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Comparison of KRAS mutation analysis of colorectal cancer samples by standard testing and next-generation sequencing

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Abstract

Aims—Based on KRAS testing, the subset of patients with metastatic colorectal cancer (CRC) that could benefit from anti-EGFR therapy can be better delineated. Though KRAS testing has become significantly more prevalent over the last few years, methods for testing remain heterogeneous and discordance has been reported between methods.

Methods—In this study, we examined a CRC patient population and compared KRAS testing done in Clinical Laboratory Improvement Amendments (CLIA) approved laboratories as part of standard clinical care and by next-generation sequencing (NGS) using the Illumina platform. Discordances were further evaluated with manual review of the NGS testing.

Results—Out of 468 CRC patient samples, 77 had KRAS testing done by both CLIA assay and NGS. There were concordant results between testing methodologies in 74 out of 77 patients, or 96% (95% CI 89% to 99%). There were three patient samples that showed discordant results between the two methods of testing. Upon further investigation of the NGS results for the three discordant cases, one sample showed a low level of the mutation seen in the standard testing, one sample showed low tumour fraction and a third did not show any evidence of the mutation that was found with the standard assay. Five patients had KRAS mutations not typically tested with standard testing.

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Conclusions—Overall there was a high concordance rate between NGS and standard testing for KRAS. However, NGS revealed mutations that are not tested for with standard KRAS assays that might have clinical impact with regards to the role for anti-EGFR therapy.

INTRODUCTION

Anti-EGFR monoclonal antibodies (mAbs) are among the first examples of successful targeted therapies in colorectal cancer (CRC). While initial data showed only modest activity of EGFR inhibitors in CRC, further analysis demonstrated that only those patients with KRAS wild-type tumours were likely to have significant benefit.[12] KRAS mutation (downstream of the EGFR protein) results in constitutive activation of the RAS-RAF-ERK pathway and is hypothesised to cause resistance to anti-EGFR therapy.[3] By current estimates, 35–45% of CRCs contain a KRAS mutation.[4]

In multiple clinical studies, KRAS mutation has been validated as a negative predictive biomarker.[5–7] ASCO provisional guidelines recommend that all patients with metastatic CRC have tumour tissue tested for KRAS mutation in a Clinical Laboratory Improvement Amendments (CLIA) approved laboratory. Patients are eligible for anti-EGFR therapy only in the absence of KRAS codon 12 or 13 mutations.[8] Based on these findings, in 2009 the Food and Drug Administration limited the indication of cetuximab (Erbitux) and panitumumab (Vectibix) to only KRAS wild-type tumours, although the type of testing to be used was not specified.

Standardised, high-accuracy sequencing techniques are vital to making appropriate clinical therapeutic decisions. A standardised assay for KRAS testing has not been established, and multiple methods of testing for KRAS mutation are used in clinical practice. In all of these methods, DNA extraction from a paraffin embedded tissue block or H&E stained section followed by PCR amplification of target sequences is performed first. KRAS mutation analysis can then be done by direct (Sanger) sequencing, high-resolution melting analysis (HRMA), pyrosequencing, cobas, TheraScreen or other techniques that have been extensively reviewed elsewhere.[9] However, the lack of quality assurance of these testing methodologies can potentially lead to both false-positive and false-negative results. Quality control studies comparing different KRAS testing methods have shown discordance depending on the method and tissue type used (FFPE vs frozen).[1011]

Given that the majority of KRAS mutations have been found on codons 12 and 13,[12] most commercially available assays use sequencing specifically targeting these areas, with some assays also testing for the less frequently mutated codon 61. However, recent work has shown that a significant number of KRAS mutations localised to other codons, including 61, 117 and 146.[1314] These extended KRAS mutations as well as mutations in NRAS have been shown to yield similarly poor clinical outcomes when patients are treated with anti-EGFR therapy.[15–18]

In addition, it has been suggested that next-generation sequencing (NGS) has a higher level of accuracy than standard KRAS testing.[1119] NGS, or high-throughput sequencing, uses

technology that produces large numbers of sequences in parallel, allowing for more data to be produced at a lower cost per sequence.[20]

KRAS mutation is a negative predictive marker for response to anti-EGFR therapy, but KRAS wild-type status does not guarantee response.[21] It is therefore important to better delineate the subgroup of patients who will respond to this potentially toxic and costly treatment. NGS can provide information about many mutations with one test and potentially provide higher accuracy but is expensive. To further investigate the utility of this technology in clinical practice for both accuracy and expanded RAS analysis, we correlated standard KRAS mutation assays with targeted exome gene sequencing using the Illumina NGS technology.

