

K-Ras Mutations in Colorectal Cancer: A Practice Changing Discovery

M. Wasif Saif, MD and Manasi Shah, MD

Dr. Saif is Associate Professor and Co-Director, Gastrointestinal Cancers Program at Yale Cancer Center and Dr. Shah is a resident in Internal Medicine at Yale Bridgeport Hospital in New Haven, Connecticut.

Address correspondence to:
Muhammad Wasif Saif
Section of Medical Oncology
Yale University School of Medicine
333 Cedar Street; FMP: 116
New Haven, CT 06520
Phone: 203-737-1568
Fax: 203-785-3788
E-mail: wasif.saif@yale.edu

Abstract: Recent insights into the molecular pathogenesis of colorectal cancer have given rise to specific target-directed therapies, including monoclonal antibodies against epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF). These drugs have been approved as second and third line therapies for metastatic colorectal cancer (mCRC). Activating mutations of the K-Ras family of genes are the most common genetic events in tumorigenesis and have been implicated as a predictive factor in determining response to anti-EGFR drugs in pivotal studies. Phase II and III trials, conducted for investigating the role of K-Ras status on anti-EGFR treatment, revealed that patients with wild-type K-Ras had better clinical response in terms of prolonged median progression-free survival and overall response rates when compared to mutant K-Ras. In contrast, patients with mCRC benefit from anti-VEGF treatment irrespective of K-Ras status. Interestingly, a combination of anti-EGFR and anti-VEGF demonstrates no added value in these patients. The studies concluded that pretreatment testing of K-Ras in patients with mCRC offers valuable information in deciding treatment options. There are several molecular methods for mutation detection that seem practical enough to apply in clinical practice. Further confirmatory prospective studies are needed to evaluate the role of K-Ras mutation detections in tumor metastases, early stage CRC, and method of sampling specimens.

Introduction

The advent of target-specific cancer therapeutics has remarkably improved the outcomes of patients with colorectal cancer. Monoclonal antibodies against 2 specific target proteins, epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF) have recently been approved for the treatment of metastatic colorectal cancer (mCRC). However, the molecular mechanisms underlying the clinical response to these drugs are not fully understood. Recent studies have shed some light on the effect of intra-cellular signaling pathways involving K-Ras on the safety and efficacy of the above drugs. This article aims to review the association of K-Ras with the safety and efficacy of drugs targeted against these receptors. We will also discuss the clinical implications of ascertaining K-Ras status in patients with mCRC.

Keywords

5-Fluorouracil, chemotherapy; colorectal carcinoma, cetuximab, panitumumab, K-Ras, genotype, oxaliplatin, irinotecan, bevacizumab

Frequency of K-Ras Mutation

K-Ras is considered one of the causal cancer genes in the Cancer Gene Census indicating that mutations in the K-Ras gene are almost certainly involved in the development of cancer. The COSMIC (Catalogue of Somatic Mutations in Cancer) database reports K-Ras mutations in a total of 8,402 samples and no mutations in 29,328 samples (ie, approximately 28.7% incidence in all human cancers).¹ Point mutations have been identified most commonly in codons 12 and 13 of the K-Ras gene and less commonly in codon 61.² These mutations occur early in the course of oncogenesis and are preserved throughout the course of tumor progression. K-Ras mutations are found in up to 65–100% of pancreatic carcinomas,³ 36% of colorectal cancers, and 20% of non-small cell lung cancers.⁵

Epidermal Growth Factor Receptor and K-Ras Mutation

The EGFR is a transmembrane tyrosine kinase that signals through at least 2 parallel intracellular pathways to regulate cellular proliferation and survival. Mitogen-activated protein kinases (MAPK) form one of the major cell-proliferation signaling pathways from the cell surface to the nucleus via a series of intermediate genes including RAS, RAF, and MEK (Figure 1). Stimulation of EGFR by various signals (EGF, amphiregulin, epiregulin, heparin-binding EGF, etc.) results in dimerization and phosphorylation of the receptor. This in turn leads to activation of RAS via adaptor molecules.^{4,5} RAS activates the cascade through phosphor-inositol kinases (PI3K) as well as RAF, and thus acts as a central distributor of the signal. Activation of PI3K via AKT inhibits apoptosis, whereas RAF activation stimulates cellular proliferation. This cascade is involved in the control of growth signals, cell survival, and invasion in cancer. Hence, mutations in the K-Ras gene lead to an independent activation of the downstream signal transduction system.⁶

