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; 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...
(Amado et al., 2008; Bokemeyer et al., 2009; Douillard et al., 2010; Liévre 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 magnets] 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 KRAS (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 (Domagala et al., 2012). DNA extraction was performed with QIAamp® DNA FFPE Tissue Kit (Qagen, 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).
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. UniMAGTM (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 mutations in codon 12 (G12S, G12C, G12R, G12D, G12V, G12A, and G12D) using TheraScreen Kit (Qiagen) according to the manufacturer’s instruction. 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).

### 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 G61H 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...
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
(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 mutation 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%, which means that the additional RAS 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 1](image1)

**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, p.G12C, p.G12R, and p.G12A.

\(^d\) NRAS p.A59T, p.A59G, p.Q61E, p.Q61P, and p.Q61R.

\(^e\) NRAS p.K117N, p.A146T, p.A146P, and p.A146V.

![Table 2b](image2)

**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 | 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.
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 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 testsings, 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 B-Raf (V600K and, V600E) and PI3Ka (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 KRAS or NRAS 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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