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%.

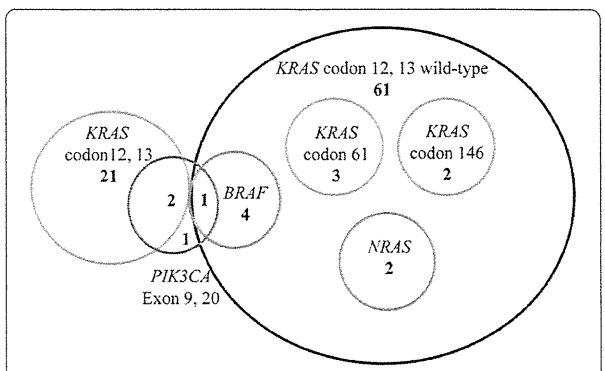

Figure 1 Associations among *KRAS*, *BRAF*, *NRAS*, and *PIK3CA* mutations. *KRAS* codon 12 and 13, *KRAS* codon 61 and 146, *BRAF*, and *NRAS* mutations were mutually exclusive. Only *PIK3CA* Exon 9 and 20 mutations overlapped *KRAS* codon 12 and 13 and BRAF mutations.

Table 3 Baseline patient characteristics

	All wild-type (*N* = 49)	*KRAS* codon 12, 13 mutations (*N* = 21)	*KRAS* codon 61, codon 146, *BRAF*, *NRAS* or *PIK3CA* mutations (any other mutations) (*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†

Table 3 Baseline patient characteristics *(Continued)*

Before bevacizumab therapy	25 (51)	9 (43)	7 (58)	
Yes (%)	24 (49)	12 (57)	5 (42)	P = 0.236[†]
No (%)	12	5	25	
Response rate for prior irinotecan-containing therapies (%)				
Pathological classification				
G1, G2 (%)	42 (86)	20 (95)	11 (92)	P = 0.481[†]
G3, G4 (%)	7 (14)	1 (5)	1 (8)	

ECOG PS Eastern Cooperative Oncology Group performance status.
[†]: Fisher's exact test.
[‡]: Kruskal–Wallis test.

Survival

The median PFS among patients with all wild-type tumors ($N = 49$), *KRAS* codon 12 or 13 mutations ($N = 21$), and *KRAS* codon 61, *KRAS* codon 146, *BRAF*, *NRAS*, or *PIK3CA* mutations ($N = 12$) 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), respectively (Table 4, Figure 2A). 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 (Table 4, Figure 2B).

We observed statistically significant differences in both PFS and OS between patients with all wild-type tumors and those with *KRAS* codon 61, *KRAS* codon 146, *BRAF*, *NRAS*, or *PIK3CA* mutations [PFS: hazard ratio (HR), 0.22; 95% CI, 0.11–0.44; $P < 0.0001$] (OS: HR, 0.30; 95% CI, 0.15–0.61; $P < 0.0001$) (Figure 2A and 2B). Differences in PFS and OS between patients with wild-type mutations and the 8 patients with *KRAS* codon 61, *KRAS* codon 146, *NRAS*, or *PIK3CA* mutations were statistically significant (PFS: P = 0.001, OS: P = 0.001), but this was not the case for the 4 patients with *BRAF* mutations. The median PFS and OS for these 4 patients were 0.9 months and 11.4 months, respectively.

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*

Table 4 Efficacy in the test population determined on the basis of gene status

	All wild-type ($N = 49$)	***KRAS* codon 12, 13 mutations ($N = 21$)**	***KRAS* codon 61, codon 146, *BRAF*, *NRAS* or *PIK3CA* mutations (any other mutations) ($N = 12$)**	
Complete response	1	0	0	
Partial response	18	1	0	
Stable disease	19	11	4	
Progressive disease	11	9	8	
Total	49	21	12	
Response rate (%)	38.8	4.8	0	P = 0.006[*] (All wild-type vs. Any other mutations)
Disease control rate (%)	77.6	57.1	33.3	P = 0.006[*] (All wild-type vs. Any other mutations)
Progression-free survival [Median (95% CI) (months)]	6.1 (3.1, 9.2)	2.7 (1.2, 4.2)	1.6 (1.5, 1.7)	P < 0.0001[**] (All wild-type vs. Any other mutations)
Overall survival [Median (95% CI) (months)]	13.8 (9.2, 18.4)	8.2 (5.7, 10.7)	6.3 (1.3, 11.3)	P < 0.0001[**] (All wild-type vs. Any other mutations)
Relative dose intensity				
Irinotecan [Median (range) (%)]	72.8 (13.0–100)	81.0 (38.4–100)	98.0 (49.3–100)	P = 0.108[***]
Cetuximab [Median (range) (%)]	86.0 (35.7–100)	86.3 (11.1–100)	100 (80.0–100)	P = 0.042[***]
Number of treatment cycles [Median (range)]	12 (1–86)	5 (1–23)	3 (1–12)	P < 0.0001[***]

[*]: Fisher's exact test.
[**]: log rank test.
[***]: Kruskal–Wallis test.

