a useful predictive marker of response to cetuximab plus irinotecan therapy in Japanese mCRC patients.

Introduction

Anti-epidermal growth factor receptor (EGFR) monoclonal antibody (mAb) therapy has been established as the standard therapy for advanced colorectal cancer (CRC). However, retrospective examination of tumors from metastatic colorectal cancer (mCRC) patients who had undergone first and subsequent lines of anti-EGFR mAb therapy has revealed that tumors with *KRAS* mutation are resistant to cetuximab or panitumumab therapy [1–8]. Based on these findings, *KRAS* mutations have been identified as negative predictors of response to anti-EGFR mAb therapy. However, the identification of *KRAS* mutations as negative markers was confirmed by the findings of prospective studies of the effectiveness of panitumumab in treating Caucasian mCRC patients, while few prospective studies have examined its effectiveness in Asian populations [6]. Moreover, a clear ethnic difference in the frequency of *EGFR* mutations, a critical predictor of response to gefitinib in patients with non-small cell lung cancer, has been found between Caucasian and Asian populations, with *EGFR* mutations identified in approximately 30–60% of Asian patients with non-small cell lung cancer compared to approximately 10–20% of Caucasian patients [9]. Despite this finding, differences between Caucasian and Asian mCRC patients in the prevalence of mutations of *KRAS* and other EGFR-downstream genes, including *BRAF, PIK3CA, NRAS,* and *AKT1,* have not been fully evaluated.

In fact, more than half of the patients with *KRAS* wild-type tumors have been found to be primarily resistant to anti-EGFR antibodies, and the results of several studies suggest that *BRAF* and *PIK3CA* mutations abrogate the efficacy of anti-EGFR mAb therapy [10, 11].

Mutation of the *BRAF* gene, which occurs in approximately only 5–10% of patients with wild-type *KRAS,* has also been shown to be both a prognostic factor and predictive factor of cetuximab response [11–13]. As such, the clinical significance of *BRAF* mutations cannot be simply evaluated. As the *BRAF* gene encodes a serine-threonine kinase function downstream of the *KRAS* gene, constitutively active *BRAF* mutations are mutually exclusive of *KRAS* mutations. Moreover, mutation of the *PIK3CA* gene, which encodes the catalytic subunit p110α of *PI3K,* occurs in approximately 15–20% of CRC patients. Because tumor-derived mutant *PI3K* stimulates the AKT pathway and promotes cell growth in several cancers, including CRC, *PIK3CA* mutations have also been associated with poor prognosis and significantly impaired response to anti-EGFR mAb therapy in mCRC patients [14, 15]. In addition, recent contradictory evidence indicates no strong rationale for using the presence of *PIK3CA* mutations as a single predictive marker of cetuximab response in chemotherapy-refractory mCRC [16]. A large-scale European study reported that the identification of *KRAS, BRAF, NRAS,* and *PIK3CA* mutation status improved the prediction of response to anti-EGFR mAb therapy [17]. Another study found that mutation of *AKT1,* a downstream gene in the PI3K/AKT pathway, occurs in 6% of mCRC patients [18]. E17K, which is a somatic hot-spot mutation in the *AKT1* gene, results in the pathological localization of AKT1 to the plasma membrane, and the constitutive activation of the downstream signal transduction.

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Although the mutation status of *KRAS* and that of other EGFR-downstream genes should be validated as a predictive marker of anti-EGFR mAb therapy in Asian populations by a prospective study, no well-designed studies have been attempted so far. To fill this research gap, this prospective study evaluated the relationship between *KRAS* mutation status and the several clinical outcomes, such as response rate (RR) to cetuximab-based therapy, progression-free survival (PFS), overall survival (OS) after cetuximab-based therapy in Japanese mCRC patients who had failed to respond to prior chemotherapies consisting of irinotecan, oxaliplatin, and fluoropyrimidine treatment and for whom no other standard anticancer therapy had been available. To contribute to the optimization of the selection of patients most likely to benefit from anti-EGFR mAb therapy, the association between *BRAF* V600E and *PIK3CA* mutations in exons 9 and 20; *NRAS* mutations in codons 12, 13, and 61, and *AKT1* E17K mutations and clinical outcome was investigated.

Materials and Methods

Patients

Enrollment of eligible patients began in October 2008 and concluded in May 2010, which is almost equivalent to the period from just after the approval of cetuximab until just before the approval of the *KRAS* examination to predict the efficacy of cetuximab-based therapy in CRC in Japan. Inclusion criteria were: (1) age 18 years or older, (2) presence of histologically confirmed EGFR-positive CRC, (3) presence of unresectable primary or metastatic tumor and presence of measurable lesions based on the Response Evaluation Criteria in Solid Tumors (RECIST) ver. 1.0 criteria, (4) Eastern Cooperative Oncology Group (ECOG) performance status score of ≤2, (5) adequate hematological function (neutrophil count ≥1,500/μl, platelet count ≥100,000/μl, and hemoglobin count ≥8.0 g/dl), (6) adequate hepatic function (aspartate aminotransferase and alanine aminotransferase levels ≤100 IU/μl and bilirubin level ≤2.0 mg/dl), (7) adequate renal function (serum creatinine level <1.5 mg/dl), (8) treatment with more than 2 rounds of oxaliplatin- or irinotecan-based chemotherapies, (9) elapsed time of the appropriate interval from a previous treatment (4 weeks from radiotherapy, 2 weeks from surgical intervention with some organ resection, 2 weeks from chemotherapy, and 4 weeks from another form of treatment provided during a clinical trial), and (10) a prognosis of more than 2 months. The protocol was approved by the Ethics Committee of Tohoku University School of Medicine and all patients provided written informed consent.

