

KRAS Mutations and Sensitivity to Epidermal Growth Factor Receptor Inhibitors in Colorectal Cancer: Practical Application of Patient Selection

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ABSTRACT

Recent retrospective evidence from several randomized studies has established that advanced colorectal cancer patients with tumors harboring a mutation in the *KRAS* gene do not derive benefit from the administration of epidermal growth factor receptor–directed monoclonal antibodies, such as cetuximab or panitumumab. This represents a paradigm-changing event and will have substantial impact on current and future anticancer drug development. These results add to the economic and ethical considerations involved in the development of novel targeted therapies and should increase our scrutiny of mechanisms of resistance and predictive biomarkers while in earlier developmental stages. In this article we will review the available clinical data, discuss the potential implications for future drug development in colorectal cancer, and provide a comprehensive overview of the technical aspects of *KRAS* mutation testing. In particular we aimed at enumerating the available procedures for mutation detection and their main characteristics, as well as comparing them from a clinical feasibility standpoint. While the true specificity and sensitivity of these methods have yet to be fully characterized, a better understanding of the differences between tests will be critical so that clinicians and pathologists can fully integrate this testing into the routine care of patients with colorectal cancer.

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INTRODUCTION

Biologic Introduction and Summary of Clinical Data

Among the most daunting challenges facing oncology today is that of patient selection, particularly for molecularly targeted agents. Colorectal cancer (CRC) has been an example of rational but ultimately inaccurate strategies to achieve that goal. The antiepidermal growth factor receptor (EGFR) monoclonal antibody cetuximab was initially granted approval only for EGFR-expressing patients,¹ but it was later evidenced that patients without detectable EGFR did obtain benefit to a similar extent than positive patients.² It was accepted that factors other than EGFR expression would better dictate efficacy (or lack of thereof) for this and similar targeted agents. After a series of nonrandomized studies reporting little or no benefit from anti-EGFR therapies as single agents or combined with chemotherapy in *KRAS* mutant subjects,³⁻⁸ evidence arising from randomized studies has become available (Tables 1 and 2). The compelling nature of this retrospective data obviates a need for prospective data collection before action is taken. CRC pa-

tients harboring a *KRAS* mutation do not derive benefit from the administration of EGFR-targeting monoclonal antibodies in the first-line,^{9,10} second-line,¹¹ or third-line settings.¹²

Biology of KRAS

The RAS proteins belong to the guanosine-5'-triphosphatase (GTase) superfamily, of which *KRAS*, *NRAS*, and *HRAS* are the most widely known members.¹³ Their role is to transduce stimuli from surface growth factor receptors, but as multiple simultaneous sources of signals (from different receptors, ligands) exist, they are increasingly considered as integrators and not just transducers. In addition, the existence of negative and positive feedback loops adds to this complexity. On activation, RAS undergoes prenylation (addition of a 15-carbon chain) of a CAAX (C, cysteine; A, aliphatic amino acid; X, serine or methionine) motif by a farnesyl transferase. This makes RAS more hydrophobic (sticky) and adherent to the inner aspect of the cytoplasmic membrane, where it activates subsequent transducers such as PI3K and MAPK. Mutations in *KRAS* permit stimuli-independent activation and perpetuate this activation because they occur in the area that regulates its level of enzymatic activity. There are a

Table 1. Results From the EGFR Inhibitor-Containing, Single-Arm Studies Analyzing the Correlation of Efficacy and *KRAS* Status in CRC

Study	Treatment	Total Patients	Response Rate (%)	
			<i>KRAS</i> MT	<i>KRAS</i> WT
Benvenuti ³	P or C or C ± CT	48	6	31
De Roock ⁴	C ± CT	113	0	40
Finocchiaro ⁵	C ± CT	81	6	26
Di Fiore ⁶	C + CT	59	0	28
Khambata ⁷	C	80	0	10
Lievre ⁸	C ± CT	89	0	40

Abbreviations: EGFR, epidermal growth factor receptor; CRC, colorectal cancer; C, cetuximab; CT, chemotherapy; MT, mutant; P, panitumumab; WT, wild-type.

limited number of mutations in the *KRAS* gene, and altogether more than 90% involve three codons (12, 13, and 61). For instance, mutations in the codon normally encoding for a glycine in position 12 will induce the insertion of some other amino acid with a side chain (glycine is the only amino acid without a chain), that will sterically interfere with the geometry state that allows the GTP to be hydrolyzed, in order for RAS to return to an inactive state.¹³ Thus, acquiring certain mutations in *KRAS* leads to a permanently active state that permits the cell to evade apoptosis and acquire a growth advantage. The fact that the number of mutations is discrete, as well as the inherent stability and detectability of mutations make *KRAS* mutations an ideal pharmacodiagnostic marker.

