Statistical analysis

Associations of continuous and categorical variables with relevant outcome variables were assessed using the Mann–Whitney *U* test and Fisher's exact test respectively. The variable skeletal muscle was not *a priori* categorized into a binary variable (sarcopenia present or not), because categorizing a continuous predictor would result in an inevitable loss of information. Instead, the multivariable fractional polynomial (MFP) approach was adopted. In the polynomial fractional model, for each continuous variable X, one or two terms of the form X^p were fitted with powers, p, chosen from (−2, −1, −0·5, 0, 0·5, 1, 2 and 3). The results of the MFP analysis revealed that the most appropriate power for skeletal muscle mass in the MFP model was given in the form of X (that is, $p = 1$), allowing expression of a final multivariable model in terms of the usual Cox regression model. Therefore, the results of the usual Cox model are reported here, giving the results of the log rank tests for the association between the presence of sarcopenia (as defined by dichotomizing skeletal muscle mass) and overall or disease-free survival[23]. To identify prognostic factors after hepatectomy, all variables were included in the overall multivariable Cox proportional model in the analyses of both overall and recurrence-free survival using the backward selection method. The overall and recurrence-free survival curves were analysed by the Kaplan-Meier method and compared with the log rank test. All analyses were performed with StatView® 5.0 software (Abacus Concepts, Berkeley, California, USA). $P < 0{\cdot}050$ was considered statistically significant.

Results

In total, 186 patients with HCC were identified from the database, of whom 75 (40·3 per cent; 50 men and 25 women) had sarcopenia. Clinicopathological characteristics of patients with and without sarcopenia are shown in *Table 1*. Women were more likely to have sarcopenia than men. Patients with sarcopenia had a significantly lower BMI than those without. Regarding liver function, serum albumin levels were significantly lower and ICGR15 values were significantly higher in patients with sarcopenia than in those without. Other host-related factors such as age, hepatitis, diabetes mellitus, Child–Pugh grade, MELD score and liver cirrhosis were not related to the presence of sarcopenia. There were no significant differences in tumour-related factors or surgical outcomes between the two groups. Operative details are shown in *Table S1* (supporting information).

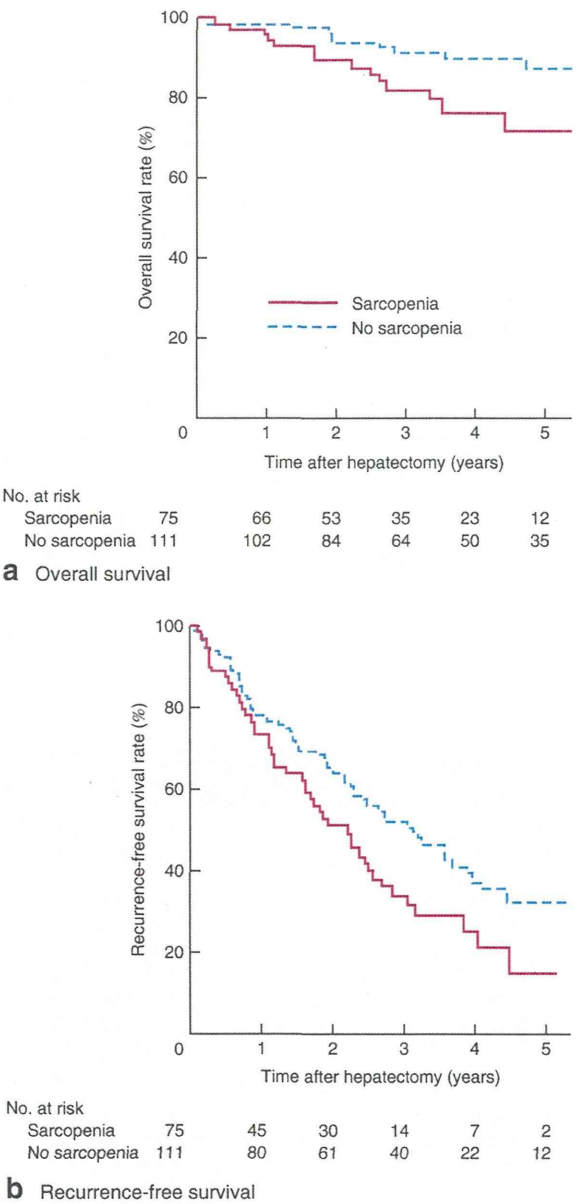

Fig. 2 **a** Overall and **b** recurrence-free survival curves after liver resection in patients with, and without sarcopenia. **a** $P = 0{\cdot}001$, **b** $P = 0{\cdot}013$ (log rank test)

