



KRAS Mutation Test in Korean Patients with Colorectal Carcinomas: A Methodological Comparison between Sanger Sequencing and a Real-Time PCR-Based Assay

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Background: Mutations in the *KRAS* gene have been identified in approximately 50% of colorectal cancers (CRCs). *KRAS* mutations are well established biomarkers in anti-epidermal growth factor receptor therapy. Therefore, assessment of *KRAS* mutations is needed in CRC patients to ensure appropriate treatment. **Methods:** We compared the analytical performance of the cobas test to Sanger sequencing in 264 CRC cases. In addition, discordant specimens were evaluated by 454 pyrosequencing. **Results:** *KRAS* mutations for codons 12/13 were detected in 43.2% of cases (114/264) by Sanger sequencing. Of 257 evaluable specimens for comparison, *KRAS* mutations were detected in 112 cases (43.6%) by Sanger sequencing and 118 cases (45.9%) by the cobas test. Concordance between the cobas test and Sanger sequencing for each lot was 93.8% positive percent agreement (PPA) and 91.0% negative percent agreement (NPA) for codons 12/13. Results from the cobas test and Sanger sequencing were discordant for 20 cases (7.8%). Twenty discrepant cases were subsequently subjected to 454 pyrosequencing. After comprehensive analysis of the results from combined Sanger sequencing–454 pyrosequencing and the cobas test, PPA was 97.5% and NPA was 100%. **Conclusions:** The cobas test is an accurate and sensitive test for detecting *KRAS*-activating mutations and has analytical power equivalent to Sanger sequencing. Prescreening using the cobas test with subsequent application of Sanger sequencing is the best strategy for routine detection of *KRAS* mutations in CRC.

Key Words: *KRAS* mutation test; Sanger sequencing; Cobas test; 454 pyrosequencing

The *KRAS* gene was recognized more than 30 years ago as the component of Kirsten sarcoma virus responsible for oncogenesis.¹ Mutations in the *KRAS* gene that lead to its constitutive activation have been identified in approximately 50% of colorectal cancer (CRC) tumors and are common in other tumor types such as pancreas (90%), lung (30%), thyroid (50%), and myeloid leukemia tumors (30%).² Most activating mutations in CRCs occur in codons 12 (~82%) and 13 (~17%) of exon 2 of the *KRAS* gene. However, mutations in codon 61 of exon 3 have also been described.³

Monoclonal antibodies against epidermal growth factor receptor (EGFR), including cetuximab (Erbix, ImClone Systems, Branchburg, NJ, USA) and panitumumab (Vectibix, Amgen, Thousand Oaks, CA, USA), have been approved for the treatment of CRC tumors.⁴ However, a number of studies have demonstrated that CRC patients with *KRAS* mutations in codons

12 and 13 do not benefit from treatment with anti-EGFR monoclonal antibodies. *KRAS* is downstream from EGFR in the *KRAS*-*BRAF*-*MEK*-*ERK* pathway, and blocking EGFR has little effect due to downstream activation of *KRAS*.⁵ Therefore, assessment of the mutational status of *KRAS* is mandatory in CRC patients to ensure appropriate treatment choice.

A number of methods for detecting *KRAS* mutations are currently in clinical use. However, it is not clear which technique offers the best performance. Sanger sequencing, which theoretically can identify all possible mutations in an exon, is a common reference method used to detect somatic mutations in tumor specimens. However, Sanger sequencing suffers from limited sensitivity for low level mutant alleles, particularly in formalin-fixed paraffin-embedded tissue specimens, and has slow turnaround time.⁶ The cobas *KRAS* mutation test (Roche Molecular Systems, Pleasanton, CA, USA) is a real-time polymerase chain

reaction (PCR)-based assay designed to identify *KRAS* mutations in codons 12, 13, and 61. This platform reveals whether a mutation is present in a specific hot spot.

