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Original Article

Clinical Validation of a Multiplex Kit for *RAS* Mutations in Colorectal Cancer: Results of the RASKET (RAS KEy Testing) Prospective, Multicenter Study

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ABSTRACT

Background: *RAS* (*KRAS* and *NRAS*) testing is required to predict anti-epidermal growth factor receptor (EGFR) treatment efficacy in metastatic colorectal cancer (CRC). Although direct sequencing (DS) with manual microdissection (MMD) is widely used, a diagnostic kit providing rapid detections of *RAS* mutations would be clinically beneficial. We evaluated the MEBGEN™ RASKET KIT (RASKET KIT), a multiplex assay using PCR-reverse sequence specific oligonucleotide and xMAP® technology to concurrently detect exon 2, 3, and 4 *RAS* mutations in a short turnaround time (4.5 h/96-specimens).

Methods: Formalin-fixed paraffin-embedded (FFPE) tissues were obtained from 308 consenting patients with histologically-confirmed CRC at six hospitals in Japan. For the RASKET KIT, we used only 50–100 ng DNA from each FFPE specimen not processed by MMD. The primary endpoint was the concordance rate between *RAS* mutations identified with the RASKET KIT and two reference assays (DS with MMD and TheraScreen® K-RAS Mutation Kit). As the secondary endpoints, we evaluated the concordance rate between DS and the RASKET KIT for *RAS* mutations in the wild-type *KRAS* exon 2 population and the genotyping performance of the RASKET KIT compared with DS.

Findings: Among 307 analyzable specimens, the reference assays detected 140 (45.6%, 140/307) *RAS* mutations: 111 *KRAS* exon 2 and 29 other (minor) *RAS* mutations. The RASKET KIT detected 143 (46.6%, 143/307) mutations: 114 *KRAS* exon 2 and 29 minor *RAS* mutations. The between-method concordance rate was 96.7% (297/307) (95% CI: 94.1–98.4%). Minor *RAS* mutations were detected in 15.7% (30/191) of the wild-type *KRAS* exon 2 population ($n = 191$); the concordance rate was 98.4% (188/191) (95% CI: 95.5–99.7%). The concordance rate of *RAS* genotyping was 100% (139/139) (95% CI: 97–100%).

Interpretation: The RASKET KIT provides rapid and precise detections of *RAS* mutations and consequently, quicker and more effective anti-EGFR therapy for CRC (Study ID: UMIN000011784).

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1. Introduction

The clinical significance of the detection of *KRAS* codon 12 and 13 mutations in patients with metastatic colorectal cancer (CRC) was established from past randomized clinical trials, and then *KRAS* mutation testing has been approved as compulsory testing before anti-epidermal growth factor receptor (EGFR) antibody treatments

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(Amado et al., 2008; Bokemeyer et al., 2009; Douillard et al., 2010; Lièvre et al., 2006; Van Cutsem et al., 2009). Recently, retrospective *RAS* mutation analyses of specimens from phase II and III studies, including panitumumab randomized trial in combination with chemotherapy for metastatic colorectal cancer to determine efficacy (PRIME) indicated that similar to *KRAS* exon 2 mutations, *KRAS* exon 3 or 4, or *NRAS* exon 2, 3, or 4 mutation predicted non-response to add-on therapy with panitumumab (Douillard et al., 2013; Heinemann et al., 2014; Schwartzberg et al., 2014). Based on these results, assessment of all *RAS* mutational status in metastatic CRC patients prior to anti-EGFR antibody therapy would be beneficial for both patients' quality of life and optimal use of healthcare resources; regulatory authorities and public health institutions, such as the National Comprehensive Cancer Network have recommended compulsory *RAS* testing (http://www.nccn.org/professionals/physician_gls/pdf/colon.pdf). Indeed, the European Medical Agency has published that the summary of product characteristics for cetuximab and panitumumab has been revised to direct prescribing these drugs for patients with both wild-type (WT) *KRAS* and *NRAS* (http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/000741/WC500047710.pdf; http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/000558/WC500029119.pdf).