Vascular Endothelial Growth Factor and K-Ras Mutation

VEGF is a potent mitogen for micro- and macrovascular endothelial cells derived from arteries, veins, and lymphatics, and it has been shown to promote angiogenesis in in vitro models.⁷ A variety of input signals upregulate VEGF expression. These include epidermal growth factor, TGF- β , mutation of p53 gene, as well as Ras mutations or amplification. Interestingly, hypoxia is noted to induce VEGF expression in solid tumors, a process that appears to involve the activation of raf kinases. Pharmacologic disruption of mutant Ras protein function in H-Ras trans-

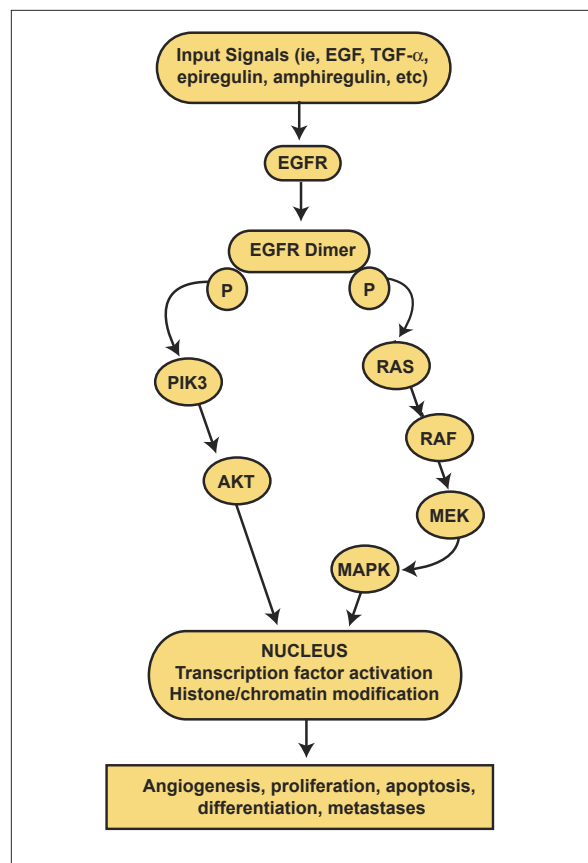


Figure 1. Downstream signaling pathway of EGFR via RAS/RAF/MAPK.

EGFR=epidermal growth factor receptor; MAPK=Mitogen-activated protein kinases.

Adapted from Raponi et al. *Curr Opin Pharmacol.* 2008;8:413-418.

formed rat intestinal epithelial cells by treatment with a protein farnesyltransferase inhibitor has been shown to cause significant suppression of VEGF. This may suggest that Ras oncogenes, in addition to having a direct effect on tumor proliferation, may also affect tumor progression indirectly by facilitation of angiogenesis.⁸

Importance Of K-Ras In Determining Response To Anti-Cancer Drugs In Colorectal Cancer

EGFR Inhibitors

Two EGFR targeted monoclonal antibodies, cetuximab (Erbix, Bristol-Myers Squibb) and panitumumab (Vectibix, Amgen), have been approved by the US Food and Drug Administration as second- and third-line agents for metastatic colorectal cancer.

Table 1. CRYSTAL Trial: K-Ras and FOLFIRI With/Without Cetuximab

| Efficacy measure | Wild-type K-Ras (n=346) | | Mutant K-Ras (n=192) | |
|---------------------|-------------------------|---------------------|----------------------|---------------------|
| | FOLFIRI (n=176) | CET/FOLFIRI (n=172) | FOLFIRI (n=87) | CET/FOLFIRI (n=105) |
| Median PFS (months) | 8.7 | 9.9 | 8.1 | 7.6 |
| | <i>P</i> =.017 | | <i>P</i> =.47 | |
| ORR(%) | 43.2 | 59.3 | 40.2 | 36.2 |
| | <i>P</i> =.0025 | | <i>P</i> =.46 | |

CET=cetuximab; FOLFIRI=leucovorin/fluorouracil/irinotecan; ORR=overall response rate; PFS=Progression-free survival

Cetuximab and K-Ras The phase III CRYSTAL trial evaluated the efficacy of cetuximab plus irinotecan based on K-Ras status.⁹ A total of 1,217 patients previously untreated for mCRC (expressing EGFR) were randomized to FOLFIRI (irinotecan 180 mg/m², 5-fluorouracil [FU] 400 mg/m² bolus, followed by 2,400 mg/m² as 46-hour continuous infusion plus leucovorin) and cetuximab plus FOLFIRI (cetuximab 400 mg/m² intravenously on day 1, then 250 mg/m²/week + FOLFIRI every 2 weeks). In the cetuximab plus FOLFIRI arm, the median progression-free survival (PFS) was 8.9 months versus 8 months in the FOLFIRI arm (*P*=.0479). There was a significant difference in the overall response rate (ORR) between the cetuximab-containing arm and the FOLFIRI only arm (46.9% vs 38.7%; *P*=.0038).