Overall and recurrence-free survival curves for patients with and without sarcopenia are shown in *Fig. 2*. Overall and recurrence-free 5-year survival rates were 71 and 13 per cent respectively in patients with sarcopenia, and 83·7 and 33·2 per cent in patients without sarcopenia (*Fig. 2*). Patients with sarcopenia had a significantly worse prognosis

Table 2 Univariable and multivariable analysis of clinicopathological factors and overall survival following partial hepatectomy with curative intent for hepatocellular carcinoma

	Univariable analysis		Multivariable analysis	
	Hazard ratio	*P**	Hazard ratio	*P*†
Age	1·02 (0·98, 1·07)	0·323		
Female sex	1·17 (0·42, 2·79)	0·746		
Skeletal muscle mass	0·92 (0·86, 0·97)	0·004	0·90 (0·84, 0·96)	0·002
Body mass index	0·92 (0·81, 1·04)	0·199		
Albumin	0·47 (0·21, 1·14)	0·092		
ICGR15	1·02 (0·97, 1·07)	0·512		
MELD score	1·08 (0·86, 1·25)	0·460		
Liver fibrosis + cirrhosis	3·97 (1·50, 13·67)	0·004		
Tumour size	1·10 (0·98, 1·22)	0·906		
Multiple tumours	1·60 (0·65, 3·64)	0·292		
TNM stage III + IV	1·62 (0·70, 3·62)	0·255		
Poor differentiation	2·26 (0·98, 5·16)	0·055	2·47 (1·05, 5·81)	0·021
Microvascular invasion	2·39 (1·05, 5·41)	0·038	3·21 (1·29, 7·94)	0·018
Intrahepatic metastases	1·67 (0·55, 4·15)	0·333		
α-Fetoprotein	1·00 (1·00, 1·00)	0·335		
DCP	1·00 (1·00, 1·00)	0·267		
Postoperative complications	2·76 (1·23, 6·28)	0·014	3·27 (1·39, 7·69)	0·007

Values in parentheses are 95 per cent confidence intervals. ICGR15, indocyanine green dye retention test at 15 min; MELD, Model for End-Stage Liver Disease; TNM, tumour node metastasis; DCP, des-γ-carboxyprothrombin. *Log rank test; †Cox proportional model.

Table 3 Univariable and multivariable analysis of clinicopathological factors and recurrence-free survival following partial hepatectomy with curative intent for hepatocellular carcinoma

	Univariable analysis		Multivariable analysis	
	Hazard ratio	*P**	Hazard ratio	*P*†
Age	1·01 (1·00, 1·04)	0·139		
Female sex	1·02 (0·63, 1·59)	0·918		
Skeletal muscle mass	0·98 (0·95, 1·00)	0·049	0·97 (0·95, 1·00)	0·016
Body mass index	0·94 (0·88, 1·02)	0·076		
Albumin	0·49 (0·33, 0·75)	0·001		
ICGR15	1·03 (1·01, 1·06)	0·048	1·02 (1·02, 1·07)	0·001
MELD score	1·03 (0·93, 1·12)	0·526		
Liver fibrosis + cirrhosis	1·98 (1·32, 3·01)	0·001		
Tumour size	1·00 (0·98, 1·11)	0·141		
Multiple tumours	1·89 (1·22, 2·84)	0·005		
TNM stage III + IV	2·44 (1·64, 3·61)	0·001	2·13 (1·38, 3·29)	0·001
Poor differentiation	1·58 (1·04, 2·35)	0·033		
Microvascular invasion	2·39 (1·05, 5·41)	0·038		
Intrahepatic metastases	2·14 (1·30, 3·38)	0·003	2·37 (1·38, 4·06)	0·018
α-Fetoprotein	1·00 (1·00, 1·00)	0·001		
DCP	1·00 (1·00, 1·00)	0·006	1·00 (1·00, 1·00)	0·001
Postoperative complications	1·11 (0·73, 1·67)	0·617		

Values in parentheses are 95 per cent confidence intervals. ICGR15, indocyanine green dye retention test at 15 min; MELD, Model for End-Stage Liver Disease; TNM, tumour node metastasis; DCP, des-γ-carboxyprothrombin. *Log rank test; †Cox proportional model.

than those without in terms of both overall ($P = 0·001$) and recurrence-free survival ($P = 0·013$).