According to several studies, hotspots of *RAS* mutations have been identified in codons 12, 13, 59, 61, 117, and 146, which are considered to induce conformational changes into constitutively active forms (Prior et al., 2012; Smith et al., 2010). In addition, these gene mutations tend to occur in a mutually exclusive manner. These findings increase the medical need for kits that can detect all *RAS* mutations occurring in these codons. During the last decade, various techniques for *RAS* testing (i.e., direct sequencing [DS], SURVEYOR®-WAVE method, pyrosequencing, allele-specific PCR, MALDI-TOF mass array, and BEAMing [bead, emulsion, amplification and magnetics] assay) have been developed (Diehl et al., 2008; Douillard et al., 2013; Heinemann et al., 2014; Maughan et al., 2011; Parsons et al., 2011; Parsons and Myers, 2013). Some of these methods require microdissected formalin-fixed paraffin-embedded (FFPE) tissues to enrich tumor cells for obtaining enough amount of tumor-derived DNA. Although real-time PCR is one of the common techniques for *RAS* testing, it requires a lot of wells and a large amount of DNA to detect all *RAS* mutations. Next-generation sequencing technologies are innovative, comprehensive and high throughput methods for *RAS* testing, which are larger-sized for *RAS* testing in the present clinical practice. On the other hand, Luminex®-xMAP® technology can provide multiplex molecular testing in a single well, and only requires a small amount of tumor-derived DNA, as previously reported (Bando et al., 2013; Fukushima et al., 2011), so that the MEBGEN™ RASKET KIT (RASKET KIT) could be considered as more cost-effective for *RAS* testing. In this study, we evaluated the RASKET KIT to detect forty-eight kinds of *RAS* amino acid mutations in CRC patients. This study is also performed as a registration trial for regulatory approval.

2. Methods

2.1. Patients

Eligibility criteria for patients were 1) patients aged ≥20 years, 2) histologically confirmed adenocarcinoma of colorectal origin, 3) availability of adequate amount of FFPE tissues, and 4) provided patients' written consent for participation in the study, between September and November 2013. The study was approved by the institutional review boards of all of six hospitals and performed in accordance with the Declaration of Helsinki and ethical guidelines for clinical research.

2.2. Study Design

The study was performed in the patients satisfying the criteria. All of the sample specimens were anonymized in a manner which only the six hospitals were able to connect to patients' personal information using a correspondence table, which was strictly controlled at each study site to avoid any disclosure to outsiders.

We sent the same anonymized FFPE sample set to three reference laboratories (G&G Science Co., Ltd., LSI Medience Corporation, and SRL Inc.) which independently generated data by one of RASKET KIT, DS, and TheraScreen® K-RAS Mutation Kit (TheraScreen Kit), respectively. Each laboratory performed *RAS* testing in blinded. The primary endpoint of the study was the concordance rate between results obtained with the RASKET KIT and the two reference assays, DS with manual microdissection (MMD) and TheraScreen Kit (for *KRAS* exon 2 mutation analysis only), in identifying *RAS* (*KRAS* and *NRAS*) exon 2, 3, and 4 mutations. For *KRAS* gene exon 2, a specimen was defined as a mutation-positive result if a mutation in codons 12 and 13 was detected by at least either one of the two reference assays. The patients confirmed as wild-type (WT) *KRAS* exon 2 were allocated for the secondary endpoint-1 analysis — the concordance rate between DS and RASKET KIT for minor *RAS* (*KRAS* exons 3 and 4, and *NRAS* exons 2, 3, and 4) mutations. All the patient samples confirmed as *RAS* mutation positive were then proceeded to the analysis for secondary endpoint-2 analysis — accuracy of *RAS* mutation genotyping through the RASKET KIT.

2.3. Histopathologic Evaluation of Specimens

We prepared hematoxylin– and eosin (HE)-stained slides using 2 μm-thick FFPE sections. One pathologist assigned for the study microscopically confirmed cancer in each patient, calculated the tumor area ratio and tumor cell ratio, and marked tumor area on the prepared HE-stained slides for MMD.

2.4. Direct Sequencing

We used 10 μm-thick FFPE serial sections for DNA extraction. After the pathological confirmation of cancer in each patient, the 10 μm-thick sections were then processed by MMD to avoid a possible case that the sensitivity is too low to adequately detect *RAS* mutations (Domagała et al., 2012). DNA extraction was performed with QIAamp® DNA FFPE Tissue Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's protocol. Briefly, each extracted DNA was amplified using six sets of primers to amplify exon 2, exon 3, and exon 4 in *KRAS* and *NRAS* (Table S1). *RAS* exon 2, 3, and 4 mutations were detected using the BigDye® Terminator Cycle Sequencing kit (Thermo Fisher Scientific, MA, USA) and analyzed on a 3130xl Genetic Analyzer (Thermo Fisher Scientific).

