A comparison of three methods for detecting KRAS mutations in formalin-fixed colorectal cancer specimens

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BACKGROUND: KRAS mutation testing is required to select patients with metastatic colorectal cancer (CRC) to receive anti-epidermal growth factor receptor antibodies, but the optimal KRAS mutation test method is uncertain.

METHODS: We conducted a two-site comparison of two commercial KRAS mutation kits – the cobas KRAS Mutation Test and the Qiagen therascreen KRAS Kit – and Sanger sequencing. A panel of 120 CRC specimens was tested with all three methods. The agreement between the cobas test and each of the other methods was assessed. Specimens with discordant results were subjected to quantitative massively parallel pyrosequencing (MPP). DNA blends were tested to determine detection rates at 5% mutant alleles.

RESULTS: Reproducibility of the cobas test between sites was 98%. Six mutations were detected by cobas that were not detected by Sanger, and five were confirmed by MPP. The cobas test detected eight mutations which were not detected by the therascreen test, and seven were confirmed by MPP. Detection rates with 5% mutant DNA blends were 100% for the cobas and therascreen tests and 19% for Sanger.

CONCLUSION: The cobas test was reproducible between sites, and detected several mutations that were not detected by the therascreen test or Sanger. Sanger sequencing had poor sensitivity for low levels of mutation.

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Retrospective analyses of pivotal clinical trials for the anti-epidermal growth factor receptor (EGFR) monoclonal antibodies, cetuximab and panitumumab, have revealed that patients with colorectal cancer (CRC) tumours containing activating mutations in the downstream KRAS gene do not receive benefit from therapy (Amado et al, 2008; Karapetis et al, 2008). The findings that KRAS mutation status is a key determinant of response to anti-EGFR therapy are now reflected in the labels for both these agents, with the European Medicines Agency labelling stating that these targeted agents are only indicated in patients with wild-type KRAS tumours, while the US Food and Drug Administration states that such treatment is not recommended in tumours with mutations in codons 12 and 13 of KRAS.

In line with the drug labels, KRAS mutation testing is now recommended by major oncology organisations. Both the American Society for Clinical Oncology and National Comprehensive Cancer Network (NCCN) specify that before treatment, tumours should be tested for the presence of mutations in codons 12 and 13 of the KRAS gene (Allegra et al, 2009; Engstrom et al, 2009), while the European Society for Medical Oncology recommends establishing that tumours are KRAS wild type, without naming specific mutations (Van Cutsem et al, 2010). To date, neither the product labels, nor the oncology guidelines delineate specific codon 12 and 13 mutations.

The recommendation to test for mutations in codons 12 and 13 of the KRAS gene reflects the high frequency of these activating mutations in colorectal tumours: 24–43% of colorectal tumours contain KRAS mutations, of which ~82% are in codon 12 and ~17% are in codon 13 (Samowitz et al, 2000; Andreyev et al, 2001). Following the initial retrospective analysis showing that patients with activating KRAS mutations did not respond to treatment, the majority of subsequent clinical trials of anti-EGFR antibodies in CRC have excluded patients with tumours harbouring codon 12 and 13 mutations (Amado et al, 2008; Douillard et al, 2010; Peeters et al, 2010; Bokemeyer et al, 2011; Van Cutsem et al, 2011).

An emerging body of data suggests that KRAS mutations in codon 61 may also be predictors of nonresponsiveness to anti-EGFR therapies. Mutations in codon 61 resulted in constitutive activation and increased the transforming activity of cells (Der et al, 1986). Limited clinical data indicate that, similarly to codon 12 and 13 mutations, codon 61 mutations also predict resistance to anti-EGFR monoclonal antibodies (Loupakis et al, 2009; De Roock et al, 2010a; Molinari et al, 2011). Furthermore, the frequency of codon 61 mutations may be greater than initially reported (Richman et al, 2009; Vaughn et al, 2011). In a recent study of CRC samples, 39% were found to be KRAS mutated and 8% of mutations were located in codon 61 (Sundstrom et al, 2010).