In univariable analysis, significant prognostic factors for overall survival were low skeletal muscle mass, and presence of liver cirrhosis, MVI and postoperative complications (*Table 2*). Significant prognostic factors for recurrence-free survival were lower skeletal muscle mass, serum albumin level, liver cirrhosis, tumour number, tumour stage, poorly differentiated HCC, MVI, intrahepatic metastases, and serum AFP and DCP levels (*Table 3*). Multivariable analysis identified four poor prognostic factors (low skeletal muscle mass, poorly differentiated HCC, MVI and postoperative complications) that influenced overall survival, and five poor prognostic factors (low skeletal muscle mass, high

ICGR15 value, high serum DCP level, presence of intrahepatic metastases, and stage III + IV disease) that influenced recurrence-free survival (*Tables 2* and *3*).

Discussion

The findings of this retrospective single-centre study suggest that sarcopenia is an independent prognostic factor for overall and recurrence-free survival in patients with HCC following partial hepatectomy. The Child–Pugh classification was the first systematic and conventional approach used to determine the severity of cirrhosis and select patients who might tolerate hepatic resection. However, it is not always a reliable indicator of hepatic reserve, and has a limited role in predicting postoperative outcome[24]. The MELD score is a reliable measure of mortality risk in patients with end-stage liver disease and is suitable for use as a disease severity index to determine organ allocation priorities. No useful, objective, easily obtained and precise marker has yet been identified to evaluate the general condition of patients before hepatectomy. The ASA grade gives an estimation of organ disease and functional status, and has been suggested as a useful prognostic factor for preoperative patients with HCC[8]. However, it has been criticized for being subjective and imprecise[16].

Sarcopenia is defined as muscle mass two standard deviations below the mean in healthy young adults[25]. Although sarcopenia is associated with ageing, it can also develop as a consequence of chronic disease and malignancy. The European Working Group on Sarcopenia in Older People[15] recommended using the presence of both low muscle mass and low muscle function for the diagnosis of sarcopenia. However, muscle function is difficult to evaluate, and thus low muscle mass was investigated in the present study. There was no correlation between sarcopenia and age, but sarcopenia was significantly correlated with liver dysfunction as indicated by abnormal serum albumin levels and ICGR15 values, as well as with reduced BMI values. There was no correlation between sarcopenia and the Child–Pugh classification, MELD score or liver cirrhosis. There are some reports that serum albumin levels are decreased in patients with sarcopenia[26], which could be an early warning sign of subclinical conditions and impending disease and disability. Montano-Loza and colleagues[12] reported that, of patients with cirrhosis, those with sarcopenia had a significantly lower BMI than patients without sarcopenia. Liver cirrhosis was observed in 50 per cent of patients in their study, in line with the present findings. There is no report concerning the relationship between ICGR15 values and sarcopenia. In one study[12], skeletal muscle area was correlated with MELD score, which would seem to contradict the present findings; however, the mean MELD score was better in the present study, perhaps explaining these findings.

CT is the standard procedure for quantifying skeletal muscle mass, enabling objective and detailed nutritional and metabolic assessment of patients. Moreover, CT is always performed before hepatectomy, allowing precise assessment of sarcopenia. There are some reports that muscle mass as measured by CT is associated with the prognosis of sarcopenia.

It has been suggested previously that surgical outcomes are worse for obese patients[27]; however, there are few reports concerning the effect of being underweight on patient outcomes following hepatectomy for HCC. In this study, lower BMI was correlated with sarcopenia but not with the prognosis. BMI was significantly lower in sarcopenic patients, although only five patients were considered to be underweight (BMI below 18·5 kg/m^2). Thus, sarcopenia is not present exclusively in underweight patients.

The molecular mechanism of sarcopenia remains poorly understood. Skeletal muscle was recently identified as an endocrine organ[28]. It has therefore been suggested that cytokines and other peptides are produced, expressed and released by muscle fibres. For example, interleukin (IL) 6 is released from skeletal muscle[28], which may subsequently affect liver metabolism. Both the level and timing of IL-6 release appear to be determining factors for the biological effect in patients with liver fibrosis and HCC[28]. Furthermore, levels of insulin-like growth factor (IGF) 1, which plays a stimulatory role in the development and regulation of skeletal muscle mass[28], are decreased in patients with sarcopenia. In some reports, serum IGF-1 levels were significantly lower in patients with cirrhosis than in healthy subjects, and were correlated with the degree of liver dysfunction. Low serum IGF-1 levels were significantly correlated with advanced clinicopathological parameters, and indicative of poor overall survival in HCC[29]. IGF-1 is produced mainly by the liver, and it may be that serum IGF-1 levels are lower in patients with sarcopenia and that low IGF-1 levels promote the progression of HCC. Further study is needed to clarify the molecular mechanism concerning muscle–liver cross-talk.