2.5. Assay with TheraScreen® K-RAS Mutation Kit

The DNA extraction from the 10 μm-thick sections was performed with QIAamp DNA FFPE Tissue Kit according to the manufacturer's protocol. Then, real-time PCR was performed to detect seven types of mutations in codons 12 and 13 of *KRAS* exon 2 (i.e., G12S, G12C, G12R, G12D, G12V, G12A, and G13D) using TheraScreen Kit (Qiagen) according to the manufacturer's instruction.

2.6. Assay with MEBGEN™ RASKET KIT

DNA extraction was performed using a modified protocol as described previously (Fukushima et al., 2011; Gilbert et al., 2007). Each extracted DNA sample was diluted to a concentration of 10–20 ng/μL with sterile TE buffer (1 mmol/L Tris–HCl [pH = 8.0], 0.1 mmol/L EDTA). Assay with RASKET KIT (MBL, Nagoya, Japan) was performed as the manufacturer's protocol. The amplification was performed in a final volume of 25 μL [20 μL master mix, including primers, Taq DNA polymerase, and Uracil-DNA-glycosylase (UDG), and 5 μL extracted DNA]. Reactions were heated for 5 min at 40 °C and 2 min at 95 °C; 10 repeating cycles of 94 °C for 20 s and 62 °C for 30 s; 45 repeating cycles of 90 °C for 20 s, 60 °C for 30 s, and 72 °C for 30 s; and then 72 °C for 1 min and 94 °C for 10 min. Each amplification product was then hybridized to mutation detection probes immobilized with color-coded beads. 5 μL of PCR products and 45 μL of hybridization solution containing probe-coupled beads were hybridized at 95 °C for 2 min followed by 55 °C for 30 min. After washing, the PCR amplification-bead complexes were reacted with streptavidin–phycoerythrin (SA–PE) at 52 °C for 15 min. Using Luminex® 100/200™ (Luminex, TX, USA), we counted median fluorescence intensity (MFI) for the color-coded beads and PE, representing types of *RAS* mutations and their signal intensities, respectively. UniMAG™ (data analysis software, MBL) was then used for analyzing raw data from Luminex® 100/200™. Thus, using RASKET KIT, we examined twelve types of *RAS* exon 2 (G12S, G12C, G12R, G12D, G12V, G12A, G13S, G13C, G13R, G13D, G13V, and G13A), eight types of *RAS* exon 3 (A59T, A59G, Q61K, Q61E, Q61L, Q61P, Q61R, and Q61H), and four types of *RAS* exon 4 (K117N, A146T, A146P, and A146V) mutations, simultaneously (Table S2).

The evaluation criterion for the RASKET KIT was ≥90% of concordance rate with the reference assays in the primary and secondary endpoint analysis-1. In case of any controversial data between RASKET KIT and the reference assays, we confirmed the results with TaqMan® Mutation Detection Assays (Thermo Fisher Scientific) (Didelot et al., 2012).

2.7. Statistical Analysis

The number of specimens required for the study was estimated as the number of specimens to satisfy that the rate of concordance between the RASKET KIT and the reference assays would be >90% at the lower limit of the 95% confidence interval (CI). Then we determined the number should be >278, using the following equation:

$$\begin{aligned}&\text{Sample ratio} - 1.96 \times \sqrt{\text{Population concordance rate} \times (1 - \text{population concordance rate})/\text{number of data}} \\ &\leq \text{Population ratio} \\ &\leq \text{Sample ratio} + 1.96 \times \sqrt{\text{Population concordance rate} \times (1 - \text{population concordance rate})/\text{number of data}}.\end{aligned}$$

3. Results

3.1. Specimen Analysis

FFPE tissues were obtained from 309 consenting patients with histologically-confirmed CRC at the six hospitals (50–55 samples per hospital) (Fig. 1). Sufficient FFPE tissues were not available in one patient, and data from one reference assay was not reportable; therefore 307 samples were included in the primary endpoint analysis. Among them, 191 patient (62.2%, 191/307) samples were confirmed as WT *KRAS* exon 2 and were then proceeded to the secondary endpoint-1 analysis. Also, 138 patient samples were confirmed as mutation positive by both the RASKET KIT and DS and were included in the secondary endpoint analysis-2.