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The association between defined mutations and response to therapy provides a clear opportunity to increase response rates and reduce the likelihood of treating patients who are unlikely to respond to certain drugs. However, there is a critical need to set standards for this type of mutation testing (van Krieken and Tol, 2009). A number of sequencing and PCR-based methods for detecting KRAS mutations are currently in clinical use. A recent methods comparison study suggested that a variety of techniques might be suitable for KRAS mutation testing. However, it is not clear which technique offers the best performance in terms of sensitivity, specificity, reproducibility, and success rates (Whitehall et al, 2009).

We performed a two-centre study to compare the analytic performance and workflow characteristics of the cobas KRAS Mutation Test to two other methods commonly used in the clinical setting: 2 × bidirectional Sanger sequencing (Sanger) and the therascreen KRAS Mutation Kit (’therascreen’, Qiagen, Hilden, Germany). We used a blinded panel of formalin-fixed paraffin-embedded tissue (FFPET) CRC specimens and DNA blends.

MATERIALS AND METHODS

Mutation testing methods

The cobas KRAS Mutation Test kit (Roche Molecular Systems, Inc., Branchburg, NJ, USA) is a CE-IVD marked TaqMelt PCR assay designed to detect the presence of 19 KRAS mutations in codons 12, 13, and 61 in FFPET CRC specimens (Supplementary Figure 1). The test requires 100 ng total DNA input which can typically be obtained by using one 5 μm section. One patient sample is tested in two wells per 96-well plate for a total throughput of 45 specimens per plate. The workflow and testing process have been described previously (Lee et al, 2011).

The therascreen KRAS PCR Kit (Qiagen, Manchester, UK) is a CE-IVD marked real-time PCR assay that combines an amplification refractory mutation system and a Scorpion fluorescent primer/probe system (ARMS) to detect seven mutations in codons 12 and 13 of the KRAS gene (Supplementary Figure 1). One patient sample is tested by one control assay and seven mutation assays for a total throughput of 10 samples per 96-well plate. All reaction assays include an exogenous internal control for the presence of inhibitors. Reactions were run in the ABI 7500 instrument (Life Technologies, Warrington, UK) and analysed using software v2.0.5 (Life Technologies). The therascreen kit requires ≥20 ng of amplifiable genomic DNA from FFPET specimens. According to the lab-validated clinical protocol 100–200 ng of total DNA, as measured by spectrophotometry, is used per PCR to account for the partial degradation of FFPET DNA without resulting in oversaturation of the reaction. The test requires a total DNA input of 800–1600 ng. The DNA volume used for each PCR is 5 μl and all samples are diluted to 20–25 ng μl⁻¹. Therascreen kit has been reported to detect mutations at 1% sensitivity in plasmids and cells lines provided the Ct value of the no template control is >29.

Sanger sequencing: Mutation screening for exons 2 and 3 of the KRAS gene was carried out using PCR conditions and 2 × bidirectional direct sequencing following previously described protocols (Fernandez et al, 2004; Conde et al, 2006). Exon 3 primers were designed specifically for this study. Tumour DNA for exon 3 was amplified using the following primers: forward primer 5’-CACCTGAATATCCGACCTGG-3’ and reverse: 5’-CCCCACCTATAATGGTAGAATATCATGCCATATT-3’. Sequencing reactions were performed in both direct and reverse directions, and electropherograms were reviewed manually to detect any genetic alteration. All variants were confirmed by resequencing-independent PCR products. Theoretically, 150 ng of DNA is used per PCR performed for a total of 300 ng total DNA input for KRAS exon 2 and exon 3.