It is important to note that, among the significant prognostic factors for overall survival, skeletal muscle mass can be evaluated before hepatectomy. Similarly, skeletal muscle mass, ICGR15, serum DCP level and stage can be evaluated before hepatectomy to prognosticate recurrence-free survival. The identification of patients with sarcopenia before hepatectomy might permit early

preventive strategies to maintain muscle mass, in order to improve prognosis and patient selection for hepatectomy. A recent study indicated that a late evening snack, as an intervention to reduce the fasting phase in patients with cirrhosis, has the potential to improve skeletal muscle proteolysis[30].

Disclosure

The authors declare no conflict of interest.

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Supporting information

Additional supporting information may be found in the online version of this article:

Table S1 Operative details in patients with hepatocellular carcinoma with, and without sarcopenia (Word document)

Snapshot quiz

Snapshot quiz 13/36

Answer: The computed tomography angiogram shows a large right popliteal aneurysm. The options for management are: radiological stenting using a covered stent; and a bypass procedure to exclude the aneurysm. The patient was managed with a bypass procedure from the superficial femoral artery to the below-knee popliteal artery using reversed saphenous vein. The aneurysm was ligated proximally and distally. This aneurysm was deemed unsuitable for radiological stenting owing to the tortuosity of the vessel. The right leg was swollen due to thrombosis of the popliteal vein caused by the pressure effect from the popliteal aneurysm. As this was at least 6 weeks old, the patient did not receive warfarin therapy.

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TECHNICAL ADVANCE Open Access

Simultaneous identification of 36 mutations in *KRAS* codons 61and 146, *BRAF*, *NRAS*, and *PIK3CA* in a single reaction by multiplex assay kit

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Abstract

Background: Retrospective analyses in the West suggest that mutations in *KRAS* codons 61 and 146, *BRAF*, *NRAS*, and *PIK3CA* are negative predictive factors for cetuximab treatment in colorectal cancer patients. We developed a novel multiplex kit detecting 36 mutations in *KRAS* codons 61 and 146, *BRAF*, *NRAS*, and *PIK3CA* using Luminex (xMAP) assay in a single reaction.

Methods: Tumor samples and clinical data from Asian colorectal cancer patients treated with cetuximab were collected. We investigated *KRAS*, *BRAF*, *NRAS*, and *PIK3CA* mutations using both the multiplex kit and direct sequencing methods, and evaluated the concordance between the 2 methods. Objective response, progression-free survival (PFS), and overall survival (OS) were also evaluated according to mutational status.

Results: In total, 82 of 83 samples (78 surgically resected specimens and 5 biopsy specimens) were analyzed using both methods. All multiplex assays were performed using 50 ng of template DNA. The concordance rate between the methods was 100%. Overall, 49 (59.8%) patients had all wild-type tumors, 21 (25.6%) had tumors harboring *KRAS* codon 12 or 13 mutations, and 12 (14.6%) had tumors harboring *KRAS* codon 61, *KRAS* codon 146, *BRAF*, *NRAS*, or *PIK3CA* mutations. The response rates in these patient groups were 38.8%, 4.8%, and 0%, respectively. Median PFS in these groups was 6.1 months (95% confidence interval (CI): 3.1–9.2), 2.7 months (1.2–4.2), and 1.6 months (1.5–1.7); median OS was 13.8 months (9.2–18.4), 8.2 months (5.7–10.7), and 6.3 months (1.3–11.3), respectively. Statistically significant differences in both PFS and OS were found between patients with all wild-type tumors and those with *KRAS* codon 61, *KRAS* codon 146, *BRAF*, *NRAS*, or *PIK3CA* mutations (PFS: 95% CI, 0.11–0.44; $P < 0.0001$; OS: 95% CI, 0.15–0.61; $P < 0.0001$).

Conclusions: Our newly developed multiplex kit is practical and feasible for investigation of a range of sample types. Moreover, mutations in *KRAS* codon 61, *KRAS* codon 146, *BRAF*, *NRAS*, or *PIK3CA* detected in Asian patients were not predictive of clinical benefits from cetuximab treatment, similar to the result obtained in European studies.