3.2. Assay Success Rates

The overall assay success rates for the three *RAS* genetic assays (RASKET KIT, DS, and TheraScreen Kit) used in this study are shown in Table S3. In all the assays, the success rates were no less than 99%. Two samples were indeterminate with the RASKET KIT, which had positive and negative results with the reference assays. The former resulted in PCR amplification failure at *NRAS* exon 4 region, even detected *KRAS* exon 2 mutation with the RASKET KIT. The latter had PCR amplification failure at multiple exons (*KRAS* exon 2, exon 3, and exon 4; and *NRAS* exon 4), and no *RAS* mutations were detected from the other exons with the RASKET KIT.

3.3. Frequency of RAS Mutations

Among the 307 specimens assayed, the RASKET KIT detected 143 (46.6%, 143/307) mutations: 114 *KRAS* exon 2 and 29 minor *RAS* mutations. The reference assays detected 140 (45.6%, 140/307) *RAS* mutations: 111 *KRAS* exon 2 and 29 minor *RAS* mutations. The population with *RAS* mutations detected by either one of the methods was 146 (47.6%, 146/307). The frequencies of mutations in exons 2, 3, and 4 were 37.8% (116/307), 2.0% (6/307) and 3.3% (10/307) in *KRAS*, and 2.0% (6/307), 2.6% (8/307), and 0% (0/307) in *NRAS*, respectively. Notably, among the 191 specimens confirmed as WT *KRAS* exon 2, the frequency of minor *RAS* mutations was 15.7% (30/191) (Table 1).

3.4. Between-kit RAS Status Concordance (Primary Endpoint Analysis)

The concordance rate between results obtained with RASKET KIT and the reference assays in assessing *KRAS* and *NRAS* exon 2, 3, and 4 mutations was 96.7% (297/307) (95% CI: 94.1–98.4%). The agreement in detecting positive *RAS* mutations was 97.9% (137/140) (95% CI: 93.9–99.6%), while that for detecting negative mutations was 95.8% (160/167) (95% CI: 91.6–98.3%) (Table 2a).

On the other hand, there were several samples with conflicting results between the RASKET KIT and the reference assays. Six samples of them were including one specimen with *KRAS* G12R, two with *KRAS* G12D, two with *KRAS* G13D, and one with *KRAS* Q61H detected by the RASKET KIT, but all the samples were negative with both the reference assays. We confirmed these samples using TaqMan Mutation Detection Assays, which is capable of detecting mutations in a sample at a level of