MATERIALS AND METHODS

Institutional Review Board approval was obtained to conduct this study. We analysed tumour samples from fresh frozen surgical biopsy specimens collected from 468 patients with CRC at Moffitt Cancer Center and affiliate community hospitals in the southeastern USA between 1998 and 2010. These patients were recruited as part of Moffitt Cancer Center's Total Cancer Care protocol. This is a multi-institutional observational study of patients with cancer in which self-reported demographic and clinical data as well as medical record information is collected prospectively. Tumour tissue is collected for research purposes. There are no exclusion criteria and every patient with CRC is eligible.

Samples were selected for sequencing based on availability of sufficient tissue for DNA extraction. Targeted exome sequencing was performed using an Illumina NGS platform resulting in a 50–100× average depth of coverage of KRAS.

All tumours were collected using snap frozen technique in liquid nitrogen within 15–20 min of extirpation. The tumours then underwent a macrodissection quality control process to ensure >80% tumour was present in the specimen that underwent sequence analysis. Normal tissue, necrotic tissue and excessive stromal tissues were dissected away from the specimen under frozen section control. DNA was then extracted for targeted gene sequencing, which was performed by Beijing Genomic Institute (Beijing, China). A total of 1321 genes, including KRAS and NRAS, were targeted using Agilent SureSelect technology (Agilent Technologies, Inc. Santa Clara, California, USA), followed by 90 base pair, paired-end sequencing on GAIIx instruments (Illumina, Inc. San Diego, California, USA).

BWA[22] was used to align sequence reads to the human reference (hs37d5). GATK[23] was used for insertion/deletion realignment, quality score recalibration and identification of single nucleotide and insertion/deletion variants. Matched normal samples were not available for comparison to identify somatic mutations, so filtering of normal variants was performed using the 1000 Genomes Project dataset.[24] Variants identified in 1000 Genomes with an minor allele frequencies (MAF) ≥0.01 were removed. ANNOVAR[25] was used to annotate variants, and both VarSifter[26] and an in-house web-based display application were used for analysis. Manual review on discrepant determinations was performed using samtools.[27]

Patient characteristics including sex, age, stage at diagnosis and primary tumour site were obtained through retrospective chart review.

KRAS mutation testing was performed on a subset of patients as part of their standard care. These assays included Sanger sequencing and the allele-specific primer extension techniques as described in table 1. KRAS mutation testing information was obtained through retrospective chart review.

RESULTS

Of 468 patients with CRC, 77 had tumours analysed by both NGS and KRAS testing in CLIA-approved laboratories. Baseline characteristics are reported in table 2. Tumours from 69 patients demonstrated equivalent results between standard testing and NGS. Among these patients, 35 tumours had KRAS wild-type tumours and 34 were KRAS mutant at codons 12 or 13. Of the KRAS mutant tumours, 29 had codon 12 mutations and 5 had codon 13 mutations.

In total, 8 of the 77 evaluable patients had a discrepant status between the two methods of testing (see table 3). Three patient tumours had mutant KRAS by standard testing but were wild type by NGS. Of these patient samples, one had G12D mutation by standard testing but was wild type by NGS. Upon review of the NGS results, it was suspected that tumour fraction was low in this sample based on overall minor allele frequencies at several positions. A second patient had a KRAS mutant (G12A) tumour by standard testing but was wild type by NGS. Further investigation of the NGS results showed that 1/147 sequence reads had the G12A mutation. A third patient had a G12A mutation by standard testing but no evidence of that mutation in NGS; however, the NGS data did show a Q61K mutation.

The five remaining patients had a KRAS mutation outside of the commonly tested codons 12 and 13, which was revealed by NGS. Of these patients, three had Q61 mutations and two had A146 mutations.

Our overall concordance rate was 74/77 or 96.1%. The CI for the concordance rate is 89.2% to 98.7%.

NRAS and HRAS mutations were also analysed as part of NGS. Of our cohort of 468 patients with CRC, two KRAS wild-type patients were found to have NRAS mutations, at Q61H and Q61K. No HRAS mutations were found.