The aim of this study was to compare the analytical performance and workflow characteristics of the cobas *KRAS* mutation test to Sanger sequencing in order to provide optimal care to metastatic colorectal cancer (mCRC) patients through optimal selection of anti-EGFR therapy. In addition, discordant specimens were subjected to next-generation 454 pyrosequencing.

MATERIALS AND METHODS

Selection of patients and tumor samples

A total of 264 patients with CRC who had undergone radical surgery at Seoul St. Mary's Hospital, The Catholic University of Korea between 2008 and 2010 were enrolled in this study. All cases were sporadic without any family history of CRC and were examined by a pathologist who specializes in gastrointestinal tract pathology. The formalin-fixed, paraffin-embedded (FFPE) tissue samples from CRC patients were tested in accordance with protocols approved by the Institutional Review Board of The Catholic University of Korea (KC12SISI0705). Estimated tumor content ranged from 50% to 90%. The study scheme is summarized in Fig. 1.

Direct sequencing technique for *KRAS* mutation

For DNA isolation, 10- μ m-thick sections from FFPE tissue samples were used for each case. The hematoxylin and eosin

sections used as references were marked with a pen to indicate the tumor-rich area, and the tumor area was scraped off with a scalpel under a dissecting microscope. For genomic DNA extraction, we used the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to manufacturer's recommendations. DNA yields were quantified using a Nanodrop spectrophotometer ND-1000 (Thermo Fisher Scientific Inc., Waltham, MA, USA). Sanger sequencing was performed using an ABI 3730 automated sequencer (Applied Biosystems Inc., Foster City, CA, USA) to detect the presence of *KRAS* exon 2 mutations with previously reported primers.⁷ The resulting PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) and the appropriate protocol on the QIAcube robotic workstation. Each chromatogram was visually inspected for abnormalities (Fig. 2).

The cobas *KRAS* mutation test

The TaqMelt PCR assay cobas *KRAS* Mutation Test (Roche Diagnostics) was used according to manufacturer's protocol. First, manual specimen preparation was conducted to obtain genomic DNA from FFPE CRC tissue samples (50 ng of DNA), and PCR amplification and detection of target DNA were performed on the cobas 4800 system. Data were automatically processed by the COBAS software.

454 Quantitative massively parallel pyrosequencing

Specimens showing discordant cobas test and Sanger sequencing results were retested using a quantitative massively parallel pyrosequencing method (454 GS Titanium, 454 Life Sciences,

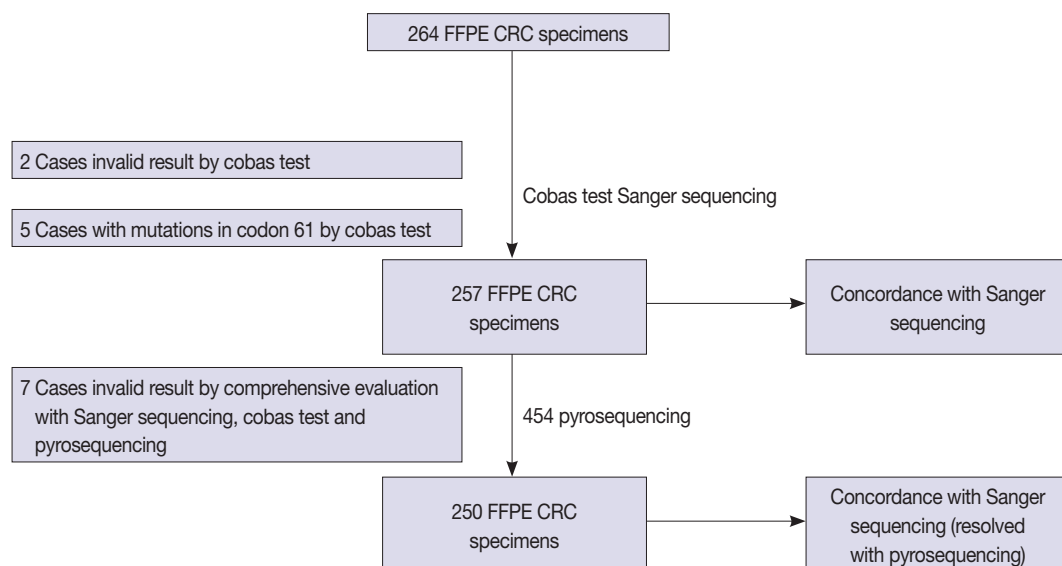


Fig. 1. Study design and specimen selection. Two hundred sixty-four formalin-fixed, paraffin-embedded (FFPE) colorectal cancer (CRC) specimens were selected and processed using Sanger sequencing and cobas test.