454 sequencing (454 Life Sciences, Branford, CT, USA) is a quantitative massively parallel pyrosequencing (MPP) method, which involves clonal amplification by emulsion PCR of target sequences followed by MPP (Margulies et al, 2005). Each specimen was amplified using primers with 454 tags. The amplicon was quantified, diluted, and pooled with 19 other specimens. Each pool underwent emulsion PCR and four pools were sequenced on a single picotitre plate.

Materials: FFPET specimens from CRC tumours were purchased from US commercial vendors: Discovery Life Sciences, Inc. (Los Osos, CA, USA); BioServe (Beltsville, MD, USA); ProteoGenex (Culver City, CA, USA); CureLine, Inc. (San Francisco, CA, USA); and Indivium, Inc. (Kensington, MD, USA).

Study design

The study was conducted using a blinded panel of FFPET tumour specimens of CRC as well as artificial DNA blends containing low percent mutant alleles as determined by 454 sequencing. The design of the study is depicted in Figure 1. From a panel of 621 vendor-purchased FFPET colorectal tumour specimens, 100 specimens were selected at random; an additional 20 specimens were chosen for challenging specimen attributes such as (a) low tumour content; (b) high levels of necrosis; and (c) presence of less frequent KRAS mutations.

The 120 tumour specimens were each sectioned into five 5 μm curls per panel member and blinded. One section was mounted on a slide and stained with haematoxylin and eosin, coded and reviewed by two pathologists (FL-R and AS-G) to assess the tumour content, lymphocyte infiltration, extent of necrosis and mucin content. Following this assessment, a panel of evaluable specimens was identified. Two 5 μm curls per panel member were sent to clinical site 1 (Hospital Universitario Madrid Sanchinarro, Madrid, Spain) for analysis on the cobas KRAS test and Sanger sequencing, and two 5 μm curls per panel member were sent to clinical site 2 (The Royal Marsden NHS Hospital, London, UK) for analysis on cobas KRAS test and therascreen KRAS PCR Kit test.

DNA for the cobas test was isolated from a single 5 μm section per tumour panel at each site using the cobas DNA Sample Preparation Kit (Roche Molecular Systems). The DNA eluate was subsequently tested using the cobas KRAS test according to the vendor-provided protocol.

DNA for Sanger and the therascreen KRAS PCR Kit was isolated from separate single 5 μm sections per tumour panel member using the QIAamp DNA DNA FFPE tissue kit and automated QIAcube robot (Qiagen, Hilden, Germany). The DNA eluate was then tested with Sanger according to a standard laboratory protocol or the therascreen KRAS PCR Kit test according to the vendor-provided protocol.

Specimens with invalid test results or with discordant results between any of the methods were retested according to manufacturer/procedure instructions. Criteria for retesting were as follows:

- cobas KRAS test: <10% tumour content; insufficient DNA concentration;
- Sanger: no PCR amplification; or difficult sequence interpretation;
- therascreen: positive controls have not amplified specific product, mixed standard delta Ct is not within specified range, no template control has Ct <38, any sample with control gene Ct >34.

Quantitative MPP (454 GS Titanium, 454 Life Sciences, Inc.) was performed on all discordant and invalid specimens.

Of the 120 FFPET specimens, 3 were excluded from analysis because pathologic review revealed ≤1% tumour content. Two additional specimens failed to generate a valid result using any of...
the three testing methods at both sites or 454 and were also excluded from the data analysis. This resulted in a panel of 115 FFPET specimens for the methods comparison analysis. The clinical and pathological features of these 115 specimens are described in Table 1.

**Study objectives**

Specific objectives of the study were to (1) compare three methods of KRAS mutation detection, by calculating positive percent agreement (PPA) and negative percent agreement (NPA) of the cobas KRAS test with the therascreen KRAS and Sanger sequencing using a panel of human FFPET CRC specimens; (2) assess the reproducibility of the cobas KRAS test at two independent laboratories; (3) assess the frequency of invalid test results for the cobas KRAS test, Sanger sequencing, and therascreen KRAS test methods; (4) assess the effects of tumour content, lymphocyte infiltration, necrosis, and mucin content on the analytical performance of the cobas KRAS test; (5) evaluate the correct call rate for each method at low percentage mutant alleles using plasmid and cell line blends of mutant and wild-type DNA from plasmids; and (6) compare turnaround times between all methods.