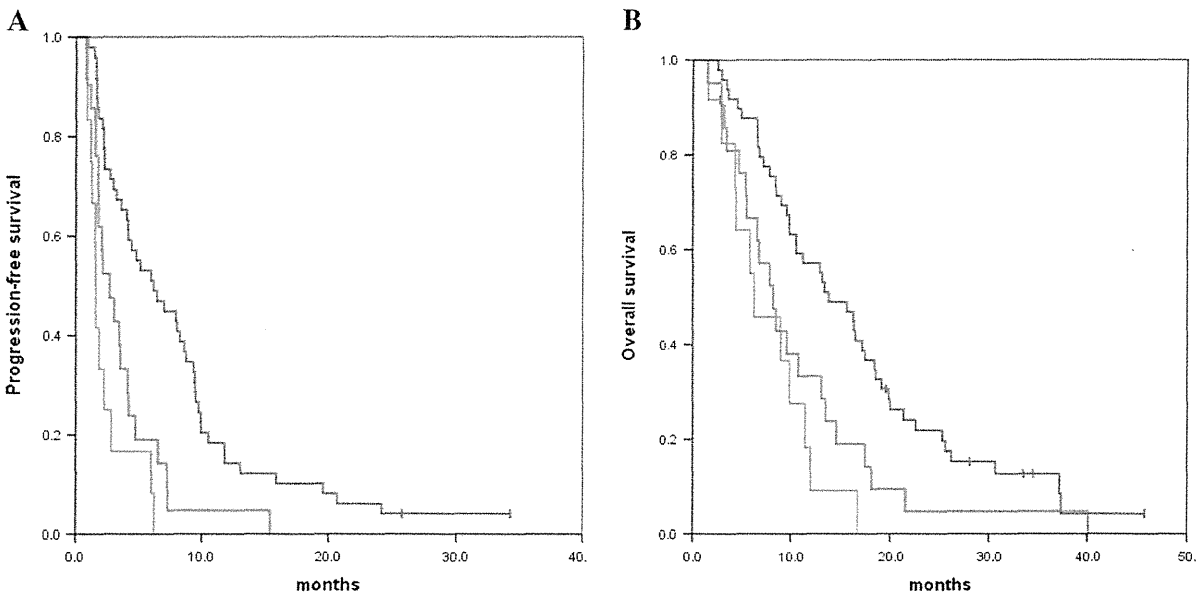

Figure 2 Kaplan–Meier plots of progression-free survival (PFS) and overall survival (OS) according to *KRAS*, *BRAF*, *NRAS*, and *PIK3CA* gene status. Figure 2A. PFS: Median PFS values were 6.1 months [95% confidence interval (CI): 3.1–9.2], 2.7 months (1.2–4.2), and 1.6 months (1.5–1.7) among patients with all wild-type tumors (*N* = 49, blue line), *KRAS* codon 12 or 13 mutant tumors (*N* = 21, green line), and *KRAS* codon 61, *KRAS* codon 146, *BRAF*, *NRAS*, or *PIK3CA* mutant tumors (*N* = 12, gray-line), respectively. Differences in PFS values between patients with all wild-type tumors and those with *KRAS* codon 61, *KRAS* codon 146, *BRAF*, *NRAS*, or *PIK3CA* mutant tumors were statistically significant (hazard ratio, 0.22; 95% CI, 0.11–0.44; $P < 0.0001$). **Figure 2B. OS:** Median OS values were 13.8 months [95% confidence interval (CI): 9.2–18.4], 8.2 months (5.7–10.7), and 6.3 months (1.3–11.3) among patients with all wild-type tumors (*N* = 49, blue line), with *KRAS* codon 12 or 13 mutant tumors (*N* = 21, green line), and with *KRAS* codon 61, *KRAS* codon 146, *BRAF*, *NRAS*, or *PIK3CA* mutations (*N* = 12, gray-line), respectively. Differences in OS values between patients with all wild-type tumors and those with *KRAS* codon 61, *KRAS* codon 146, *BRAF*, *NRAS*, or *PIK3CA* mutant tumors were statistically significant (hazard ratio, 0.30; 95% CI, 0.15–0.61; $P < 0.0001$).

codon 146, *BRAF*, *NRAS*, or *PIK3CA* mutations (PFS: $P = 0.091$, OS: $P = 0.236$) (Figure 2A and 2B).

We also analyzed the differences in PFS and OS between patients with *KRAS* codon 12 mutations and those with *KRAS* codon 13 mutations. Similar to our previous study in a different population [17], there were no statistically significant differences between these groups (median PFS: *KRAS* codon 12, 2.1 months vs. *KRAS* codon 13, 3.4 months, P = 0.682; median OS: *KRAS* codon 12, 6.8 months vs. *KRAS* codon 13, 9.6 months, P = 0.147).