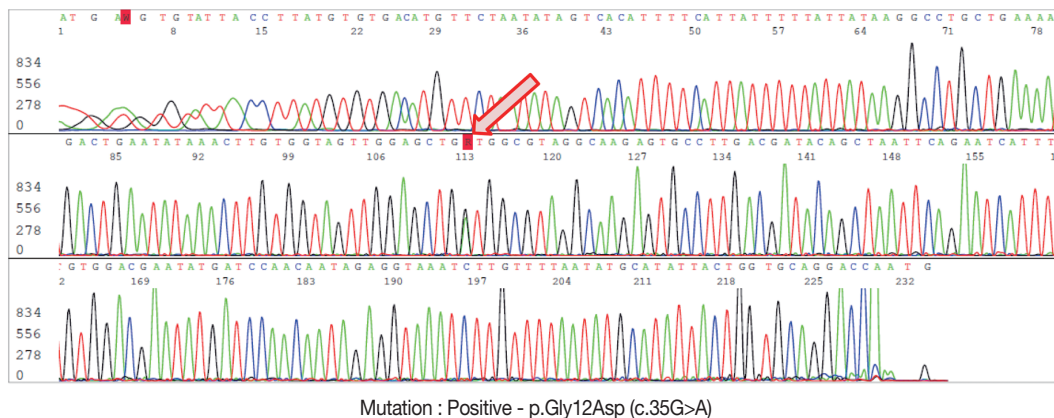


Fig. 2. Electropherogram from Sanger sequencing. Representative sample shows mutant codon 12 with GGT>GAT (arrow).

Branford, CT, USA) by Roche R&D Center China (RRDCC, Shanghai, China) using a validated protocol.

Statistical analysis

Data were analyzed using the SPSS statistical software ver. 21.0 (IBM Corp., Armonk, NY, USA) for Windows. The analytical performance of the cobas test compared with Sanger sequencing for the detection of *KRAS* mutations was evaluated by positive percent agreement (PPA), negative percent agreement (NPA), positive predictive value, and negative predictive value with two-sided 95% confidence intervals.

RESULTS

Patients and samples characteristics

The characteristics of the patients and samples are summarized in Table 1. Patients included 161 men (61.0%) and 103 women (39.0%) with a median age of 62 years (range, 32 to 93 years). Most tissue samples were from primary tumors, while the remaining 1.5% of samples (4 of 264) were metastatic. A total of 262 patients were diagnosed with adenocarcinoma and two patients were diagnosed with neuroendocrine tumor, grade 1 (carcinoid tumor) and gastrointestinal stromal tumor. The median DNA concentration was 199.59 (range, 8.35 to 1,180.87).

Frequency of mutations in *KRAS* exon 2 by Sanger sequencing and the cobas test

The detailed mutation status of 264 cases using Sanger sequencing and the cobas test is summarized in Table 2 and Supplementary Table S1. *KRAS* mutations in codons 12 and 13 were detected in 114 cases (43.2%) by Sanger sequencing. *KRAS* mutations in codons 12, 13, and 61 were revealed in 123 cases (46.6%) by the cobas test. Among the 123 mutation cases on the cobas test,

Table 1. Patients and samples characteristics

Characteristic	No. (%)
Total patients	264
Age, median (range, yr)	62 (32–93)
Gender	
Male	161 (61.0)
Female	103 (39.0)
Sites	
Primary	260 (98.5)
Metastasis	4 (1.5)
DNA concentration (ng/ μ L)	199.59 (8.35–1,180.87)
DNA purity (260/280)	1.99 (1.59–3.01)