Study Design

Before enrollment, the tumor specimens collected from all patients had been confirmed to exhibit EGFR expression in more than 1% of malignant cells, as determined by immunohistochemistry with the Dako EGFR PharmDx kit (DakoCytomation, Glostrup, Denmark), and all patients confirmed not to have undergone previous anti-EGFR mAb therapy. After enrollment, patients were intravenously administered cetuximab at a standard dosage of 400 mg/m^2 over 2 h on day 1 of treatment, followed by 250 mg/m^2 over 1 h on 1 day per week, and intravenously administered irinotecan at two standard dosages which were approved for CRC treatment in Japan based on the phase II study (150 mg/m^2 every 2 weeks or 100 mg/m^2 weekly for 3 consecutive weeks, followed by 1 week of rest) [19]. Patients were evaluated for tumor response or progression every 8 weeks by means of radiologic imaging. If severe skin toxicity (more than grade 3) happened, cetuximab treatment was postponed until the skin toxicity went down to less than grade 2 and was reduced to 200, 150, and 100 mg/m^2 in each event. If a severe hematological (neutropenia more than grade 4, thrombocytopenia more than grade 2) or nonhematological adverse event (more than grade 3) was observed, irinotecan treatment was postponed until these events recovered and was reduced to 80, 60 and 50% of doses in each event. Cetuximab-based treatment was continued until disease progression or unacceptable toxicity occurred. Even though *KRAS* mutant patients would be expected to show a lower RR in Japanese as well as Caucasian populations, we did not plan an interim analysis or two-stage design because the study period was limited from the approval of cetuximab treatment to that of *KRAS* gene analysis in Japan. Only in this period were cetuximab treatments for *KRAS* mutant patients allowed ethically, since cetuximab treatments were performed without *KRAS* analyses in clinical practice.

Tumor Collection and Processing

Formalin-fixed paraffin-embedded samples of tumor tissue from archival specimens that had been collected at the time of diagnosis were stored at Tohoku University Hospital. The extent of *KRAS, BRAF, PIK3CA, NRAS,* and *AKT1* mutation was assayed at the Department of Clinical Oncology, Institute of Development, Aging and Cancer, Tohoku University. All patients' samples were screened for *KRAS* mutations in codons 12, 13, and 61; *BRAF* V600E and *PIK3CA* mutations in exons 9 and 20; *NRAS* mutations in codons 12, 13, and 61; *AKT1* mutations in codon 17, and *PIK3R1* mutations in exon 16. All available tissue samples were categorized into either a *KRAS* mutant or a *KRAS* wild-type subgroup depending on the results.

Nucleotide Sequence Analysis

Mutation analysis was performed by extraction of genomic DNA from macro-dissected formalin-fixed paraffin-embedded tissue slides or sections. DNA was extracted using the QIAamp DNA formalin-fixed paraffin-embedded tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The appropriate sites of each gene were amplified using nested primer sets under specified cycle and temperature conditions and analyzed by 1.0% agarose gel electrophoresis. Reactions were performed using the automated CEQ2000XL DNA analysis system (Beckman Coulter, Fullerton, Calif., USA) and including the appropriate positive and negative controls for each gene to be analyzed. To minimize bias, the researchers who performed the mutation analyses remained blinded to the clinical outcomes.

Statistical Analysis

Statistical analysis of categorical variables was performed using the χ^2 test and RR was defined according to RECIST ver. 1.0 criteria. An independent review committee assessed response and the time to progression during the study according to RECIST criteria and categorized patients as either responders who had achieved complete response (CR) or partial response (PR) or as nonresponders who showed stable disease (SD) or progressive disease (PD). To determine the sample size, the expected RR was set at 30%, the RR threshold at 10%, the significance level for the results of one-sided testing at 5%, and the power at 80% for the *KRAS* wild-type subgroup. After estimating the proportion of patients in each *KRAS* subgroup (wild-type and mutant) and the number of patients in the *KRAS* wild-type subgroup (approximately 24), it was determined that the sample should consist of at least 40 patients.

To evaluate the RR, the primary endpoint, the study began by testing the hypothesis that the RR of the *KRAS* wild-type subgroup is equal or less than 10% assuming the efficacy of cetuximab to be similar between Japanese and Caucasian populations. If a significant treatment effect ($p \leq 0.05$) as measured by RR was identified for the *KRAS* wild-type subgroup, then the *KRAS* mutant subgroup would be analyzed similarly. PFS was defined as the time from the beginning of chemotherapy until the first objective evidence of disease progression or death from any cause and was determined using the Kaplan-Meier method. The survival curves developed from the results of the determination of PFS of each subgroup were compared using the log-rank test. The level of statistical significance was set at 0.05 for the results of one-sided testing for RR and the results of two-sided testing for other variables.

Results

Patient Characteristics

The clinical characteristics of the patients are listed in table 1. As can be observed, of the 43 patients who had undergone cetuximab-based treatment, 28 were categorized as ECOG performance status 0 and 15 as ECOG performance status 1. All patients had failed to respond to prior chemotherapy, including irinotecan, oxaliplatin, and 5-fluorouracil treatment, and none had previously been treated with anti-EGFR mAb therapy. The 41 patients who had undergone oxaliplatin-based therapy were treated only by the FOLFOX regimen (infusion and bolus of 5-fluorouracil plus oxaliplatin). Treatment of the 42 patients who had undergone irinotecan-based therapies was based on the FOLFIRI regimen (infusion and bolus of 5-fluorouracil with irinotecan) for 34 patients, S-1 plus irinotecan for 6 patients and irinotecan monotherapy for 2 patients. Bevacizumab therapy had been administered as first-line, second-line, or both lines of treatment to 26 patients.