Keywords: Luminex assay, *KRAS*, *BRAF*, *NRAS*, *PIK3CA*, Epidermal growth factor

Background

The clinical significance of *KRAS* codon 12 and 13 mutation tests in the selection of patients with colorectal cancer who might benefit from anti-epidermal growth factor receptor (EGFR) antibodies is well established, and regulatory authorities in Europe, the United States, and Japan have recommended compulsory *KRAS* mutation testing before treatment [1-6]. Although conventional *KRAS* tests are useful to decrease treatment to nonbeneficiary populations, the efficacy of determining beneficiary populations requires improvement. The response rate to anti-EGFR antibody monotherapy among pretreated patients with tumors harboring *KRAS* codons 12 and 13 wild-type is 13%–17% [1,2], and that of combination anti-EGFR antibody and cytotoxic agent therapy is 11%–35% [5,7]. One explanation for such relatively low efficacy is that molecular alterations other than *KRAS* codon

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12 and 13 mutations might confer resistance to anti-EGFR antibody therapies. Recent retrospective studies have revealed that mutations in *KRAS* codons 61 and 146, *BRAF*, *NRAS*, and *PIK3CA* are also related to resistance to anti-EGFR antibodies [8-13].

Several issues should also be considered to establish the clinical utility of expanded genome biomarker tests for anti-EGFR antibodies. First, information about the relation between mutation status and efficacy of treatment, especially among Asian populations, is still limited. Second, efficacious quality-controlled *in vitro* diagnostic kits and systems suitable for multiple genome biomarker detection are needed.

In Japan, a *KRAS* mutation assay kit based on the ARMS–scorpion method that detects seven frequently observed mutations in *KRAS* codons 12 and 13 (TheraScreen® K-RAS Mutation Kit; QIAGEN) was first approved for *in vitro* diagnostic use, and a kit using Luminex (xMAP) assay (MEBGEN KRAS Mutation Detection Kit, MBL) followed [14,15]. We recently developed another Luminex-based research-use kit, GENOSEARCH Mu-PACK, which simultaneously detects 36 mutations in *KRAS* codons 61 and 146, *BRAF*, *NRAS*, and *PIK3CA*. In addition to the hitherto approved *KRAS* codon 12 and 13 mutation kit, the multiplex kit identifies mutations by a single tube reaction using 50 ng of template DNA from formalin-fixed paraffin-embedded (FFPE) specimens.

In this study, we examined the feasibility and robustness of this multiplex kit using routine clinical samples collected from multiple hospitals. Meanwhile, we collected precise clinical data for these cases and retrospectively analyzed the relation of the mutation profiles of expanded markers to clinical outcomes following cetuximab therapy.

Methods

Patients

We screened and selected clinical and pathological data from consecutive patients who were administered either cetuximab monotherapy or cetuximab plus irinotecan between July 2008 and April 2010.

Patients who met all of the following inclusion criteria were retrospectively included in the analyses: (1) age ≥20 years; (2) histologically confirmed adenocarcinoma of the colon or rectum; (3) presence of unresectable metastatic disease; (4) baseline computed tomography (CT) performed within 28 days of initial cetuximab administration; (5) initial CT evaluation performed within 3 months of initial cetuximab administration; (6) previously documented as refractory or intolerant to fluoropyrimidines, oxaliplatin, and irinotecan; (7) Eastern Cooperative Oncology Group performance status score ≤2; and (8) adequate hematological, hepatic, and renal functions.

In the monotherapy regimen, cetuximab was administered at an initial dose of 400 mg/m^2 followed by weekly infusions of 250 mg/m^2. In the cetuximab plus irinotecan regimen, cetuximab was administered at the same dose as for monotherapy and followed by biweekly infusions of 150 mg/m^2 irinotecan, as per the manufacturer's instructions for irinotecan in Japan.

The study was conducted with the approval of the National Cancer Center Institutional Review Board, Cancer Institute Hospital of Japanese Foundation for Cancer Research Review Board, National Hospital Organization Shikoku Cancer Center Review Board, Shizuoka Cancer Center Review Board, Saitama Cancer Center Review Board, Hokkaido University Review Board, and the Ethics Committee of the University of Toyama. Written informed consent was obtained from as much patients who were alive as possible. For the deceased patients and their relatives, we also disclosed the study design at the website of National Cancer Center and gave them chances to express their wills in accordance with Epidemiological Study Guideline of Ministry of Health, Labour and Welfare in Japan.