Table 2. Frequency of mutations in *KRAS* exon 2 by Sanger sequencing and the cobas test

	Sanger sequencing	The cobas test
Mutation		
Codon 12	90 (34.1)	118 (44.7)
Codon 13	24 (9.1)	
Codon 61	-	5 (1.9)
No mutation	150 (56.8)	139 (52.7)
N/A specimen	0	2 (0.8)
Total	264 (100)	264 (100)

Values are presented as number (%).
N/A, not available.

five cases were revealed to have mutations in codon 61 that were not detected by Sanger sequencing, but instead only by the cobas test. Therefore, we excluded five cases of codon 61 mutation from comparison. Two cases had invalid results on the cobas test due to inadequate specimen and were excluded from comparison. In total, we evaluated 257 cases for comparison.

Overall concordance for detecting *KRAS* mutation of codons 12/13 by Sanger sequencing and the cobas test

KRAS mutations for 257 evaluable cases were detected in 112 cases (43.6%) by Sanger sequencing and 118 cases (45.9%) by

the cobas test. Concordance between the cobas test and Sanger sequencing for each lot was 93.8% PPA and 91.0% NPA for codons 12/13. Discordant ratio of Sanger sequencing and cobas test was 7.7% (20 out of 257 cases) (Table 3).

Analysis for 20 discrepant cases with 454 pyrosequencing

For further analysis of 20 discordant cases, we performed 454 pyrosequencing. Among 20 cases, seven cases showed inconclusive results due to low allele frequency on 454 pyrosequencing (Nos. 32 and 82), different mutation types among test modalities (Nos. 26, 100, and 104), and inadequate specimen for 454 pyrosequencing (Nos. 34 and 116).

In one case (No. 54), the result of Sanger sequencing coincides with that of 454 pyrosequencing. In the remaining cases, 10 cases (Nos. 49, 80, 85, 86, 88, 103, 125, 149, 159, and 221) had no mutation with Sanger sequencing, whereas a single nucleotide

variant (SNV) was identified by the cobas test and 454 pyrosequencing. In those cases, mutations with high allele frequency by 454 sequencing were regarded as the final results. In two cases (Nos. 97 and 223), no mutation was identified by the cobas test while different mutation types were shown between Sanger sequencing and 454 sequencing. Considering repetitive failure for PCR amplification or inconsistent results on the cobas test, this discrepancy might be due to extensive DNA fragmentation and/or FFPE-related artifacts. Thus, mutations with a high allele frequency by 454 sequencing were considered as the final results in these cases (Table 4, Supplementary Tables S2, S3).

Overall concordance for detecting KRAS mutation of codons 12/13 (the cobas test and combined Sanger and 454 sequencing resolution)

After discordant resolution with 454 pyrosequencing, we ana-

Table 3. Summary of overall concordance for detecting KRAS mutation of codon 12/13

The cobas test	Sanger sequencing		
	Mutation detected	Mutation not detected	Total
Mutation detected	105 (40.9)	13 (5.1)	118 (45.9)
Mutation not detected	7 (2.7)	132 (51.3)	139 (54.1)
Total	112 (43.6)	145 (56.4)	257 ^a (100)

Values are presented as number (%). Positive percent agreement (sensitivity): 93.8% (95% confidence interval [CI], 87.6 to 97.5). Negative percent agreement (specificity): 91.0% (95% CI, 85.2 to 95.1). Positive predictive value: 89.0% (95% CI, 81.9 to 94.0). Negative predictive value: 95.0% (95% CI, 89.9 to 98.0). Positive likelihood ratio: 10.5 (95% CI, 6.2 to 17.6). Negative likelihood ratio: 0.1 (95% CI, 0.0 to 0.1).

^aOf 264 cases, two cases showed invalid result and five cases revealed KRAS mutations in codon 61 by the cobas test.