KRAS As a Prognostic Factor in CRC

A definitive impact of *KRAS* mutations on outcome that is independent of treatment is controversial in CRC, as reports are conflicting. A large series of 3,439 CRC patients found that of the 12 possible mutations on codons 12 and 13, only the glycine to valine mutation on codon 12 (8.6%) had a statistically significant impact on outcome.¹⁴ Smaller series have shown somewhat similar results,¹⁵ but retrospective data from other large randomized studies has failed to consistently demonstrate a meaningful effect of *KRAS* mutation on outcome in CRC, such as the study that evaluated adding bevacizumab to first-line irinotecan, fluorouracil (FU), and leucovorin (IFL).¹⁶ However, in this study all arms included treatment, thus confounding a pure prognostic effect. In the large randomized phase III study of panitumumab versus best supportive care no difference was observed in PFS in the untreated patients, which was 7.3 weeks in both wild-type (WT) and mutant groups.¹² The National Cancer Institute of Canada reported

only in poster format a study that randomized refractory patients to cetuximab or best supportive care (BSC)¹⁷; in the BSC arm patients with *KRAS* WT and mutant tumors survived 4.8 and 4.6 months ($P = .97$), respectively.

KRAS As a Predictive Factor in CRC

Several studies have recently provided evidence indicating *KRAS* mutation status dictates response to anti-EGFR monoclonal antibodies in CRC. The CRYSTAL trial was a randomized phase III trial assessing cetuximab with the irinotecan-containing infusional FU regimen, bolus and infusional FU + leucovorin + irinotecan as first-line treatment in patients with EGFR-expressing metastatic CRC. A statistically significant improvement in the overall response rate and median progression-free survival (PFS) in the patients enrolled on the cetuximab-containing arm was found. However, in the subset of patients that could be analyzed for *KRAS* mutational status, the benefit of cetuximab appeared to be restricted to patients without mutations in the *KRAS* gene.⁹ This was a retrospective study conducted on a subset of the intent-to-treat population, but the *KRAS* mutant and *KRAS* WT patients were similar in terms of demographics and disease characteristics. Similarly, in the OPUS phase II study that evaluated FOLFOX ± cetuximab in first-line, *KRAS* WT patients obtained benefit from receiving cetuximab in addition to FOLFOX compared to those receiving FOLFOX alone both in terms of response rate (61% v 37%; $P = .01$) and PFS (7.7 v 7.2 months; $P = .02$).¹⁰

The second-line EVEREST study tested whether a rash-based cetuximab dose escalation would increase the response rate combined with irinotecan.¹¹ In addition to assessing rash as a presumed surrogate for pharmacokinetics or pharmacodynamics, the predictive value of *KRAS* was tested. In patients with *KRAS* WT tumors there were no differences and response rates ranged from 30% to 42% in low- and high-dose groups, respectively; a 0% response rate was documented in patients with *KRAS* mutant tumors. In third-line, panitumumab was compared to BSC in CRC, demonstrating a survival benefit.¹² In the subset analysis, the treatment effect on PFS in the group with WT *KRAS* tumors was significantly greater than in the group with tumor *KRAS* mutations (HR, 0.45 v 0.99; $P < .0001$). Median PFS in the group with WT *KRAS* tumors was 12.3 weeks for panitumumab and 7.3 weeks for BSC, and responses were 17% and 0%, respectively. On the basis of these results the European Medicines Agency (EMA) has approved panitumumab only for patients with tumors that are WT *KRAS*. This was the first approval of an agent for CRC based on the presence/absence of a gene mutation, and opens a new era in biomarker-driven therapy in this disease.