**Invalid test rate and workflow measures**

The number of invalid test results from the evaluable tumour panel was recorded and compared across the three testing methods. Assay turnaround time from DNA isolation to results reporting was compared for all methods, assuming one 8-h shift per day.

**Methods correlation**

The PPA and NPA of the cobas KRAS test was compared with the two other testing methods (Sanger and therascreen) in sections of evaluable FFPET colorectal tumours. Discrepent analysis by 454 was performed on all specimens for which the cobas KRAS test and the comparison method gave discordant results and/or for which one of the two testing methods gave an invalid result.

**Reproducibility**

The reproducibility of the cobas KRAS test was evaluated by comparing the results from testing the evaluable FFPET tumour
panel at the two independent clinical laboratory sites. Discrepant analysis using 454 was performed on all specimens for which the cobas KRAS test gave discordant results and/or when an invalid result was obtained.

Impact of specimen attributes on analytic performance

The following pathological characteristics - tumour content, lymphocyte infiltration, necrosis, and mucin content - were assessed and graded according to the following criteria:

- Tumour content was graded as high ($\geq 10\%$) or low ($< 10\%$);
- Lymphocyte infiltration was graded as high ($\geq 10\%$) or low ($< 10\%$);
- Tumour necrosis was graded as high ($\geq 50\%$) or low ($< 50\%$);
- Mucin content was graded as high ($\geq 50\%$) or low ($< 50\%$).

Correct call rate at low percentage mutant alleles

Two DNA blends with 5% mutant alleles (as determined by 454) were prepared. One blend was prepared using a composite KRAS mutant plasmid containing both a codon 12 (G12D) and codon 61 (Q61H) mutation and WT genomic DNA. The other blend was prepared from genomic DNA derived from the CCL221 cell line containing a codon 13 (G13D) mutation and WT genomic DNA. In all, 48 replicates (21 replicates of each of the two 5% mutant allele blends and 6 wild-type specimens) were tested by all three methods to assess correct call rate at low percentage mutant alleles.

RESULTS

Invalid test rate

Of the 230 specimens that were evaluated by the cobas KRAS test (115 at each site), 2.2% (5 out of 230) gave an initially invalid test result, and 4 (1.7%) were invalid upon retesting. Of the 115 specimens evaluated by Sanger sequencing, 8 specimens (7.0%) were initially invalid; upon retest, 0.9% (1 out of 115) remained invalid. Of the 115 specimens evaluated by therascreen test, 1.7% (2 out of 115) gave initially invalid test results and remained invalid upon retesting.

Methods correlation with Sanger sequencing

Of the 115 specimens tested at Site 1 using the cobas KRAS test and Sanger sequencing, two specimens were invalid by either cobas or Sanger, leaving 113 evaluable specimens for comparison. The initial agreement analysis showed a PPA of 98.2%, an NPA of 90.2%, and an OPA of 96.3% (Table 3A), leaving 106 evaluable specimens for comparison. Of these six specimens, two were G13R, one was G13V, and three were codon 61 mutations. The initial agreement analysis showed a PPA of 100%, an NPA of 96.3%, and an OPA of 98.1% (Table 3B).

Methods correlation with the therascreen KRAS PCR Kit

Of the 115 specimens tested at Site 2, using the cobas KRAS test and the therascreen KRAS PCR Kit, three specimens were invalid by either cobas test or therascreen, six specimens were mutations that the therascreen kit was not designed to detect, including codon 61 mutations, and were therefore removed from the analysis (Table 3A), leaving 106 evaluable specimens for comparison. Of these six specimens, two were G13R, one was G13V, and three were codon 61 mutations. The initial agreement analysis showed a PPA of 100%, an NPA of 96.3%, and an OPA of 98.1% (Table 3B).