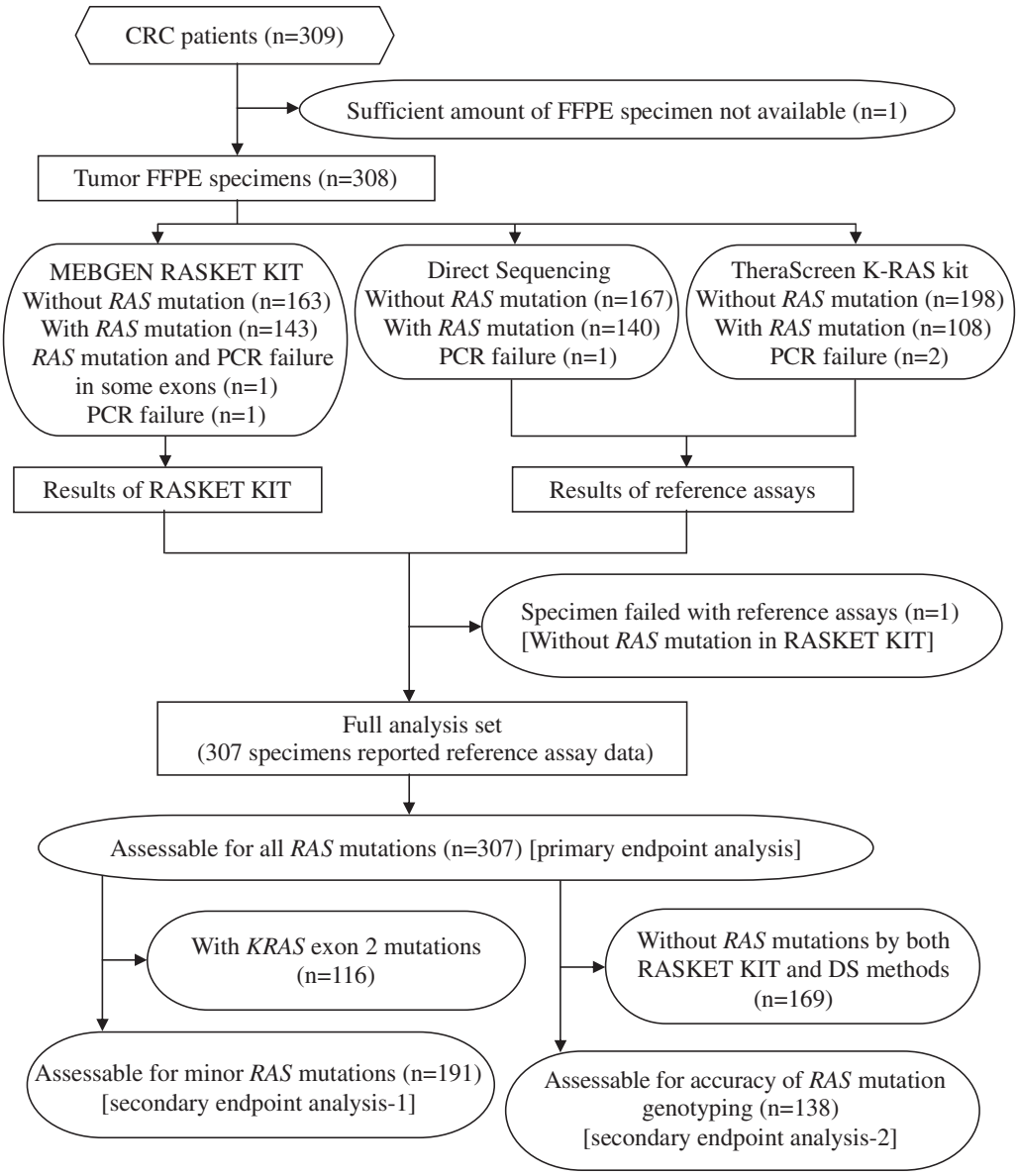

Fig. 1. Study design and patients eligible for primary and secondary endpoint analyses.
Among 307 assessable samples, 191 samples having *RAS* mutations other than those in exon 2 were forwarded to secondary endpoint analysis-1. Also, 138 samples with *RAS* mutation resulted by at least one assay were included to secondary endpoint analysis-2.

0.1–1%, and the analyses of all the six samples resulted in indeed mutation positive (Table 3). The other two conflicting samples were shown as mutation negative with the RASKET KIT and positive by the DS method. One sample had a mutation in *KRAS* codon 11 as well as *KRAS* G12C. *KRAS* codon 11 is located in the region of mutation detection probes immobilized *KRAS* exon 2 color-coded beads, so that the PCR amplification product with the codon 11 mutation did not bind even to G12C beads. The other sample was a *KRAS* exon 3 mutation (A59E), which is a mutation outside the detectable range of the RASKET KIT.

3.5. Between-kit RAS Status Concordance in WT KRAS Exon 2 Population (Secondary Endpoint Analysis-1)

A total of 191 patients were confirmed as WT *KRAS* exon 2 and were included in the secondary endpoint analysis-1. The concordance rate between RASKET KIT and DS for secondary endpoint analysis-1 was 98.4% (188/191) (95% CI, 95.5–99.7%) (Table 2b).

3.6. Accuracy of RAS Mutation Genotyping (Secondary Endpoint Analysis-2)

Specimens confirmed as mutation positive by both the RASKET KIT and DS were included in the analysis of secondary endpoint analysis-2. The concordance of each genotype for the overall population as assessed by the RASKET KIT and DS was 100% (139/139) (95% CI, 97%–100%) (Table 4).

4. Discussion

This study is the first to demonstrate a clinical usefulness of RASKET KIT, a CE-marked and approved by the Ministry of Health, Labour and Welfare of Japan *in vitro* diagnostics (IVD) kit for determination of all *RAS* mutation status in FFPE tissues of CRC patients. For the purpose, we prospectively compared the RASKET KIT to TheraScreen Kit and DS with MMD which are the gold standard for *RAS* testing (Massarelli et al., 2007). Our data revealed that the overall concordance rate

Table 1
Frequency of all *RAS* mutations detected in colorectal cancer patients.