Discussion

This study is the first to verify the relevance of the mutation status of *KRAS* codons 61 and 146, *BRAF*, *NRAS*, and *PIK3CA*to the clinical efficacy of anti-EGFR antibody therapy among Asian patients. As reported in a pooled analysis from a European population, patients with the aforementioned less-frequent mutations exhibited statistically significant worse outcomes equivalent to those of *KRAS* codon 12 and 13 mutants [8]. Though systemically analyzed studies have not been reported since the first European analysis, our results strongly support the usefulness of the expanded pretreatment test for anti-EGFR therapies.

Because our aim was to compare the outcomes of *KRAS* codon 12 and 13 mutant cases with those characterized by other mutations, clinical data and FFPE specimens of the patients treated with cetuximab-containing regimens at seven Japanese cancer centers from July 2008 to April 2010 were collected. At that time, the Japanese authorities did not require pretreatment *KRAS* tests, and patients with *KRAS* codon 12 and 13 mutations were eventually treated with cetuximab. However, the proportion of patients with *KRAS* codon 12 or 13 mutant tumors in this study (25.6%) was slightly lower than that in previous reports of Western and Asian study populations [18], supposedly because several participating institutions had established lab-based tests and used the data for selecting nonbeneficiary populations. Among *KRAS* codon 12 and 13 wild-type cases, the proportion with mutations of overall tested genes (12/61, 19.7%) was similar to that of previous reports, suggesting that such expanded testing would be equally useful in Western and Asian countries.

Because the potential usefulness of multiplex mutation analyses is demonstrated, the development of robust *in vitro* diagnostic systems is needed for clinical application. The application of multiplex mutation detection systems in colorectal cancer specimens has been reported.

Lurkin I. et al. reported the validity of multiplex assays using a SNaPshot® Multiplex kit (Life Technologies), which detects 22 mutations in *KRAS*, *BRAF*, *NRAS*, and *PIK3CA* [19]. Here we evaluated a quality-controlled kit detecting 36 mutations of *KRAS* codons 61 and 146, *BRAF*, *NRAS*, and *PIK3CA* using Luminex (xMAP) technology. Data obtained by this kit were fully concordant with those by conventional direct sequencing, regardless of any variation in fixation methods between participating institutes (unpublished data).

This kit has several advantages with regard to its development for routine clinical use. It is manufactured under the same quality as the hitherto approved *in vitro* diagnostic kit detecting mutations in *KRAS* codons 12 and 13. Design of the hands-on operations is simple and easy; detection of the 36 mutations is performed in a single reaction of multiplex PCR followed by Luminex bead assay, with an overall hands-on time of 4.5 h. In addition, the requirement for template DNA is as low as 50 ng. We collected a median of 370 ng (range: 154–889) DNA per 10-μm biopsy slice in this study, which is sufficiently large to perform the test and to reserve backup DNA. Meanwhile, the ARMS–Scorpion assay, another approved *in vitro* diagnostic kit, requires larger amounts of template DNA. The currently approved *KRAS* codons 12 and 13 kit consists of 8 (1 control and 7 mutations) PCR reactions. A total of 80–160 ng of template DNA (10–20 ng for each PCR reaction) are needed to examine a sample [20], and it would be difficult to expand the PCR reactions because of the limitation of template DNA.

It has been estimated that approximately 10%–20% of all patients with colorectal cancer have either *KRAS* codon 61, *KRAS* codon 146, *BRAF*, *NRAS*, or *PIK3CA* gene mutations, suggesting that approximately 60,000–120,000 patients (10%–20% of the 600,000 who die annually from colorectal cancer) worldwide could be screened by this expanded mutation test. Furthermore, because the usefulness of regular administration of aspirin for patients with mutated *PIK3CA* colorectal cancer and the possibility of combining EGFR and BRAF inhibitors for patients with mutated *BRAF* colorectal cancer have been reported, detection of those mutations could become of greater importance in many ways [21,22]. Once further studies with larger sample sizes and a range of clinical samples provide evidence of its clinical utility, this technique might advance the precision of colorectal cancer treatment.

Conclusions

Our newly developed multiplex kit is practical and feasible for investigating various types of FFPE samples. 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.

Abbreviations

EGFR: Anti-epidermal growth factor receptor; PFS: Progression-free survival; OS: Overall survival; CI: Confidence interval; FFPE: Formalin-fixed, paraffin-embedded; CT: Computed tomography; H-E: Hematoxylin–eosin; PCR: Polymerase chain reaction; RR: Response rate; DCR: Disease control rate.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TY and KT conceived the study design. HB carried out the majority of molecular genetic studies and analyses of the clinical data. ES, TN, KY, KY, SY, and SK provided clinical data and helped collect tumor tissues. SF carried out the pathological diagnoses. TY statistically analyzed the clinical data. AO coordinated the study and helped to draft the manuscript. All authors have read and approved the final manuscript.

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