Table 2. Results From the EGFR Inhibitor-Containing Arms From Randomized Studies Analyzing the Correlation of Efficacy and *KRAS* Status in CRC

Study	Treatment	Total Patients	<i>KRAS</i> MT		<i>KRAS</i> WT	
			Duration	HR	Duration	HR
Amado ¹²	P v BSC (third line)	427	7.4 weeks	0.99	12.3 weeks	0.45
Van Cutsem ⁹	FOLFIRI ± C (first line)	540	7.6 months	1.07	9.9 months	0.68
Bokemeyer ¹⁰	FOLFOX ± C (first line)	233	5.5 months	1.83	7.7 months	0.57

Abbreviation: EGFR, epidermal growth factor receptor; CRC, colorectal cancer; BSC, best supportive care; C, cetuximab; FOLFIRI, bolus and infusional fluorouracil + leucovorin + irinotecan; FOLFOX, bolus and infusional fluorouracil + leucovorin + oxaliplatin; HR, hazard ratio; MT, mutant; P, panitumumab; WT, wild-type.

IMPACT AND IMPLICATIONS

These data have general implications. First, it generates a corollary hypothesis: are *KRAS* mutations responsible for the poorer outcomes observed in subsets of patients treated with conventional therapy plus EGFR inhibitors compared to conventional therapy? Medically, it also opens a new avenue as novel treatment alternatives are clearly needed for *KRAS* mutant CRC patients. Pragmatically, implementing this selection parameter in the general CRC population poses important logistic challenges, as *KRAS* mutation detection (as opposed to prior experiences with HER2 and EGFR immunohistochemical testing) has not been consistent between studies and there is currently no US Food and Drug Administration-approved standardized test.

***KRAS* As a Potential Deleterious Factor for Anti-EGFR Therapies**

Can the presence of *KRAS* mutations be a potential deleterious factor for anti-EGFR therapies? Although in the CRYSTAL study a clear-cut deleterious effect of EGFR inhibitors in *KRAS* mutant subjects was not documented, in the smaller OPUS trial *KRAS* mutant patients receiving FOLFOX plus cetuximab fared worse than those receiving FOLFOX alone in terms of PFS (5.2 v 8.6 months; $P = .02$) and marginally worse in response rate (33% v 49%; $P = .1$). These results are in line with earlier data from non-small-cell lung cancer (NSCLC) studies where subset analyses indicated that patients with *KRAS* mutations treated with chemotherapy and an EGFR inhibitor (erlotinib) had a significantly worse outcome than those treated with chemotherapy alone in an advanced setting.¹⁸ In a subsequent study that assessed maintenance therapy with gefitinib versus placebo in patients with lung cancer, the gefitinib-treated group had a worse outcome that could not be explained by toxicity, but that was attributed to increased tumor progression.¹⁹ The results of the biologic evaluation of those patients are eagerly awaited, but it clearly raises the question of whether unselected pathway inhibition not only leads to lack of benefit, but also to inferior outcome. Currently, there are no clear explanations at hand for these observations. These hints of contradictory effects, however, are consistent with the observed complexity of signaling pathways in cancer, and suggest that pathway linearity is only a conceptual model. As demonstrated by the phospho-Akt activation after nonselective mammalian target of rapamycin inhibition, initially puzzling and counterintuitive findings may result in the identification of compensatory feedback loops.^{20,21} The ultimate outcome of a pharmacologic intervention will rely on the specific cellular context and dynamic equilibrium, which in turn is likely dictated by a complex genetically altered background.²²

How to Increase Response Rate in *KRAS* WT Patients?

Having an intact *KRAS* is necessary but not sufficient to derive benefit from EGFR inhibition in CRC. This is similar to the situation in NSCLC where *KRAS* mutations also indicate a lack of benefit from EGFR inhibitors (although the evidence is less compelling), and are mutually exclusive with the positive predictor, *EGFR* mutations.¹⁸ For the *KRAS* WT population, positive predictive markers that are currently being evaluated include an increase in *EGFR* gene copy number (GCN); patients whose tumors had elevated *EGFR* GCN obtained more benefit from cetuximab.²³ A recent report has confirmed that

EGFR GCN testing provided significant information independent of the *KRAS* status to predict response and overall survival²⁴; however reproducibility concerns regarding the cutoff points for GCN are still problematic. The identification of further negative predictive factors would also increase the efficacy of the treated population. A genetic-based grid approach should be studied and tested, where multiple subgroups are generated to further refine the responsive population of patients, mimicking what is standard of care in the management of hematologic malignancies management such as chronic myeloid leukemia (CML).