RAS status	No. of cases	Proportion among 307 cases
WT *RAS*	162	52.8%
KRAS exon 2 mutant	116	37.8%
p.G12S	5	1.6%
p.G12C	8	2.6%
p.G12R	4	1.3%
p.G12D	46	15.0%
p.G12V	23	7.5%
p.G12A	6	2.0%
p.G12R, p.G12A	1	0.3%
p.G13C	1	0.3%
p.G13D	20	6.5%
p.G12D, p.G13D	2	0.7%
Other *KRAS* exon 2 mutant[a]	0	0%
KRAS exon 3 mutant	6	2.0%
p.A59E	1	0.3%
p.Q61H	5	1.6%
Other *KRAS* exon 3 mutant[b]	0	0%
KRAS exon 4 mutant	10	3.3%
p.K117N	2	0.7%
p.A146T	6	2.0%
p.A146P	1	0.3%
p.A146V	1	0.3%
NRAS exon 2 mutant	6	2.0%
p.G12D	4	1.3%
p.G12V	2	0.7%
Other *NRAS* exon 2 mutant[c]	0	0%
NRAS exon 3 mutant	8	2.6%
p.Q61K	2	0.7%
p.Q61L	5	1.6%
p.Q61R	1	0.3%
Other *NRAS* exon 3 mutant[d]	0	0%
NRAS exon 4 mutant[e]	0	0%

Prevalence of each *RAS* mutation presented as n and %.
[a] *KRAS* p.G13S, p.G13R, p.G13V, and p.G13A.
[b] *KRAS* p.A59T, p.A59G, p.Q61K, p.Q61E, p.Q61L, p.Q61P, and p.Q61R.
[c] *NRAS* p.G12S, pG12C, p.G12R, and p.G12A.
[d] *NRAS* p.A59T, p.A59G, pQ61E, p.Q61P, and Q61H.
[e] *NRAS* p.K117N, p.A146T, p.A146P, and p.A146V.

(96.7%, 297/307) was satisfied with the predefined criterion (>90%). As a useful kit detecting all *RAS* mutations, the capability of detecting minor *RAS* mutations other than *KRAS* exon 2 is required. Our study also showed a high concordance rate of 98.4% (188/191) between mutational status obtained with the RASKET KIT and DS with MMD, in WT *KRAS* exon 2 population ($n = 191$). Similar to the overall concordance rate, the positive and negative concordance rates were no less than 95%. The frequency of *RAS* mutations detected with the RASKET KIT in our study agreed with those reported in the several past studies (Bokemeyer et al., 2009; Douillard et al., 2010, 2013; Heinemann et al., 2014; Watanabe et al., 2013). For example, 37.1% (114/307) in exon 2 detected in this study corresponded with the 37.6% reported in a large-scaled Japanese study (Watanabe et al., 2013).

KRAS testing to determine exon 2 status prior to anti-EGFR treatment for CRC patients has been widely used (Normanno et al., 2009). Many *KRAS* exon 2 mutation detection kits such as TheraScreen Kit are approved as IVD in Japan, USA, and Europe. Recent studies suggested that additional *RAS* mutation as well as *KRAS* exon 2 could predict an efficacy of anti-EGFR treatment, and consequently the indication of anti-EGFR therapeutic antibodies has been revised to direct the treatment of patients with wild-type *RAS* (both *KRAS* and *NRAS*) metastatic CRC (Douillard et al., 2013; Heinemann et al., 2014; Schwartzberg et al., 2014). Thus, expanded *RAS* testing using an approved IVD kit is recommended.