Table 1. Patient characteristics

	All	*KRAS* mutant	*KRAS* wild-type
Total number of patients	43	13	30
Median age (range)	68 (50–82)	67 (50–81)	68 (52–82)
Gender			
Male	27	7	20
Female	16	6	10
ECOG performance status			
0	28	10	18
1	15	3	12
Prior chemotherapy for advanced disease			
FOLFOX	41	13	28
FOLFIRI/IRIS/irinotecan	34/6/2	11/2/0	23/4/2
Bevacizumab	26	8	18
Primary tumor			
Cecum	1	0	1
Ascending colon	9	3	6
Transverse colon	4	0	4
Descending colon	2	1	1
Sigmoid colon	9	4	5
Rectum	18	5	13
Metastatic sites			
Lung	30	10	20
Liver	28	11	17
Intra-abdominal lymph nodes	12	2	10
Peritoneum	5	2	3
Bone	2	0	2
Others	8	3	5

FOLFOX = 5-Fluorouracil, leucovorin, oxaliplatin; FOLFIRI = 5-fluorouracil, leucovorin, irinotecan; IRIS = irinotecan, S-1.

Concerning the sites of metastases, lung metastases were found in 30 patients (69.8%), followed by liver metastases found in 28 (65.1%), intra-abdominal lymph node metastases found in 12 (27.9%), and peritoneum metastases found in 5 (11.6%) patients. No significant differences were seen regarding clinical characteristics between the *KRAS* wild-type and *KRAS* mutant subgroups.

Toxicity

Table 2 provides a summary of the toxicity data. As can be observed, grade 3 or 4 neutropenia was identified in 12 patients (29.3%) and grade 3 or 4 anemia in 4 (9.8%). Although skin toxicity, including acneiform rash, dry skin and pruritus, was observed in the majority of patients (82.9%), grade 3 or 4 skin toxicity was observed in only 2 patients (4.9%). Other conditions for which grade 3 or 4 toxicity was identified were diarrhea (4.9%), stomatitis (2.4%), and hypomagnesemia (4.9%). No significant differences were found between the toxicity profiles of the *KRAS* wild-type and *KRAS* mutant subgroups.

Mutation Analysis of KRAS, BRAF, PIK3CA, NRAS and AKT1

Table 3 provides a list of the mutations detected by direct sequencing. The common *KRAS* mutations in codons 12 and 13 and rarer mutations in codon 61 were analyzed to increase the sensitivity of mutation detection. Of the 13 tumors (30.2%) that harbored *KRAS* muta-

Table 2. Toxicity profile in 43 mCRC patients

Event	All (n = 41)		*KRAS* mutant (n = 13)		*KRAS* wild-type (n = 28)	
	grade 1-4	grade 3-4	grade 1-4	grade 3-4	grade 1-4	grade 3-4
Leukopenia	21 (51.2, 35.1–67.1)	9 (22.0, 10.6–37.6)	5 (38.5, 13.9–68.4)	3 (23.1, 5.0–53.8)	16 (57.1, 37.2–75.5)	6 (21.4, 8.3–41.0)
Neutropenia	20 (48.8, 32.9–64.9)	12 (29.3, 16.1–45.5)	5 (38.5, 13.9–68.4)	4 (30.8, 9.1–61.4)	15 (53.6, 33.9–72.5)	8 (28.6, 13.2–48.7)
Anemia	36 (87.8, 73.8–95.9)	4 (9.8, 2.7–23.1)	11 (84.6, 54.6–98.1)	1 (7.7, 0.2–36.0)	25 (89.3, 71.8–97.7)	3 (10.7, 2.27–28.2)
Thrombocytopenia	13 (31.7, 18.1–48.1)	2 (4.9, 0.6–16.5)	5 (38.5, 13.9–68.4)	0 (0.0, 0.0–24.7)	8 (28.6, 13.2–48.7)	2 (7.1, 0.88–23.5)
Diarrhea	23 (56.1, 39.8–71.5)	2 (4.9, 0.6–16.5)	6 (46.2, 19.2–74.9)	1 (7.7, 0.2–36.0)	17 (60.7, 40.6–78.5)	1 (3.6, 0.09–18.4)
Skin toxicity	34 (82.9, 67.9–92.9)	2 (4.9, 0.6–16.5)	12 (92.3, 64.0–99.8)	0 (0.0, 0.0–24.7)	22 (78.6, 59.1–91.7)	2 (7.1, 0.88–28.2)
HFS	11 (26.8, 14.2–42.9)	1 (2.4, 0.06–12.9)	2 (15.4, 1.92–45.4)	0 (0.0, 0.0–24.7)	9 (32.1, 15.9–52.4)	1 (3.6, 0.09–18.4)
Stomatitis	14 (34.1, 20.1–50.6)	1 (2.4, 0.06–12.9)	6 (46.2, 19.2–74.9)	0 (0.0, 0.0–24.7)	8 (28.6, 13.2–48.7)	1 (3.6, 0.09–18.4)
Nausea	19 (46.3, 30.7–62.6)	2 (4.9, 0.6–16.5)	7 (53.8, 25.1–80.8)	1 (7.7, 0.2–36.0)	12 (42.9, 24.5–62.8)	1 (3.6, 0.09–18.4)
Vomiting	4 (9.8, 2.7–23.1)	0 (0.0, 0.0–8.6)	0 (0.0, 0.0–24.7)	0 (0.0, 0.0–24.7)	4 (14.3, 4.0–32.7)	0 (0.0, 0.0–12.3)
Fatigue	22 (53.7, 37.4–69.3)	3 (7.3, 1.54–19.9)	9 (69.2, 38.6–90.9)	0 (0.0, 0.0–24.7)	13 (46.4, 27.5–66.1)	3 (10.7, 2.27–28.2)
Anorexia	26 (63.4, 46.9–77.9)	6 (14.6, 5.57–29.2)	9 (69.2, 38.6–90.9)	1 (7.7, 0.2–36.0)	17 (60.7, 40.6–78.5)	5 (17.9, 6.1–36.9)
Hypomagnesia	19 (46.3, 30.7–62.6)	2 (4.9, 0.6–16.5)	7 (53.8, 25.1–80.8)	1 (7.7, 0.2–36.0)	12 (42.9, 24.5–62.8)	1 (3.6, 0.09–18.4)