DISCUSSION

KRAS mutation status is widely acknowledged to provide important information with regards to guiding anti-EGFR therapy for patients with metastatic CRC. However, testing methodologies have not been standardised. In this study, we compared standard KRAS mutation testing performed in community practice to NGS.

We found that a subset of patients in our cohort had disparate results in KRAS status between standard testing and NGS. As expected, some patients were found to have KRAS

Q61 and A146 mutations, which are not routinely assessed by standard KRAS testing. We also found additional two patients with NRAS mutations that have been shown to be unlikely to respond to anti-EFGR inhibitors.[15] Recent work has shown that a significant number of relevant mutations in KRAS 61, 117 and 146, HRAS 61 and NRAS codons 12, 13, 61, 117 and 146[1528] are missed with the testing currently required (KRAS codons 12 and 13) by ASCO guidelines.

This is a critical issue, as patients with tumours harbouring these additional mutations are unlikely to respond to treatment with anti-EGFR agents. Recent data from a large phase III trial by Douillard and colleagues showed that panitumumab (a fully humanised monoclonal antibody to EGFR) was beneficial in patients with wild-type RAS as expected, but was detrimental in patients with non-KRAS 12 and 13 and NRAS mutations.[15] Similarly, in data presented by Heinemann at the European Society for Medical Oncology meeting in 2013, patients with mCRC who were wild type for all RAS mutations (not only KRAS codons 12 and 13, but also KRAS codons 61, 117 and 146 and NRAS codons 12, 13, 61, 117 and 146) were shown to have improved overall survival with 5-fluorouracil, leucovorin and irinotecan chemotherapy (FOLFIRI) plus cetuximab compared with FOLFIRI plus bevacizumab in the first-line setting.[2829] However, as with the Douillard study, patients who were wild type for KRAS codons 12 and 13 but had other RAS mutations were found to have decreased benefit.

In our study, three samples had discordant results between testing methodologies. In the case of patient 1, manual review of NGS showed that there was low tumour fraction in the sample, indicating that the biopsy used for NGS might have had primarily non-cancer tissue. For patient 2, standard testing showed a G12A mutation that was not reported on NGS. Upon manual review of the NGS data, this mutation was seen in 1/ 147 sequence reads but did not meet standard NGS programme filters to be considered mutant. This discrepancy can be accounted for by tumour heterogeneity or possibly by increased sensitivity of standard testing compared with NGS. The sample for patient 3 showed a codon 12 mutation by standard testing that was not found on NGS, though there was a codon 61 mutation. Manual review of NGS showed an appropriate amount of tumour tissue and no evidence of a codon 12 mutation.

As mentioned, one major potential explanation for assay discordance is intratumoral heterogeneity. In addition, the specimens obtained for standard testing and NGS were occasionally obtained at different times and from different sites. Though KRAS is an early mutation in carcinogenesis, tumours may comprise heterogeneous cells and tumour heterogeneity maybe greater than 10% within the same sample.[30] Biopsies can also contain varying degrees of tumour cellularity, which may result in different KRAS mutation signal strengths.

Another potential source of discordance is differences in the assay methodologies. KRAS testing by standard assays can be subjective as it relies on a pathologist to make a determination regarding the changes in signal peak heights reported with Sanger sequencing. Another limitation of current testing is that the raw data are more difficult to review in cases of discrepancy.

NGS may also lead to incorrect results and has not yet been established as a gold standard. There is an intrinsic error rate with different types of NGS, and adjusting analysis sensitivity too high will allow for the inclusion of false-positive mutations. Additionally, due to tumour heterogeneity, mutations can be present at varying frequencies within the tumour. Adjusting sensitivity to identify low-frequency mutations can return results that are not clinically meaningful (ie, those with low frequency in the tumour), while setting it too high will miss mutations that might have significance even though they are in a minor fraction of tumour cells. A potential benefit of the NGS approach is that it can be reviewed in cases of clinical discrepancy, such as a patient who is not responding to anti-EGFR therapy. Mutation frequencies could eventually be included in the pathology report to allow clinicians to take advantage of this information. Reporting a low mutation frequency, as in the case of patient 1, could allow the treating practitioner to consider anti-EGFR therapy as an option if the patient had progressed through other therapies, but perhaps not as a first-line therapy due to a potentially resistant population of tumour cells. More research is needed to determine at which mutation frequency cut-offs a tumour should be considered ‘mutated’ for therapeutic purposes.