We had several inconsistent results between the RASKET KIT and the reference assays. Six specimens were determined as positive with RASKET KIT and negative with the reference assays. According to the confirmation study using TaqMan Mutation Detection Assays, all of the six specimens were *RAS* mutation positive. Such discrepancy may be caused by a smaller amount of mutant DNA. Indeed, as shown in Table 3, the ratios of mutant *RAS* DNA to WT *RAS* DNA were 0.1–1% in two specimens and 1–5% in three out of the six specimens. Another conflicting data was found in a sample with over 5% of mutant *RAS* DNA. In this study, although FFPE sections were equally distributed to three reference laboratories, it could not be completely denied a possibility of intratumoral heterogeneity. Bando et al. reported that the results by TheraScreen Kit whose sensitivity seems to be 1–5% were correlated with anti-EGFR therapeutic efficacy more than those by DS (Bando et al., 2011). On the other hand, a recent data suggested that patients with low-frequency (<1%) *KRAS* mutations may benefit of targeted anti-EGFR therapies (Laurent-Puig et al., 2014). The detection sensitivity of the RASKET KIT would also be at least 1–5%, which means that the

Table 2a
Primary endpoint analysis/concordance of mutations detected with RASKET KIT versus reference assays ($n = 307$).

Concordance		Reference assays		
		Mutation positive	Mutation negative	Total
MEBGEN RASKET KIT	Mutation positive	137	6	143
	Mutation negative	2	160	162
	Not reportable	1	1	2
	Total	140	167	307
Overall percent agreement		96.7% (95% CI, 94.1%–98.4%)		
Positive percent agreement		97.9% (95% CI, 93.9%–99.6%)		
Negative percent agreement		95.8% (95% CI, 91.6%–98.3%)		

Results of concordance rate in *KRAS* and *NRAS* exon 2, exon 3, and exon 4. Data are number of samples and percentage. Each percent agreement is calculated with the numbers.

Table 2b
Secondary endpoint analysis-1/concordance of mutations detected with RASKET KIT versus direct sequencing in *KRAS* exon 2 mutation-negative patients ($n = 191$).

Concordance		Reference assay — direct sequencing		
		Mutation positive	Mutation negative	Total
MEBGEN RASKET KIT	Mutation positive	28	1	29
	Mutation negative	1	160	161
	Not reportable	0	1	1
	Total	29	162	191
Overall percent agreement		98.4% (95% CI, 95.5%–99.7%)		
Positive percent agreement		96.6% (95% CI, 82.2%–99.9%)		
Negative percent agreement		98.8% (95% CI, 95.6%–99.9%)		

Results of concordance rate in *KRAS* exon 3, and exon 4, and *NRAS* exon 2, exon 3, and exon 4. Data are number of samples and percentage. Each percent agreement is calculated with the numbers.

Table 3
Discrepancy samples between RASKET KIT and reference assays confirmed by TaqMan Mutation Detection Assays.

RASKET KIT	Reference assays	Percentage of mutant DNA in TaqMan Mutation Detection Assays
KRAS p.G12D	WT	2.8% (p.G12D)
KRAS p.G12D	WT	1.8% (p.G12D)
KRAS p.G12R	WT	0.3% (p.G12R)
KRAS p.G13D	WT	0.4% (p.G13D)
KRAS p.G13D	WT	2.0% (p.G13D)
KRAS p.Q61H	WT	38.9% (p.Q61H)
WT	*KRAS* p.G12C[a]	Not tested
WT	*KRAS* p.A59E	Not tested

Eight samples are in conflicting between MEBGEN RAKET KIT and reference assays. Data are shown as each result of those assays and percentage of amount of mutated DNA among total sample.
[a] *KRAS* p.A11A (c.33T>C) and *KRAS* p.G12C (c.34G>T) positive.

Table 4
Secondary endpoint analysis-2/genotyping performance of the RASKET KIT and direct sequencing.

			Direct sequencing																	
			KRAS													*NRAS*				
			G12S	G12C	G12R	G12D	G12V	G12A	G13C	G13D	Q61H	K117N	A146T	A146P	A146V	G12D	G12V	Q61K	Q61L	Q61R
RASKET KIT	*KRAS*	G12S	5																	
		G12C		7																
		G12R			4															
		G12D				46														
		G12V					23													
		G12A						6												
		G13C							1											
		G13D								19										
		Q61H									4									
		K117N										2								
		A146T											6							
		A146P												1						
		A146V													1					
	NRAS	G12D														4				
		G12V															2			
		Q61K																2		
		Q61L																	5	
		Q61R																		1

All of mutational data obtained by MEBGEN RASKET KIT was identical to those done by direct sequencing.

detection of each *RAS* mutation can be thoroughly secured in samples with 5% *RAS* mutant allele in wild-type *RAS* genes and some of those alleles could be detected even in case of 1% (Table S4a and S4b). Thus, this kit can provide clinically appropriate detection of *RAS* mutations, although not being able to quantify the mutations can be a limitation in using this kit to screen the *KRAS* mutations for metastatic CRC patients. Further investigation will be needed to clarify the most appropriate detection sensitivity of *RAS* mutation as a companion diagnostics prior to anti-EGFR therapies.