Figures in parentheses are percentages and 95% confidence intervals. HFS = Hand-foot syndrome.

Table 3. *KRAS, BRAF* and *PIK3CA* mutation frequencies (n = 43)

Gene	Codon	Nucleotide substitution	Amino acid substitution	Number	
KRAS	12	GGT→G<u>A</u>T	G12D	3 (7.0)	13 (30.2)
		GGT→G<u>T</u>T	G12V	7 (16.2)	
	13	GGC→GAC	G13D	3 (7.0)	
BRAF	600	GTG→G<u>A</u>G	V600E	3 (7.0)	3 (7.0)
PIK3CA	542	GAA→<u>A</u>AA	E542K	1 (2.3)	6 (14.0)
	545	GAG→G<u>G</u>G	E545G	2 (4.7)	
	1,047	CAT→CGT	H1047R	1 (2.3)	
		CAT→TAT	H1047Y	1 (2.3)	
		CAT→CTT	H1047L	1 (2.3)	
NRAS	12	GGT→GAT	G12D	2 (4.7)	2 (4.7)
AKT1	17	GAG→AAG	E17K	2 (4.7)	2 (4.7)

Figures in parentheses are percentages.

tions in codons 12 and 13, none harbored mutations in codon 61. Of the 13 detected mutations in codons 12 and 13, the most frequent mutation was G12V (16.3%), followed by G13D (7.0%), and G12D (7.0%). Three tumors (7.0%) harbored *BRAF* mutations in codon 600 (V600E). Six tumors (14.0%) had *PIK3CA* mutations in exon 9 (E542K and E545G) and in exon 20 (H1047R, H1047Y, and H1047L). Two tumors showed *NRAS* mutations in codon 12. Two tumors harbored *AKT1* E17K mutations. Analysis of the findings indicated that *KRAS* mutations are mutually exclusive of *BRAF* and *NRAS* mutations (fig. 1). Two of the 13 *KRAS* mutant tumors harbored *PIK3CA* exon 20 mutations but not exon 9 mutations. One of the 3 *BRAF* mutant tumors showed *AKT1* mutations. None of the tumors harbored *PIK3R1* mutations.

Cetuximab Efficacy

The RR of the *KRAS* wild-type subgroup was found to be 17.9% (exact 90% confidence interval = 7.3–33.9%). As no significant treatment effect was obtained for the *KRAS* mutant subgroup with one-sided testing (p = 0.083), exploratory analysis was conducted for this

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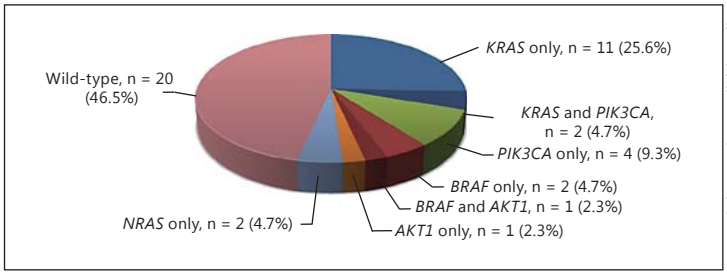

Fig. 1. Prevalence of mutation in EGFR-related genes. Numbers refer to the numbers of tumors harboring single or double mutations in the indicated genes.

Table 4. Response to cetuximab according to the presence or absence of gene mutation in the 41 patients

Tumor response	*KRAS*		Genetic status of *KRAS* and *BRAF*		Genetic status of *KRAS, BRAF, PIK3CA, NRAS* and *AKT1*		All patients
	mutant	wild-type	mutant of any genes	wild-type of all genes	mutant of any genes	wild-type of all genes	
Total	13	28	15	26	22	19	41
CR	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0
PR	0 (0)	5 (17.9)	0 (0)	5 (19.2)	0 (0)	5 (26.3)	5
SD	2 (15.4)	9 (32.1)	2 (12.5)	9 (34.6)	5 (22.7)	6 (31.6)	11
PD	9 (69.2)	12 (42.9)	11 (78.5)	10 (38.5)	13 (59.1)	8 (42.1)	21
NE	2 (15.4)	2 (7.1)	2 (14.3)	2 (7.7)	4 (18.2)	0 (0)	4
RR	0.0	17.9[a]	0.0	19.2	0.0	26.3	12.2
90% CI	0.0–20.6	7.3–33.9	0.0–18.1	7.9–36.3	0.0–12.7	11.0–47.6	4.9–23.9
DCR	15.4	50.0	13.3	39.6	22.7	57.9	39.0
90% CI	2.8–41.0	33.3–66.7	2.4–36.3	36.2–70.8	9.4–42.0	36.8–77.0	27.6–55.4
mPFS, months	1.6	3.7	1.6	5.2	1.8	5.2	2.5
95% CI	1.3–2.6	2.1–5.8	1.5–2.2	2.1–6.2	1.5–2.6	2.5–6.5	1.8–5.2
p value[b]	0.0039		0.0008		0.0042		
mOS, months	7.5	10.3	7.4	11.8	7.4	11.8	8.47
95% CI	3.6–8.9	5.9–13.7	3.6–8.5	6.0–14.4	4.3–9.1	6.0–14.4	6.0–11.6
p value[b]	0.02		0.0026		0.24		