Our study has several limitations. We did not have access to clinical data regarding the usage of anti-EGFR therapy. We therefore could not evaluate whether those patients who had non-KRAS codon 12 and 13 mutations had a change in outcome compared with the rest of the cohort. In addition, we did not have matched normal samples to identify somatic mutations. However, while the lack of matched samples does make precise identification of somatic mutations challenging, common driver mutations are not found in normal tissue of otherwise healthy individuals. The KRAS mutations described in this study would not be present as germ line variants in otherwise healthy individuals. Also, sample enrichment was not performed in our series, which may have prevented some of the discrepancy. Finally, the KRAS testing method varied from patient to patient; however, this is reflective of current community practice.

Widespread use of NGS itself has some limitations. In addition to the direct costs of sequencing, which include upfront machine costs and the associated maintenance and technical expertise to run the samples, there is also a cost for appropriate analysis and storage of the data obtained,[631] which might mean the necessity for a dedicated bioinformatics department. These difficulties could be partially overcome by using targeted gene panels, which allow for lower cost and decreased data burden, while targeting the currently known clinically relevant genes.

CONCLUSIONS

To our knowledge, this is the largest series comparing standard KRAS testing to NGS. Concordance with standard methods to NGS was high for codons 12 and 13, which confirms that these methods are generally reliable. However, NGS had several advantages over standard testing methods as it yielded data on multiple genes of interest from a single test without the added time and expense of running subsequent analyses when new information is published or if a patient is not responding to treatment with anti-EGFR drugs. This is quite timely as recent studies have demonstrated that extended RAS testing is needed to avoid

giving anti-EGFR drugs that can be ineffective and possibly detrimental to patients with RAS mutations. New data regarding tumour evolution during treatment might demonstrate an increased role for NGS.

Although there are additional upfront costs, with proper tumour cell enrichment NGS can yield additional information. This can help clinicians more effectively use a toxic and expensive medication. In the future, NGS could be used to better refine personalised treatment regimens for patients with meta-static CRC.

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Take home messages

- Standard KRAS testings are sensitive and reliable.
- Main drawback with standard KRAS testing may miss mutations that might help more effectively tailor treatment with anti-EGFR agents for patients with advanced colorectal cancers.
- Next-generation sequencing is increasingly feasible in clinical practice but must be validated prior to widespread adoption.

Table 1

Types of KRAS testing by CLIA

Testing type	Description	No. of patients
RedPath	PCR followed by dideoxy chain termination sequencing	24
Genzyme	Allele-specific primer extension technique for any mutation of codons 12 and 13	6
Caris	DNA was amplified with primers flanking KRAS exon 2 (codons 12 and 13) and KRAS exon 3 (codon 61)	2
LabCorp	7 KRAS mutations on 12 and 13 using ARMS (Amplification refractory mutation system) and real-time PCR using Scorpions™ technology	4
BayCare Molecular Pathology Lab	Real-time PCR of codons 12 and 13	2
Mayo Clinic 7 Mutation Panel	Sanger sequencing, allele-specific PCR, tests for seven common mutations in codons 12 and 13	1
Clinical Laboratory Partners	PCR for 12 and 13	1
Phenopath	PCR for 12 and 13	2
Clarient	Real-time PCR for 12 and 13	1
Testing type unavailable		34

Table 2

Baseline characteristics

Number of patients	77
Median age (years)	62
Gender	
Male	43
Female	34
Primary site	
Right sided	23
Left sided	50
Transverse	3
Unknown	1
AJCC stage at diagnosis	
1	3 (4%)
2	9 (13%)
3	26 (34%)
4	39 (49%)

AJCC, American Joint Committee on Cancer.

Table 3

Discrepancies between standard testing and next-generation sequencing (NGS)

Sample	Standard testing	NGS	Explanation
1	G12A mutant	Wild type (WT)	1/147 reads show mutation
2	G12D mutant	WT	Low tumour fraction
3	G12A mutant	Q61K mutant	
4	WT	Q61K mutant	
5	WT	Q61K mutant	
6	WT	Q61H mutant	
7	WT	A146T mutant	
8	WT	A146V mutant	