Are There Novel Therapeutic Alternatives to *KRAS* Mutant Patients?

RAS has proven to be one of the most challenging targets in anticancer drug development. A variety of approaches primarily inhibiting farnesyl transferase activity have failed in multiple RAS-mutated cancers including CRC.²⁵ Also the inhibition of targets downstream of RAS has failed in tumors such as pancreatic cancer where RAS mutations are virtually universal.²⁶ So what makes RAS such an elusive target? The fact that altered proteins have a physical and geometrical impediment to return to their inactivated state may account why it is so difficult to counterbalance that activation. Simultaneously targeting multiple downstream pathways, accelerating the turnover of mutant proteins via heat shock protein 90 inhibitors, tackling alternative activation steps other than farnesylation, or taking advantage of the differential immunogenicity of mutated RAS are being explored as alternatives. In addition, MEK inhibitors have the potential to exert this role as they inhibit downstream effectors of *KRAS*.²⁷

KRAS TESTING: ASSAY CONSIDERATIONS

A tremendous logistical challenge lies in the actual testing of tumors in metastatic CRC patients, a test that will conceivably be conducted on all CRC patients in the near future. At a time when regulatory bodies and cooperative groups are adapting to this new scenario, the question of which test to use for validation but also for clinical decisions in the general population is of foremost relevance. While the fact that mutations are binary events (absent or present) with less room for interpretation than protein or mRNA expression-based tests, assay-specific considerations are important to define the optimal balance between accuracy and practicality.

Specimen Selection: Challenges of Using the Most Clinically Available Source

Typically, the specimens available for mutational analysis are formalin-fixed paraffin-embedded (FFPE) tissue blocks. Until recently, DNA extracted from FFPE has been viewed as difficult and time consuming to work with and of low quality and yield.²⁸ Initial studies directly comparing mutation detection rates in frozen and paraffin embedded samples of the same tissue have found a mutation rate in FFPE samples approximately half that detected in frozen samples.²⁹ However, refinements of techniques have compensated for the limitations of FFPE tissue and have enhanced the sensitivity of DNA testing in formalin-fixed material. Fixation damages DNA as formaldehyde breaks hydrogen bonds and unstacks double-stranded DNA, facilitating covalent reactions between formaldehyde and DNA

bases.³⁰ The result is sequence alterations that may introduce artificial mutations into conventional PCR procedures. Fortunately, this is an infrequent event if sufficient cellular material is available for sequencing and in one study was not observed when the equivalent of 300 or more cells were available for analysis.³¹ It is therefore of some importance to use sufficient DNA to avoid this type of artifact and a minimum of 30 ng of template DNA is suggested for *KRAS* testing, an amount that is easily obtained from most FFPE tissue blocks.

Tissue Enrichment

A second source of potential error in *KRAS* testing is the dilution of tumor DNA with that of DNA from reactive cells around tumor (such as fibroblasts, leukocytes, or endothelial cells) that do not harbor mutations but may compete with mutant DNA in amplification reactions. Some form of tumor cell enrichment increases the sensitivity of mutation testing and this may take the form of micro- or macrodissection or selective sampling of the paraffin block by needle core as is widely practiced in the construction of tissue microarrays. Care should be taken in the application of these procedures so that sufficient DNA for amplification (> 30 ng) is available, thus avoiding the artificial mutations mentioned above.