Figures in parentheses are percentages. DCR = Disease control rate.
[a] One-sided p = 0.083 (z test); the null hypothesis (the true RR of the *KRAS* wild-type group was more than 0.1) was tested.
[b] log-rank test.

subgroup to identify an RR of 0% (90% confidence interval = 0.0–20.6%). As can be observed in table 4, which shows the RR and median PFS (mPFS) according to the presence or absence of gene mutations, the *KRAS* mutant subgroup was found to have a lower RR than the *KRAS* wild-type subgroup. Combined analysis of *KRAS, BRAF, PIK3CA, NRAS,* and *AKT1* mutation status indicated that none of the 23 patients with mutations in any of these 5 genes, whose objective RR was 0.0%, had responded to treatment, whereas 5 of the 18 patients with no mutations, whose objective RR was 27.8%, had responded, indicating that *KRAS, BRAF, PIK3CA, NRAS,* and *AKT1* mutations were associated with a lack of response. Combined

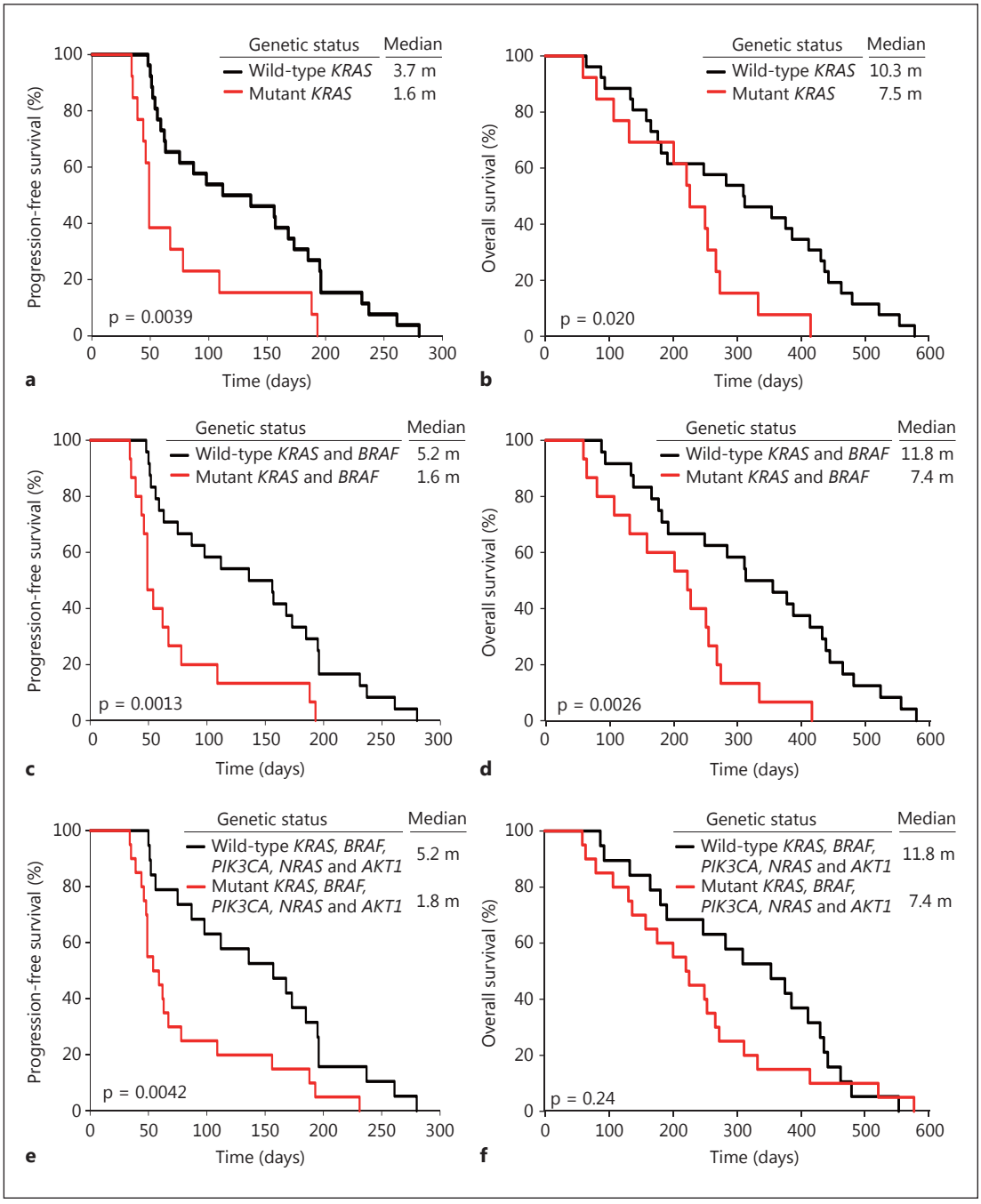

Fig. 2. Kaplan-Meier analysis of cumulative PFS and OS based on *KRAS, BRAF* and *PIK3CA* mutational status in mCRC patients treated with cetuximab. PFS (**a**) and OS (**b**) of patients with wild-type *KRAS* versus mutant *KRAS*. PFS (**c**) and OS (**d**) of patients with wild-type *KRAS* or *BRAF* versus mutant *KRAS* or *BRAF*. PFS (**e**) and OS (**f**) of patients with wild-type *KRAS, BRAF, PIK3CA, NRAS,* or *AKT1* versus mutant *KRAS, BRAF, PIK3CA, NRAS,* or *AKT1*. m = Months.