Tissue samples and DNA extraction

Genomic DNA was obtained from primary and metastatic colorectal cancer tissues of all patients treated with cetuximab. Tissue samples harvested by biopsy or surgical resection at the participating hospitals were collected and sent to the research institution (MBL, Japan). A 2-μm hematoxylin-eosin (HE) slide and a 10-μm unstained slide were obtained from the FFPE tissue blocks; the latter was subsequently sliced into 3–10 sections. Pathological diagnoses were confirmed by a pathologist (Satoshi Fujii), with reference to the 4th edition of the WHO classification. The tumor area, determined by examining HE slides, was macroscopically dissected. Genomic DNA was isolated as described previously [16].

Luminex (xMAP) tests

A total of 36 mutations of *KRAS* codon 61 (Q61K, Q61E, Q61L, Q61P, Q61R, Q61H), *KRAS* codon 146 (A146T, A146S, A146P, A146E, A146V, A146G), *BRAF* codon 600 (V600E), *NRAS* codon 12 (G12S, G12C, G12R, G12D, G12V, G12A), codon 13 (G13S, G13C, G13R, G13D, G13V, G13A), codon 61 (Q61K, Q61E, Q61L, Q61P, Q61R, Q61H), *PIK3CA* exon 9 codon 542 (E542K), codon 545 (E545K), codon 546 (E546K), and exon 20 codon 1047 (H1047R, H1047L) were analyzed using Luminex (xMAP) technology (GENOSEARCH Mu-PACK, MBL, Japan).

First, 50 ng of template DNA collected from FFPE tissue samples was amplified by polymerase chain reaction (PCR) using a biotin-labeled primer. Thereafter, the PCR products and fluorescent Luminex beads (oligonucleotide probes complementary to wild and mutant genes were bound to the beads) were hybridized and labeled with streptavidin–phycoerythrin. Subsequently, the products

were processed by Luminex assay and the collected data analyzed using UniMAG software (MBL, Japan). The procedure time was approximately 4.5 h.

We also used the Luminex assay kit (MEBGEN KRAS Mutation Detection Kit, MBL, Japan) currently approved for clinical use by the Ministry of Health, Labour and Welfare of Japan [16] to detect *KRAS* codon 12 and 13 mutations.

Direct sequencing methods

In addition, to confirm the mutations detected by the Luminex assays, the same mutations of *KRAS* codons 61 and 146, *BRAF*, *NRAS*, and *PIK3CA* were analyzed by direct sequencing. A total of 700 ng of template DNA was used for these PCR reactions and the PCR products were directly sequenced with the same primers used for PCR. A BigDye Terminator v3.1 Cycle Sequencing Kit and an ABI PRISM 3730xl DNA Analyzer (Life Technologies) were used. Analyses of DNA sequences were performed using Sequencher (GeneCodes).

Statistical analysis

Response rates (RRs) and disease control rates (DCRs) (including complete or partial response and stable disease) were evaluated as per the Response Evaluation Criteria in Solid Tumors (RECIST) (version 1.0). Progression-free survival (PFS) was defined as the time from initial administration of a cetuximab-containing regimen to either the first objective evidence of disease progression or death from any cause. Overall survival (OS) was defined as the time from initial administration of a cetuximab-containing regimen to death from any cause. RRs, DCRs, PFS, and OS of all patients were re-evaluated by the principal investigators at each institution. The relative dose intensity was defined as the ratio of the actual dose administered to the planned dose.

Fisher's exact test and the Kruskal–Wallis test were used to compare patient characteristics, relative dose intensity, and treatment response. PFS and OS data were plotted as Kaplan–Meier curves, and differences among the groups according to *KRAS*, *BRAF*, *NRAS*, and *PIK3CA* gene status were compared using the log-rank test and hazard ratio calculated from a Cox regression model with a single covariate. All analyses were performed by a biostatistician (Takeharu Yamanaka), using IBM SPSS® Statistics 21 package software (SPSS Inc., Tokyo, Japan).

Results

Concordance between Luminex and direct sequencing

From September 2008 to April 2010, 376 patients were treated with a cetuximab-containing regimen at seven institutions. Of these, 83 patients met the inclusion criteria and specimens were collected from them for analysis (232 patients did not meet the inclusion criteria and 61 specimens could not be collected). We collected 78 surgically resected specimens and 5 biopsy specimens, from which the median amount of template DNA collected was 25,114 ng (range: 2740–84,738) and 1691 ng (range:1469–2668), respectively (Table 1).

One patient's gene status could not be detected by either Luminex or direct sequencing because DNA harvested from the resected metastatic liver specimens could not be amplified by PCR. In the remaining 82 patients, the concordance rate for mutations between the 2 methods was 100% (Table 2).