The two specimens with discordant test results – reporting ‘mutation detected’ with the cobas KRAS test and ‘mutation not detected’ with the therascreen KRAS PCR Kit – were subsequently subjected to 454 sequencing. One of these was subsequently reported as ‘mutation not detected’. Following discrepant resolution with 454 sequencing, where the 454 result was used as the arbiter, the PPA was 100%, the NPA was 98.1%, and the OPA was 99.1% (Table 3B).

Reproducibility

In total, 112 specimens were evaluable at each site (224 tests in total) using the cobas KRAS test. Of these, 110 (98.2%) produced concordant results.

Impact of specimen attributes on analytic performance

Pathological assessment of the 115 FFPE specimens revealed specimens with varying degrees of tumour content, lymphocyte infiltration, necrosis, and mucin content (Table 1). None of these pathologic characteristics had a significant effect on agreement analysis, reproducibility, and invalid rate of the cobas KRAS test (data not shown). The mutation results for the 20 specimens
Table 3  Methods correlation based on reportable results (A) between (B) cobas KRAS test and therascreen KRAS test at Site 2 and (C) after 454 sequencing of discrepant specimens

(A) Mutation | Base change | Cosmic ID
---|---|---
Gly12Ala | GGT > GCT | 522
Gly12Asp | GGT > GAT | 521
Gly12Arg | GGT > GCT | 518
Gly12Cys | GGT > TGT | 516
Gly12Ser | GGT > AGT | 517
Gly12Val | GGT > GCT | 520
Gly13Asp | GGC > GAC | 532

(B) therascreen KRAS

| Method | MD | MND | Totals |
|---|---|---|---|
cobas KRAS | 52 | 2 | 54 |
MD | 0 | 52 | 52 |
MND | 52 | 54 | 106 |
Total | 53 | 3 | 54 |
Positive agreement: 100% (95% CI: 93.1–100)
Negative agreement: 96.3% (95% CI: 87.5–99.0)
Overall agreement: 98.1% (95% CI: 93.4–99.5)

(C) therascreen KRAS post 454 testing

| Method | MD | MND | Totals |
|---|---|---|---|
cobas KRAS | 53 | 1 | 54 |
MD | 0 | 52 | 52 |
MND | 53 | 53 | 106 |
Total | 53 | 3 | 54 |
Positive agreement: 100% (95% CI: 93.2–100)
Negative agreement: 98.1% (95% CI: 90.1–99.7)
Overall agreement: 99.1% (95% CI: 94.8–99.8)

Abbreviations: CI = confidence interval; MD = mutation detected in codons 1/2/3; MND: wild-type or non-codons 1/2/3.

DISCUSSION

The recognition that activating mutations in the KRAS gene render cancer patients non-responsive to therapy with anti-EGFR monoclonal antibodies has resulted in the need for tumour mutation status to be ascertained before treatment. Treating patients who are unlikely to respond to a therapy is both costly and exposes them unnecessarily to potential adverse effects. In the case of the anti-EGFR monoclonal antibodies, panitumumab and cetuximab, treating patients with activating KRAS mutations may result in detrimental effects on progression-free survival (Bokemeyer et al., 2009; Douillard et al., 2010), further emphasising the need to maximise the detection of KRAS activating mutations. The detection of KRAS activating mutations in CRC has largely been focused on mutations in codons 12 and 13; however, mutations in codon 61 are known to result in activation of KRAS, which is also activating (Der et al., 1986; Loupakis et al., 2009; De Roock et al., 2010a; Molinari et al., 2011), although additional clinical data are needed to confirm these observations.