Table 4. Analysis for 20 discrepant cases with 454 pyrosequencing

Sample ID	Sanger sequencing	The cobas test	454 Pyrosequencing		Combined result
			Variant	Frequency	
026	G12D	Not detected	G13D	0.65	N/A
054	G12D	Not detected	G12D	0.69	G12D
082	G12D	Not detected	G12D/G12V	0.68/1.78	N/A
097	G12D	Not detected	G12V	10.92	G12V
100	G12D	Not detected	G12A/G12S/G12V	3.13/0.45/13.2	N/A
104	G13D	Not detected	G12D/G12S/G12V/G13D	1.06/0.79/3.62/0.36	N/A
223	G12D	Not detected	G12V	17.58	G12V
032	Negative	Codon 12/13	G12C/G13D	2.43/0.38	N/A
034	Netative	Codon 12/14	N/A	N/A	N/A
049	Negative	Codon 12/13	G12V/G13S	23.56/0.34	G12V
080	Negative	Codon 12/13	G12C/G12V/G13D/G13S	15.25/6.5/1.62/1.29	G12C
085	Negative	Codon 12/13	G12D/G12V	4.2/13.53	G12V
086	Negative	Codon 12/13	G12V/G13D	9.72/1.09	G12V
088	Negative	Codon 12/13	G12V	17.5	G12V
103	Negative	Codon 12/13	G12D/G12V/G13D	1.86/4.47/0.81	G12V
116	Negative	Codon 12/13	N/A	N/A	N/A
125	Negative	Codon 12/13	G12V/G13D	15.66/3.88	G12V
149	Negative	Codon 12/13	G12V	24.9	G12V
159	Negative	Codon 12/13	G12D/G12S/G12V	0.4/1.41/6.7	G12V
221	Negative	Codon 12/13	G12V/G13S	8.92/1.64	G12V

N/A, not available.

Table 5. Summary of overall concordance for detecting *KRAS* mutation (the cobas test and combined Sanger and 454 sequencing resolution)

The Cobas test	Sanger sequencing and 454 pyrosequencing		
	Mutation detected	Mutation not detected	Total
Mutation detected	115 (46.0)	0	115 (46.0)
Mutation not detected	3 (1.2)	132 (52.8)	135 (54.0)
Total	118 (47.2)	132 (52.8)	250 ^a (100)

Values are presented as number (%). Positive percent agreement (sensitivity): 97.5% (95% confidence interval [CI], 92.8 to 99.5). Negative percent agreement (specificity): 100% (95% CI, 97.2 to 100). Positive predictive value: 100% (95% CI, 96.9 to 100). Negative predictive value: 97.8% (95% CI, 93.7 to 99.5). Negative likelihood ratio: 0.03 (95% CI, 0.0 to 0.1).

^aOf 257 cases, seven cases showed invalid result by comprehensive evaluation with Sanger sequencing, the cobas test and 454 pyrosequencing.

lyzed 250 cases for concordance between the cobas test and combined Sanger sequencing–454 pyrosequencing. *KRAS* mutations for 250 evaluable cases were detected in 118 cases (46.0%) by combined Sanger sequencing–454 pyrosequencing and 115 cases (46.0%) by the cobas test. Concordance between the cobas test and combined Sanger sequencing–454 pyrosequencing was 97.5% PPA and 100% NPA. The discordant ratio of the Sanger sequencing and the cobas test was 1.2% (3 out of 250 cases) (Table 5).