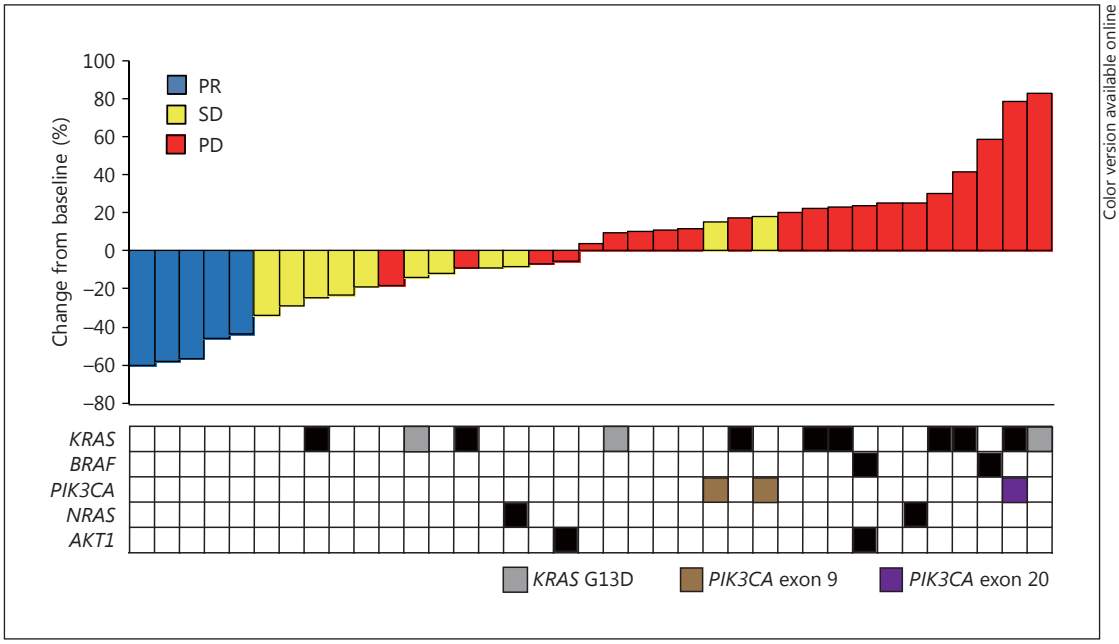

Fig. 3. Waterfall plots showing maximal reduction in size of target lesions based on analysis of *KRAS, BRAF, PIK3CA, NRAS*, and *AKT1* mutational status in mCRC patients treated with cetuximab.

analysis of *KRAS, BRAF, PIK3CA, NRAS,* and *AKT1* mutations improved RR (26.3%) of the subgroups expected to show response compared to the analysis of *KRAS* mutations alone (17.9%). Whereas the combined analysis of *KRAS, BRAF, PIK3CA, NRAS,* and *AKT1* mutations did not reduce the disease control rate (22.7%) of the subgroup expected to have no response compared to the analysis of *KRAS* mutations alone (15.4%), the combined analysis of *KRAS* and *BRAF* mutations did reduce it (12.5%).

As can be seen in figure 2a and b, the mPFS and median overall survival (mOS) of the *KRAS* wild-type subgroup were found to be significantly longer than those of the *KRAS* mutant subgroup (mPFS = 3.7 vs. 1.6 months, respectively, p = 0.0039; mOS = 10.3 vs. 7.5 months, respectively, p = 0.02). However, as shown in figure 2c and d, the difference in mPFS and mOS between both the *KRAS* and *BRAF* wild-type subgroups and the mutant subgroups was found to be greater (mPFS = 5.2 vs. 1.6 months, respectively, p = 0.0013; mOS = 11.8 vs. 7.4 months, respectively, p = 0.0026). The difference in mPFS and mOS between the subgroup with all 5 wild-type genes *(KRAS, BRAF, PIK3CA, NRAS,* and *AKT1)* and the subgroup with mutations in any one of the 5 genes was found to be less significant (mPFS = 5.2 vs. 1.8 months, respectively, p = 0.0042; mOS = 11.8 vs. 7.4 months, p = 0.24; fig. 2e, f).

The analysis of a waterfall plot of the best response in target lesions and mutation status indicates a similar tendency to RR and mPFS analysis. As shown in figure 3, almost all patients with *KRAS, BRAF,* and/or *PIK3CA* mutations failed to respond to cetuximab-based treatment. Whereas 23% (3/13) of the patients in the *KRAS* mutant subgroup experienced tumor reduction, 57.7% (15/26) of the patients in the *KRAS* wild-type subgroup showed tumor reduction, including patients with PR, SD, and PD. In contrast, 28% (5/18) of patients in the *KRAS, BRAF* or *PIK3CA* mutant subgroup and 72% (13/19) of patients in any of the wild-type subgroup experienced tumor reduction. These results indicate the clinical relevance of the analysis of mutation status of these genes in predicting the efficacy of cetuximab-based treatment in patients with mCRC.