At present, there are numerous ways of testing for KRAS mutations, and there have been comparative studies and analyses of the sensitivity of these assays in the clinical setting (Whitehall et al., 2009; Angulo et al., 2010; Franklin et al., 2010; Tol et al., 2010). There remains an unmet need to set standards in the field of mutation testing. In this current study, the analytical performance of the cobas KRAS Mutation Test was compared with Sanger sequencing and the ARMS-Scorpion therascreen test using a blinded panel of FFPE tumour specimens of CRC as well as artificial DNA blends. The study was conducted at two independent clinical laboratories, one comparing the cobas KRAS test with Sanger sequencing and the second site comparing the cobas KRAS test with the therascreen KRAS test.

The cobas KRAS test was highly reproducible (>98%) between sites. Reproducibility of mutation test results between different clinical laboratories sites is a critical clinical diagnostic issue. The current use of a variety of testing methodologies to detect specific mutations, both commercially available and in-house designed, increases the potential variability of results in different laboratories with different levels of expertise, especially when labour-intensive methods that require subjective analysis and interpretation are used. A recent analysis of KRAS testing at 59 laboratories from 8 European countries found that only 70% of laboratories correctly identified the mutational status in all samples (Bellon et al., 2011); similar findings were observed in a study of KRAS and EGFR mutation testing in non-small cell lung cancer (Beau-Faller et al., 2011).

Both the cobas KRAS test and therascreen tests had short turnaround times (1 day) and were more sensitive for detecting KRAS mutations at 5% mutant alleles compared with Sanger sequencing.

The cobas KRAS test requires 100 ng of total DNA input and detects 19 mutations in codon 12/13 and codon 61. The therascreen test required >160 ng of amplifiable DNA (equivalent to 800 ng of total DNA input in our experience) and detects seven mutations in codon 12/13. With the Sanger sequencing method, two PCRs were performed (one reaction for exon 2 and one reaction for exon 3) using, when possible, ~150 ng DNA per PCR to theoretically detect any mutation in these two exons.

Analysis of discordant results suggests that the cobas KRAS test is more sensitive than both Sanger and therascreen for detecting KRAS mutations. In some instances, the increased rate of detection by the cobas KRAS test reflected the ability of the cobas KRAS test to detect more mutations (19) in codons 12, 13, and 61, than the therascreen assay, which is designed to detect seven mutations. In addition, macrodissection of the tissue sample before DNA extraction can increase the sensitivity of Sanger sequencing, although this also introduces a higher chance of human error.
Current guidelines for KRAS mutation testing in CRC do not delineate specific mutations within codons 12 and 13. However, one group has suggested, based on retrospective studies of small cohorts, that patients with tumours harbouring G13D mutations may benefit from therapy with anti-EGFR antibody therapy, suggesting that all codon 12 and 13 mutations may not be equal in terms of their clinical impact (De Roock et al., 2010b). These findings have been challenged by a recent retrospective analysis of patients treated with panitumumab in three large randomised controlled trials (Peeters et al., 2011). In this study, there was no evidence that patients with G13D mutations benefitted from anti-EGFR antibody treatment. Thus, the treatment guidelines for selecting patients for anti-EGFR antibodies are unchanged and recommend that patients with any mutations of codons 12 and 13 should not be treated with these therapies.

In summary, we have presented a methods comparison study of three assays for the detection of KRAS mutation in FFPET specimens of CRC. The three methods had comparable invalid rates. The cobas and therascreen assays had greater sensitivity for low levels of mutation than Sanger sequencing. The cobas test detected a number of KRAS mutations that the therascreen test was not designed to detect. The cobas KRAS test was highly reproducible, had the most rapid turnaround time, and was the only test that offered automated results reporting.

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Conflict of interest

FS, MV, VHB, and HJL are employees of Roche Molecular Systems, Inc. The remaining authors declare no conflict of interest.

Supplementary Information accompanies the paper on British Journal of Cancer website (http://www.nature.com/bjc)

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