DISCUSSION

There are various testing assays available for *KRAS* mutations, and several comparative studies on the analytical power of these methods in the clinical context have been conducted.^{3,8-10} In this study, we compared the performance of two platforms for detecting *KRAS*-activating mutations in Korean CRC cohorts. Determination of the *KRAS* mutation status is now obligatory for treatment with anti-EGFR monoclonal antibodies such as cetuximab and panitumumab in patients with metastatic CRC, as only patients with wild-type *KRAS* may respond to treatment.^{11,12} Furthermore, in patients with metastatic CRC and *KRAS*-activating mutations, treatment with anti-EGFR monoclonal antibodies may result in deleterious effects on progression-free survival, which highlights the necessity of maximizing the detection of *KRAS* mutations.^{13,14} However, validated and standardized procedures for *KRAS* somatic mutation testing were lacking until recently. Sanger sequencing remains the gold standard assay for detecting all possible mutations, but is generally considered to be less sensitive than other methods.¹⁵

The cobas (*KRAS* mutation) test is a CE-IVD validated method based on real-time PCR and TaqMelt technology.¹⁶ The accuracy of this method is similar to that of massively parallel pyrosequencing. In this study, the cobas test showed a $\geq 95\%$ correct mutation call rate for the recommended DNA input of ≥ 50 ng. It has been designed to detect 19 common *KRAS* mutations in codons 12, 13, and 61 in FFPE-derived DNA from

CRC samples.¹⁷ Moreover, the cobas test has been reported to detect mutations in tissue samples containing a minimal amount of 5% of tumor DNA and showed comparable and greater sensitivity for low-level mutant allele burden than Sanger sequencing.¹⁷ The results indicate the presence or absence of *KRAS* mutations without detailed information regarding the exact mutated site. While 454 pyrosequencing also shows a sensitivity of 5%, this method did not confirm the results obtained from other test assays. We used 454 pyrosequencing as a reference method to evaluate discrepant results between the cobas test and Sanger sequencing.

In this study, there were 20 discordant results (7.8%) between the cobas test and the Sanger sequencing, excluding two cases with invalid results and five cases with *KRAS* mutations in codon 61 by the cobas test. For further evaluation of samples with discrepant mutation results, each sample was run three times on Sanger sequencing and the cobas test. If invalid results are obtained, a new sample from a different tissue block was used if possible, which might result in different mutation types from intratumoral heterogeneity. In those cases, we made a great effort to choose the tissue section with the higher tumor portions ($> 70\%$), and no necrotic or mucinous areas.

In five cases (Nos. 26, 97, 100, 104, and 223), different mutation types were identified between Sanger sequencing and 454 sequencing. In addition, two cases (Nos. 32 and 82) showed low allele frequency on 454 pyrosequencing. Considering repetitive failure for PCR amplification in Sanger sequencing and/or invalid results in the cobas test, this discrepancy among test modalities might come from extensive DNA fragmentation from old formalin-fixed tissues. FFPE-related artifacts, i.e., spontaneous deamination of cytosine bases, might have a great effect on this inconsistency. Recently, uracil lesions in which hydrolytic deamination of cytosine bases to uracil takes place have been identified as major sources of sequence artifacts in FFPE DNA, leading to artifactual C:G>T:A variants.¹⁸ These sequence artifacts are known to be detected more frequently when low copy numbers of FFPE DNA are provided.¹⁹

In ten cases (Nos. 49, 80, 85, 86, 88, 103, 125, 149, 159, and 221), no mutation was detected by Sanger sequencing, whereas SNVs were identified by the cobas test and 454 sequencing. These discrepancies might result from low analytical sensitivity of Sanger sequencing. Although Sanger sequencing is considered to be the gold standard in mutation testing, it has a modest limit of detection that can be highly variable depending on the specific mutation sequence. A recent study has shown that Sanger sequencing yielded 11.1% false-positives and 6.1% false-negatives for *KRAS* mutation detection using an automated interpretation algorithm with a 10% threshold, highlighting the need for manual review of all Sanger sequencing data (Table 4, Supplementary Tables S2, S3).²⁰

In addition to the above mentioned factors, artifactual mutations from FFPE tissues can be caused by various sources, including over-fixation in 10% non-buffered formalin, oxidative DNA damage during sample preparation, DNA polymerase error, pseudogene amplification, sequencing chemistry, and errors of alignment and/or annotation.^{19,21-24} Several studies have shown that the numbers of SNV identified in formalin-fixed tissues were higher than those in matched frozen tissues, which suggests the possibilities of artifactual sequence changes, thus increasing the risk of false-positive mutation calls.^{25,26} Additionally, the discrepant results may have also come from uncommon *KRAS* mutations that cannot be detected using the cobas design. Understanding these issues is important for accurate interpretation of various mutations detected.