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Discussion

The RR of the *KRAS* wild-type subgroup in this study (17.9%) was found to be lower than would be expected according to the results of previous studies. Several studies have shown that the RR of *KRAS* wild-type patients to cetuximab therapy with or without chemotherapy in second-line or later lines of treatment was higher (27–41%) [2, 3, 5, 8, 20, 21]. One European study found that the RR of the *KRAS* wild-type subgroup to cetuximab plus irinotecan in third-line therapy was 37% [22]. Whereas the RR of all patients in this study was 12.2%, the RR of the 206 patients in the third-line subgroup in the BOND study, who had undergone cetuximab plus irinotecan therapy, was 22.2% [23]. The lower RR in this study might have arisen from bias in several steps, such as during *KRAS* testing, patient sampling, and/or evaluation of efficacy. Specifically, while this study performed direct sequencing to determine *KRAS* status, several other studies used the Scorpion-Arms method, which is more sensitive to detect mutations. However, as the frequency and spectrum of *KRAS* mutation in this study were similar to those of previous studies, the sensitivity of *KRAS* examination was not likely to have differed greatly between this and previous studies [24, 25]. Retrospective analysis of the *KRAS* status of 43 other chemorefractory patients who had undergone cetuximab treatment in our previous study that we had conducted using the same method as that used in the present study revealed that the RR of the *KRAS* wild-type subgroup had been higher than that of the patients examined in the present study (22%) [26]. Using the single-base primer extension method, the direct sequencing results were validated in this previous study. Specifically, the characteristics of the patients in both studies were similar except the total mutation rates of the *BRAF, PIK3CA, NRAS,* and *AKT1,* which were higher in the present study (23% in the present study vs. 9% in the previous study). One explanation for this difference may be a high rate of tumors with mutations in the EGFR pathway in the present study's cohort, which is associated with a putative poor prognosis or nonresponse to cetuximab-based therapy. However, this explanation is not completely acceptable, since this mutation rate was not significantly higher than that identified in studies of Caucasian patients [17]. In addition to our previous study, a South Korean study found that the RR of Korean patients with refractory *KRAS* wild-type CRC to cetuximab plus irinotecan treatment was similar (33%) to that of comparable Caucasian patients, indicating that the existence of ethnic differences in response to cetuximab therapy is unlikely [20].

Exploratory analysis of the study data confirmed that *KRAS* mutation is a negative predictive marker of cetuximab efficacy in Japanese as well as Caucasian patients. The RR, the primary endpoint of this study, was higher and the mPFS and mOS were significantly longer in the *KRAS* wild-type subgroup compared to the *KRAS* mutant subgroup. The rate of *KRAS* mutation identified in this study (30.2%) was consistent with that reported by previous studies of various populations (30–40%), and the spectrum of the *KRAS* mutation was very similar to that reported by previous studies of Caucasian populations [24, 25]. All of the 3 common *KRAS* mutations examined in the RASCAL and RASCAL II studies – the G12V, G13D, and G12D mutations – were frequently detected in this study. Based on these findings, the *KRAS* mutation, in terms of both frequency and the nature of the mutation spectrum, was concluded not to differ significantly between Japanese and Caucasian populations. Recently, the *KRAS* G13D mutation has been shown to be associated with better outcome after cetuximab treatment compared to that associated with other mutations [27]. However, the 3 patients with *KRAS* G13D-mutated tumors examined in this study had no better response to cetuximab-based therapy than had patients with other mutations (fig. 3). The sample size was too small to evaluate the relationship between the efficacy of cetuximab-based therapy and the *KRAS* G13D mutation.

In this study, 42.9% of patients, even those in the *KRAS* wild-type subgroup, were found to show PD after cetuximab treatment. Several studies have revealed that analysis of mutation status of EGFR-related downstream genes, such as *BRAF, PIK3CA,* and *NRAS,* improves the specificity of prediction of cetuximab efficacy [17]. In addition to analysis of mutation status of these 3 genes, this study analyzed that of *AKT1* and *PIK3R1,* for which mutations have been identified in between 6 [18] and 8.3% [28] of CRC patients and found to result in the activation of the AKT pathway. However, the prevalence of mutations in these genes in the patients of this study was found to be low (*BRAF* = 7.0%, *PIK3CA* = 14.0%, *NRAS* = 4.7%, *AKT1* = 4.7%, and *PIK3R1* = 0%), indicating that these mutations can hardly be evaluated as independent predictors. Therefore, future studies with larger sample sizes are required to analyze the clinical significance of these mutations as predictive markers for the efficacy of cetuximab. To obtain suggestive evidence, we evaluated which combinations of these genes produce the most significant differences in the clinical outcome of cetuximab treatment between the wild-type and mutant subgroups. The most significant difference in RR was obtained by combined analysis of all 5 genes. However, the more significant differences in disease control rate, PFS and OS were obtained by combined analysis of only *KRAS* and *BRAF*. These findings indicate that the combined analysis of *KRAS* and *BRAF* genes clearly contributes to detecting the responders to cetuximab treatment by evaluating the various clinical outcomes but that the contribution of additional analyses of other EGFR-related genes is uncertain. Therefore, in the assessment of predictive markers, careful evaluation of the results of combined analysis of EGFR-related genes must be performed to prevent erroneously identifying patients who may benefit from cetuximab therapy as nonresponders.