Among the 82 specimens, 3 *KRAS* codon 61 mutations (3.6%), 2 *KRAS* codon 146 mutations (2.4%), 4 *BRAF* mutations (4.9%), 2 *NRAS* mutations (2.4%), and 4 *PIK3CA* mutations (4.9%) (1 in exon 9 and 3 in exon 20) were detected using both the expanded kit and direct sequencing. Moreover, we identified 15 *KRAS* codon 12 mutations (18.3%) and 6 *KRAS* codon 13 mutations (7.3%); in total, 21 samples (25.6%) with *KRAS* codon 12 or 13 mutations were detected by using the *KRAS* Luminex assay kit. All mutations except for *PIK3CA* were mutually exclusive (Table 2, Figure 1).

Patient characteristics

Clinical data were collected from 83 patients. We used data from 82 patients whose genomic DNA could be successfully examined using both the expanded kit and direct sequencing. Six of the 82 patients were treated with cetuximab monotherapy, while the remaining 76 were treated with a regimen of cetuximab plus irinotecan.

Of these 82 patients, 49 had tumors with no mutation (all wild type), 21 had tumors with mutation of either *KRAS* codon 12 or 13, and 12 had tumors with mutation of either *KRAS* codon 61, *KRAS* codon 146, *BRAF*, *NRAS*, or *PIK3CA*. No significant difference was observed in the characteristics of these three groups except for the ratio of refractoriness to intolerance of prior oxaliplatin (Table 3).

Table 1 Template DNA harvested from FFPE specimens

	Surgically resected	Biopsy	Total
Number of specimens	78	5	83
Total amount of template DNA (ng) [median (range)]	25,114 (2,740–84,738)	1,691 (1,469–2,668)	22,591 (1,469–84,738)
Amount of template DNA per slice (ng) [median (range)]	8,371 (914–28,246)	370 (154–889)	7,530 (154–28,246)

Table 2 Concordance between Luminex and direct sequencing

Gene	Direct sequencing (DS)	Luminex	Concordance rate	Mutation rate
***KRAS* codon 61**	**3**	**3**	**100%**	**3.6%**
Q61K	0	0	100%	0%
Q61E	0	0	100%	0%
Q61L	0	0	100%	0%
Q61P	0	0	100%	0%
Q61R	0	0	100%	0%
Q61H	3	3	100%	3.6%
***KRAS* codon 146**	**2**	**2**	**100%**	**2.4%**
A146T	2	2	100%	2.4%
A146S	0	0	100%	0%
A146P	0	0	100%	0%
A146E	0	0	100%	0%
A146V	0	0	100%	0%
A146G	0	0	100%	0%
***BRAF* codon 600**	**4**	**4**	**100%**	**4.9%**
V600E	4	4	100%	4.9%
***NRAS* codon 12**	**2**	**2**	**100%**	**2.4%**
G12S	0	0	100%	0%
G12C	0	0	100%	0%
G12R	0	0	100%	0%
G12D	2	2	100%	2.4%
G12V	0	0	100%	0%
G12A	0	0	100%	0%
***NRAS* codon 13**	**0**	**0**	**100%**	**0%**
G13S	0	0	100%	0%
G13C	0	0	100%	0%
G13R	0	0	100%	0%
G13D	0	0	100%	0%
G13V	0	0	100%	0%
G13A	0	0	100%	0%
***NRAS* codon 61**	**0**	**0**	**100%**	**0%**
Q61K	0	0	100%	0%
Q61E	0	0	100%	0%
Q61L	0	0	100%	0%
Q61P	0	0	100%	0%
Q61R	0	0	100%	0%
Q61H	0	0	100%	0%
***PIK3CA* Exon 9**	**1**	**1**	**100%**	**1.2%**
E542K	1	1	100%	1.2%
E545K	0	0	100%	0%
E546K	0	0	100%	0%
***PIK3CA* Exon 20**	**3**	**3**	**100%**	**3.7%**
H1047R	1	1	100%	1.2%
H1047L	2	2	100%	2.4%

Response to treatment

RRs of patients with all wild-type tumors ($N = 49$), *KRAS* codon 12 or 13 mutations ($N = 21$), and mutations of *KRAS* codon 61, *KRAS* codon 146, *BRAF*, *NRAS*, or *PIK3CA* ($N = 12$) were 38.8%, 4.8%, and 0%, respectively (Table 4). Partial response was observed in one patient with a *KRAS* codon G12C mutation. In addition, DCRs were 77.6%, 57.1%, and 33.3%, respectively, for these patient groups (Table 4). Differences for both RRs and DCRs between patients with all wild-type tumors and those with *KRAS* codon 61, *KRAS* codon 146, *BRAF*, *NRAS*, or *PIK3CA* mutations were statistically significant (Fisher's exact test, RRs: $P = 0.006$, DCRs: $P = 0.006$). On the other hand, there were no statistically significant differences between patients with *KRAS* codon 12 or 13 mutations and those with *KRAS* codon 61, *KRAS* codon 146, *BRAF*, *NRAS*, or *PIK3CA* mutations (Fisher's exact test, RRs: $P = 0.636$, DCRs: $P = 0.170$).