Assay Selection: Balancing Sensitivity and Accuracy With Clinical Applicability

Finally, the testing procedure itself may be variably sensitive and specific. Several mutation detection procedures have been described, a representative list of reported methods is provided in Table 3. All methods are based on the polymerase chain reaction (PCR). The most widely accessible method for *KRAS* testing is direct sequencing of *KRAS* PCR products, which has long been the gold standard for mutation detection.³² This method detects all mutations in amplified DNA sequences, but requires that mutant copies have a concentration that is at least 20% to 50% of any accompanying WT sequences. Obtaining sufficient high quality DNA for this procedure in FFPE blocks can be difficult and it is direct sequencing that was the method

employed in studies that have reported diminished sensitivity of mutational testing in FFPE tissue.^{28,29}

The low sensitivity and expense of direct sequencing has stimulated the development of feasible technologies that are more suitable for application to clinical samples. Methods for detection of point mutation were reviewed over a decade ago³² but since that time new more sensitive and specific assays been applied to assess *KRAS* in clinical samples. These methods employ restriction fragmentation length polymorphism (RFLP)³³⁻³⁵; allele specific oligonucleotide (ASO) hybridization; high resolution melting analysis (HRMA); and amplification refractory mutation system (ARMS). The classic method, gene sequencing, can be summarized as obtaining nucleotide by nucleotide the sequence of the gene (or of the areas of the gene more frequently affected by mutations), and comparing to the normal sequence of the gene. RFLP methods are based on differences between mutant and WT *KRAS* DNA in susceptibility to digestion by restriction enzymes, that only insert a cut in the DNA if a defined sequence exists. Most of these methods rely on the introduction of a restriction site into normal, but not mutant, DNA by mismatching single bases in PCR primers. This is based on selecting a restriction enzyme that would only induce a break in the DNA if not mutated; if there is a mutation then the nucleotide changes and the restriction enzyme will not recognize that section of the gene, and the mutated sample will then be cut in bigger pieces. For example, a *BstNI* restriction site can be introduced into codon 11 in normal but not mutant DNA.³⁵ Digestion of PCR products with *BstNI* reduces the concentration of WT DNA, resulting in preferential amplification of mutant copies that can be detected by gel electrophoresis³⁴ and more recently by Bioanalyzer instrumentation (Agilent Technologies, Santa Clara, CA). While this method is reported to be highly sensitive, it is complex and requires tight control of PCR and restriction digestion conditions to avoid replication errors and artificial mutations. An important disadvantage of the RFLP technique is the fact that in case of a mutation the specific nucleotide cannot be detected. The method typically requires confirmation by direct sequencing and does not appear to be practical for

Table 3. *KRAS* Mutation Analysis Methods

Method	Principle	Sensitivity (MT/WT; %)	Turnaround	Disadvantages
Direct sequencing	Non-mutation-specific determination of test case nucleotide sequence and comparison with normal sequence	20-50	Slow turnaround (4 days to 2 weeks from paraffin)	Poorly quantitative, insensitive, prolonged turnaround
Restriction fragmentation length polymorphism, confirmed by direct sequencing	Mutation presence induces or eliminates specific sites where DNA-targeting enzymes insert cuts in DNA	0.10	Slow turnaround (4 days to 2 weeks from paraffin)	Complicated; requires sequencing confirmation; considerable manual input required; non-quantitative; questionable specificity
Allele specific probe	Polymerase chain reaction/selective detection	10	Rapid (< 2 days from paraffin)	Relatively low sensitivity
High resolution melting analysis, confirmed by direct sequencing	Sequences with mutations hybridize at different, fixed temperatures	5	Slow turnaround (4 days to 2 weeks from paraffin)	Complicated; requires sequencing confirmation; considerable manual input required
Amplification refractory mutation system	Mutation specific polymerase chain reaction/detection	1	Rapid (< 2 days from paraffin)	Detects only single specific mutation per reaction; requires specially engineered primer/probe

Abbreviations: MT, mutant; WT, wild-type.

high-volume clinical application. Concerns about the specificity of this approach hamper a routine clinical application.