The RASKET KIT is designed to detect a total of forty-eight *RAS* amino acid mutations in codons 12, 13, 59, 61, 117, and 146. These codons are recognized as mutational hotspots for *RAS* genes (Prior et al., 2012; Smith et al., 2010). In this study, there were two false negative results in the RASKET KIT, not caused by a lower DNA amount. One was the sample with *KRAS* A59E mutation, which is not included in the RASKET KIT, due to a rare mutation. Indeed, this mutation was not reported in the PRIME study, but A59E was not at all (Douillard et al., 2010). The other false negative sample had double mutations in *KRAS* codon 11 and codon 12 (G12C). The codon 11 region is located at the next to codons 12 and 13, so that the probes for detecting codons 12 and 13 mutations also include the sequence for codon 11. Based on the assay principle, PCR amplifications including codon 11 mutation would not be able to hybridize to any detection beads for codons 12 and 13. Anyway, according to past reports, these results would have little impact on clinical performance of the RASKET KIT so far, as both cases would hardly occur in clinical practice (Amado et al., 2008; Bokemeyer et al., 2009; Douillard et al., 2010; Heinemann et al., 2014; Schwartzberg et al., 2014).

Luminex®-xMAP® technology has been widely applied for not only protein assays, but also several multiplex molecular testings, such as HLA genotyping and virus genotyping, which require differential detections from a number of related sequencing (Itoh et al., 2005; Ozaki et al., 2014). Hence, this technique could be suitable for molecular testing for personalized cancer medicine. Indeed, several studies previously reported usefulness of assay kits using this technique (Bando et al., 2013; Kawamoto et al., 2012; Shinozaki et al., 2014). For example, GENOSEARCH™ Mu-PACK detects mutations in *BRAF* (V600K and, V600E) and *PIK3CA* (E542K, E545K, Q546K, H1047R, and H1047L) as well as some regions of *RAS* (Bando et al., 2013). This kit would also be expected to be approved as an IVD kit. In the point of view of such multiplex molecular assay, any possible cross reactions should be avoided for appropriate assays. Actually, the RASKET KIT offers a PCR reaction of eight regions simultaneously, in a single well. For performing *RAS* testing by DS, it is required to visually confirm to distinguish a significant peak and a noise on chromatogram. The RASKET KIT can provide us an objective detection of *RAS* mutation using cut-off values in a short turnaround time (approximately 4.5 h) and regardless of the number of samples less than 96. This specification may be a benefit for pathological laboratories of any size, especially centralized reference laboratories. Physicians struggle to clinically diagnosis for starting early treatments for any diseases, so that *RAS* mutation detections by the RASKET KIT would be beneficial for CRC patients, as well. Furthermore, the amount of DNA required for detection of all *RAS* mutation is quite a few (50–100 ng/48 mutations/well) in the RASKET KIT, unlike other techniques which perform reactions as one mutation/well. Even for expansion of testing from *KRAS* to all *RAS*, RASKET KIT could require the same amount of DNA and consequently keep the same testing cost as well. Thus, the RASKET KIT is well-designed for rapid, high throughput and multiplex detection of all *RAS* mutations.

In conclusion, the clinical evaluation study of the MEBGEN™ RASKET KIT met the predefined primary and secondary endpoints and showed a high concordance rate with existing *RAS* or *KRAS* mutation assays. The RASKET KIT provides rapid and precise detection of *RAS* mutations from FFPE CRC tissue, allowing oncologists to more quickly and effectively target treatment to individual patients.

Author Contributions

TY was responsible for drafting the report and critical revision of important intellectual content. All authors contributed to the study concept, design, and supervision, and to the interpretation of the data and writing of the report.

Conflicts of Interest

TY, KM, KY, TN, TD, TKu, WO, HT, KA, TKa, and TS have received honoraria from MBL during the conduct of the study. SH has nothing to disclose.

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Appendix A. Supplementary Data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2015.02.007.

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