The relative dose intensity of cetuximab was significantly higher among patients with *KRAS* codon 61, *KRAS* codon 146, *BRAF*, *NRAS*, or *PIK3CA* mutations. However, the number of treatment cycles was significantly greater among patients with all wild-type tumors (Table 4).

RR for all patients included in the study was 24.4%, whereas that for patients with *KRAS* codon 12 or 13 wild-type tumors was 31.1%. Furthermore, RR for patients with all wild-type tumors was 38.8%.

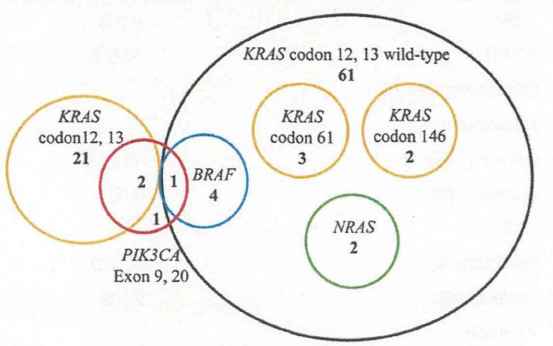

Figure 1 Associations among *KRAS*, *BRAF*, *NRAS*, and *PIK3CA* mutations. *KRAS* codon 12 and 13, *KRAS* codon 61 and 146, *BRAF*, and *NRAS* mutations were mutually exclusive. Only *PIK3CA* Exon 9 and 20 mutations overlapped *KRAS* codon 12 and 13 and BRAF mutations.

Table 3 Baseline patient characteristics

	All wild-type	*KRAS* codon 12, 13 mutations	*KRAS* codon 61, codon 146, *BRAF*, *NRAS* or *PIK3CA* mutations (any other mutations)	
	(*N* = 49)	(*N* = 21)	(*N* = 12)	
Treatment				
Cetuximab + irinotecan (%)	47 (96)	19 (90)	10 (83)	P = 0.212†
Cetuximab monotherapy (%)	2 (4)	2 (10)	2 (17)	
Age				
Median (range)	61 (29–78)	65 (51–80)	65 (43–76)	P = 0.605‡
Gender				
Male (%)	31 (63)	16 (76)	6 (50)	P = 0.312†
Female (%)	18 (37)	5 (24)	6 (50)	
ECOG PS				
0 (%)	34 (69)	13 (62)	5 (42)	P = 0.185†
1–2 (%)	15 (31)	8 (38)	7 (58)	
Primary lesion				
Colon (%)	28 (57)	15 (71)	9 (75)	P = 0.416†
Rectum (%)	21 (43)	6 (29)	3 (25)	
Site of Metastasis				
Liver				
Yes (%)	33 (67)	13 (62)	8 (67)	P = 0.945†
No (%)	16 (33)	8 (38)	3 (33)	
Lung				
Yes (%)	34 (69)	15 (71)	9 (75)	P = 1.000†
No (%)	15 (31)	6 (29)	3 (25)	
Lymph node				
Yes (%)	26 (53)	7 (33)	9 (75)	P = 0.068†
No (%)	23 (47)	14 (67)	3 (25)	
Peritoneum				
Yes (%)	11 (22)	3 (14)	2 (17)	P = 0.791†
No (%)	38 (78)	18 (86)	9 (83)	
No. of metastatic sites				
1 (%)	9 (18)	9 (42)	3 (25)	P = 0.106†
>2 (%)	40 (82)	12 (58)	9 (75)	
Prior chemotherapy				
Fluoropyrimidine				
Refractory (%)	49 (100)	21 (100)	12 (100)	
Intolerant (%)	0 (0)	0 (0)	0 (0)	
Oxaliplatin				
Refractory (%)	40 (82)	10 (48)	9 (75)	P = 0.017†
Intolerant (%)	9 (18)	11 (52)	3 (25)	
Irinotecan				P = 1.000†
Refractory (%)	48 (98)	21 (100)	12 (100)	
Intolerant (%)	1 (2)	0 (0)	0 (0)	P = 0.669†