ASO hybridization methods are based on the principle that a single base mismatch caused by a mutation results in a reduction of the melting point temperature of the double stranded hybrid due to a lower binding energy.³⁶ The difference in the melting point between matched and single-base mismatched sequences can be used to detect single-base mismatches between WT and mutant sequences.³⁷ ASO may be deployed in a dot blot format³⁸⁻⁴⁰ but also is the principle around which oligonucleotide microarray DNA chips and high resolution melting point analyses have been developed. However, the problem of finding rare copies of rare mutant alleles in a DNA mixture is a daunting one particularly in preparations that contain a high level of normal alleles, as is characteristic of clinical samples. In addition, this testing platform requires specialized equipment and specialized software for analysis, making the overall technique expensive. Nonetheless, a DNA microarray mutational platform for diagnostics has been validated for clinical oncology samples.⁴¹

HRMA measures differences in melting point temperatures between matched and mismatched double stranded DNA, either caused by polymorphisms or by somatic mutations.⁴² This is achieved by comparing the melting point temperatures to a known reference scale, and is based on the principle that the affinity of two DNA chains is disrupted by the presence of a mutation, the chains will bind with less energy and will be more easily separated by heat. HRMA is performed following PCR and is inexpensive and fast. The sensitivity of the assay is high with the ability to detect a DNA mismatch at a mutant allelic concentration of 5%.⁴³ Preliminary studies of *KRAS* mutation in colon carcinoma⁴³ and NSCLC^{44,45} suggest that the specificity of the test will be high, particularly in light of the fact that melting point patterns may be associated with particular allelic mismatches. However, this assay does not specifically identify individual mutations. Because any DNA alteration may produce an abnormal melting point curve, abnormal curves need to be confirmed by sequencing. This increases turnaround time and expense of the method and reduces its advantage over direct sequencing alone. HRMA may thus be a rapid screening method but the need for confirmation by sequencing may limit the utility of this methodology in the clinical setting.

ARMS is based on the observation that oligonucleotides with a single base mismatch at the 3' base will not function as primers.⁴⁶ With proper primer design at the 3' base, mutant alleles can be preferentially amplified in specimens where mutant copies are at a low concentration. Its mode of action is based on the fact that the reaction only finalizes (ie, copies the whole sequence and emits a signal that is automatically detected) if the abnormal (mutated) sequence exists. ARMS was used to document *KRAS* mutations in colon carcinoma a decade ago.⁴⁷ More recently it has been used in a quantitative PCR platform⁴⁰ and linked with a bifunctional fluorescent primer/probe molecule⁴⁸ (scorpion⁴⁹). The combined assay uses seven primer/probes for seven different mutations in *KRAS* in a single kit that directly detects the presence of *KRAS* mutations in heterogenous specimens at a low allelic concentration (1%) without the need for confirmation by direct sequencing. This assay has been successfully deployed in a phase III trial of metastatic colon carcinoma in which patients were treated with the anti-EGFR antibody, panitumumab.¹² The liability of this assay is

that only known mutations are detected. It nevertheless seems likely that this assay with its simplicity and rapidity is likely to be valuable in clinical practice.

New technologies can be expected to address *KRAS* mutation in clinical samples. For example, pyrosequencing⁵⁰ is a robust technology that is now used in high throughput sequencing platforms has demonstrated feasibility for the detection of *KRAS* mutations in colon carcinoma.⁵¹ Another approach that has shown promise is the detection of *KRAS* mutations in peripheral blood.⁵²

CONCLUSION

Administering EGFR-targeting monoclonal antibodies, such as cetuximab or panitumumab, to unselected metastatic CRC patients can no longer be considered the standard of care, as those agents will be ineffective in patients with activating mutations in *KRAS*. These results add to the economic and ethical considerations involved in the development of novel targeted therapies and should increase our scrutiny of mechanisms of resistance and predictive biomarkers while in earlier developmental stages. The true specificity and sensitivity of the methods at hand is yet to be determined and at the moment costs and instrumentation challenges are formidable. It is likely though that the costs will decline and high throughput technology that provides a globally comprehensive assessment of the cancer genome will allow individualized therapy that coincides with the molecular pathology of tumor as well as the histology and cell type.

In this interval period where CRC patients are negatively selected for EGFR-based therapy without biologic alternatives it is important to convey to patients with *KRAS* mutations that the current chemotherapy regimens for this disease are active and such decisions are intended to improve the risk to benefit ratio for them. Likewise, clinical trial participation should be placed at the forefront of our treatment recommendations for these patients. Adequate banking of tissue samples is critical, not only to offer our current patients the best standard of care treatment, but also to enhance our readiness for the increasing array of genetic and proteomic biologic markers that will be tested and incorporated in our daily routines in the near future.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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