Although a “mutated in codon 12/13 or 61” result in the cobas *KRAS* mutation test is a sufficient basis for the application of anti-EGFR monoclonal antibodies in mCRC, several studies suggest that different mutation sites have different clinical impacts on cetuximab and/or panitumumab efficacy. The potential differential sensitivity of *KRAS* G13D mutation to anti-EGFR monoclonal antibodies has been reported in a small number of studies, suggesting that the addition of cetuximab may be beneficial to CRC patients with a *KRAS* G13D mutation.^{27,28} However, no significant difference between *KRAS* G13D and other *KRAS* mutated CRCs was detected in terms of treatment benefit from anti-EGFR monoclonal antibodies for mCRC.²⁹

Detection of *KRAS*-activating mutations in CRC has largely only focused on mutations in codons 12 and 13. However, recent data has shown that clinicopathological features and gene expression profiles of CRCs harboring non-traditional *KRAS* mutations appear to be similar to those of tumors with *KRAS* mutations in codons 12 and 13.³⁰ *KRAS* mutations in codon 61 and/or 146 are additional hotspots in CRC, and the available data from

a small number of studies suggest that resistance to anti-EGFR therapy is associated with *KRAS* mutation at these sites.^{31,32} Pre-screening using the cobas *KRAS* mutation test, which accurately detects all main *KRAS* mutations in codons 12, 13, and 61, with further evaluation by Sanger sequencing enables the identification of codon 12 and 13 mutations. This may be an easy and reliable approach for routine diagnostic purposes regarding *KRAS* mutations in mCRC. In addition, combining both mutation assays greatly reduces the probability of obtaining false-negative or false-positive results.

This study also emphasized that the preanalytical step must be strictly controlled because DNA degradation was the main cause of “not available” results, which leads to non-conclusive results, and samples cannot be evaluated using other genetic tests.

Several techniques for detecting *KRAS* mutations are currently available, but there is limited data supporting the analytical performance of individual methods compared with other methods.³³⁻³⁵ In addition, recent studies demonstrated the variability of mutation tests resulting from different clinical laboratories.³⁶ In contrast to other *KRAS* mutation detecting kits, such as the Therascreen *KRAS* mutation test, the cobas test can detect 12 additional mutation sites, representing approximately 1% of all *KRAS* mutation cases of CRC based on data from the Catalogue of Somatic Mutations in Cancer (COSMIC) database.³ However, several studies revealed that *KRAS* mutations in codon 61 are more prevalent than reflected by the COSMIC data in CRC.^{3,37,38} Thus, more comprehensive *KRAS* mutation coverage may be helpful for selecting patients for anti-EGFR target therapies.

A limitation of this study is that the study design was retrospective and the sample size was unsatisfactory to evaluate the significance of infrequent *KRAS* mutation subtypes. Subsequent translational prospective studies from different cohorts are needed to confirm our data. In addition, some invalid and discrepant results were not sufficiently resolved. Nevertheless, we demonstrated that the cobas *KRAS* mutation test is a reproducible companion diagnostic test in patients with CRCs under consideration for anti-EGFR target therapies when used with Sanger sequencing.

In conclusion, we confirmed that the cobas *KRAS* mutation test is an accurate and sensitive test for detecting *KRAS*-activating mutations and has equivalent analytical power to the Sanger direct sequencing method. Therefore, pre-screening using the cobas *KRAS* mutation assay with subsequent application of Sanger sequencing is the best strategy for routine diagnostic purposes regarding *KRAS* mutations in mCRC.

Electronic Supplementary Material

Supplementary materials are available at Journal of Pathology and Translational Medicine (<http://jpatholtm.org>).

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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