In this study, analysis of *BRAF* mutations was found to contribute to the identification of additional nonresponders, as only 2 (7.0%) patients with *BRAF* mutations were found to have PD. *BRAF* mutations were observed at a rate comparable to that found in Caucasian populations (5–10%) and mutually exclusive of *KRAS* mutation, as previously shown [29]. Although *BRAF* mutations have been shown to be negative predictive markers of response to anti-EGFR mAb therapy, they have also been found to be strong prognostic factors [11]. Moreover, recent studies have indicated that even patients with *BRAF* mutations may benefit from cetuximab treatment, having found better clinical outcomes for cetuximab-treated compared to non-cetuximab-treated patients with *BRAF* mutations, although the difference in outcome between these 2 groups did not reach a level of statistical significance [30, 31]. While the results of the present study reveal that the analysis of *BRAF* mutation status contributes to better identification of responders to cetuximab treatment, the possibility that patients with a poor prognosis will be erroneously identified as nonresponders, regardless of resistance to cetuximab, cannot be ruled out in a one-arm phase II study. To evaluate the benefit of cetuximab in *BRAF* mutant patients, large-scale randomized studies are required. A recent study found that treatment with EGFR inhibitors, including cetuximab, has a synergistic effect with treatment with BRAF inhibitors in *BRAF* mutant CRC patients. This indicates that BRAF inhibitor monotherapy achieves only a limited effect because it causes rapid feedback activation of EGFR [32]; moreover, it shows that genetic analysis of *BRAF* status will become more useful if combination therapies inhibiting both BRAF and EGFR are developed in the future.

Of the 2 *AKT1* mutant patients showing PD in this study, 1 patient was found to have an overlapping *BRAF* mutation. As such, only the findings for 1 of these patients can be considered representative of the impact of *AKT1* mutation on cetuximab efficacy. Nevertheless, it indicates that hot-spot somatic mutations such as *AKT1* E17K, which is reported in various tumors, including breast, colorectal and ovarian cancers, may be a novel predictive marker of cetuximab efficacy. Even though *AKT1* E17K has been shown to be oncogenic in both in vitro and in vivo analysis, it has not been found to be associated with the efficacy of anti-EGFR mAb treatment [18]. Previous studies have found no association between phosphorylated AKT

level and clinical outcome after anti-EGFR mAb treatment [33]. In contrast to the report observing absence of tumors with *AKT1* mutations in Asian populations, 2 patients (7.0%) were found to have *AKT1* mutations in this study of Japanese patients [34].

The prevalence of *PIK3CA* mutations in the patients of this study (14.0%) was similar to that in studies of Caucasian populations (10–20%). The clinical relevance of *PIK3CA* mutations in the prediction of response to anti-EGFR therapy remains controversial. Among the 5 *PIK3CA* mutant patients examined in this study, only 2 patients with exon 9 mutations were available for evaluation of clinical outcome. Both *PIK3CA* mutant patients showed SD, suggesting that *PIK3CA* analysis did not contribute to improving the specificity of the prediction of response to cetuximab. This finding is consistent with a recent large European study that found that *PIK3CA* mutations in exon 20 but not exon 9 are associated with resistance to anti-EGFR mAb therapy [17]. Nevertheless, the spectrum of *PIK3CA* mutation could differ by ethnicity; both *PIK3CA* mutations detected in *KRAS* mutant tumors were located in exon 20, which contrasts with a previous report saying that *PIK3CA* exon 9 mutations are found more frequently in *KRAS* mutant tumors than exon 20 mutations. Unfortunately, no data were collected in the present study that could provide more information regarding exon 20 mutations. Of the 5 detected mutations, E542K and H1047R are among the 3 most frequent mutations (E542K, E545K, and H1047R), whereas E545G, H1047Y, and H1047L are relatively rare mutations [17]. Especially, E545G was detected in 2 patients of this study in contrast to the reports saying that E545G is quite rare in Caucasian populations [16].

NRAS mutation, which was detected in 2 patients, has been associated with poor response to cetuximab treatment [17]. However, *NRAS* analysis did not clearly contribute to an improvement of the sensitivity of the prediction of treatment response, with the 2 *NRAS* mutant patients showing SD and PD. These 2 *NRAS* mutations were found in codon 12, whereas *NRAS* mutations are more commonly seen in codon 61 in Caucasian populations, suggesting ethnic differences may have led to the differences in the sensitivity of prediction. *NRAS* mutations were mutually exclusive of *KRAS* mutations, as shown previously. Unfortunately, no data regarding *KRAS* codon 61 and *PIK3R1* mutations could be collected because no patients with these mutations were identified in this study.

This is a prospective study to evaluate the relationship between *BRAF, PIK3CA,* and *KRAS* mutation status and response to cetuximab therapy in Japanese patients with mCRC. Despite the study's focus on mutation status, almost 42.1% of the patients for whom no *KRAS, BRAF, PIK3CA, NRAS,* or *AKT1* mutations were detected were found not to have responded to cetuximab therapy and to have suffered tumor progression. This finding suggests that other as yet unidentified biomarkers are important determinants of response, including the tumor suppressor PTEN protein, a negative regulator of PI3-kinase-initiated signaling for which loss of expression has been associated with lack of response to cetuximab [33, 35].

In conclusion, the results of this study prospectively confirmed that cetuximab-based treatment is effective and well tolerated in both Japanese and Caucasian mCRC patients with *KRAS* wild-type mutations who have failed to respond to prior chemotherapy consisting of irinotecan, oxaliplatin, and fluoropyrimidine administration. The results also indicate the clinical relevance of analysis of not only *KRAS* mutation status in predicting the efficacy of cetuximab-based treatment in Asian patients with mCRC but also *BRAF* mutation status in improving identification of patients most likely to benefit from anti-EGFR mAb therapy. Nevertheless, as the maximum RR to cetuximab-based treatment was found to be only 26.6%, even with genetic analysis, they also indicate the need for the development of new diagnostics to enable the identification of anti-EGFR mAb-sensitive mCRC patients without any known mutations to improve the cost-effectiveness of providing anti-EGFR mAb therapy.

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