A retrospective analysis of the study reported by van Cutsem and colleagues⁹ investigated the impact of K-Ras mutation in the efficacy of the intention-to-treat arm. A total of 540 patient samples were available for K-Ras mutation detection, of which 35.6% of patients had mutant K-Ras (Table 1).

In patients with a wild-type K-Ras, the addition of cetuximab to FOLFIRI significantly improved the median PFS (*P*=.017) as well as the ORR (*P*=.0025; Table 1). However, patients with mutant K-Ras did not derive any clinical benefit with the addition of cetuximab as determined by the median PFS (*P*=.47) or the ORR (*P*=.46).

The safety profiles were similar for patients in both arms independent of K-Ras status. Grade 3/4 acne-like rash (16% and 17% in wild-type and mutant K-Ras, respectively) and infusion reactions (1.7% and 3.8%, respectively) were more common in the cetuximab treatment arm. Patients with mutant K-Ras, in the cetuximab arm, had a higher incidence of febrile neutropenia (3.8%) and fatigue (9.5%) compared to patients with wild-type K-Ras.

Another study looking into the implication of K-Ras mutation on mCRC outcomes was conducted by

Bokemeyer and coauthors.¹⁰ OPUS, a phase II randomized study performed in Europe, randomized 337 previously untreated patients expressing EGFR with FOLFOX-4 (oxaliplatin 85 mg/m², 5-FU 400 mg/m² bolus, followed by 2,400 mg/m² as 46-hour continuous infusion, FA 200 mg/m² on day 1 every 2 weeks) and cetuximab (400 mg/m² initial dose then 250 mg/m²/week) plus FOLFOX-4.¹¹ The median PFS was 7.2 months in both arms with a trend towards increased ORR in the cetuximab arm compared to the FOLFOX only arm (45.6% vs 35.7%; *P*=.063).

A retrospective analysis of the study investigated 233 patients evaluable for K-Ras status; 134 patients (58%) had wild-type K-Ras and 99 patients (42%) had mutant K-Ras (Table 2). In patients with wild-type K-Ras, the addition of cetuximab to FOLFOX resulted in a longer median PFS (*P*=.016) and a significant improvement in ORR (*P*=.011). Patients with mutant K-Ras had a decreased median PFS compared to patients with wild-type K-Ras (5.5 vs 8.6 months; *P*=.0192) and a trend towards decrease in ORR (32.7% vs 48.9%; *P*=.106).

The safety profile differed in terms of K-Ras status in the 2 treatment arms. In the cetuximab arm, patients with a mutant K-Ras had a higher incidence of infusion reactions compared to wild-type K-Ras (7.7% vs 1.4%) but a lower incidence of any grade 3/4 events (67.3% vs 83.6%).

A phase II trial (EVEREST) was conducted by Tejpar and colleagues to determine the link between cetuximab and K-Ras response.¹² Patients with grade 0/1 skin reactions after 22 days of treatment with irinotecan and standard-dose cetuximab were randomized to receive standard dose (250 mg/m²) or escalated (up to 500 mg/m²) doses of cetuximab. Of the 86 samples available for K-Ras analysis, 62% were wild-type and 37.2% were mutant K-Ras. Patients with wild-type K-Ras enjoyed considerable benefit from irinotecan plus dose-escalated cetuximab treatment (Table 3). They had a higher ORR when compared to patients with mutant K-Ras. Patients with K-Ras

Table 2. OPUS Trial: K-Ras and FOLFOX With/Without Cetuximab

| Efficacy measure | Wild-type K-Ras (n=134) | | Mutant K-Ras (n=99) | |
|---------------------|-------------------------|-------------------|---------------------|-------------------|
| | FOLFOX (n=73) | CET/FOLFOX (n=61) | FOLFOX (n=47) | CET/FOLFOX (n=52) |
| Median PFS (months) | 7.2 | 7.7 | 8.6 | 5.5 |
| | P=.016 | | P=.0192 | |
| ORR (%) | 37 | 60.7 | 48.9 | 32.7 |
| | P=.011 | | P=0.106 | |

CET=cetuximab; FOLFOX=leucovorin/fluorouracil/oxiplatin; ORR=overall response rate; PFS=progression-free survival.

Table 3. EVEREST Trial: K-Ras and Irinotecan Plus Cetuximab Versus Dose-escalated Cetuximab

| Efficacy measure | Wild-type K-Ras (n=54) | | Mutant K-Ras (n=32) | |
|------------------|------------------------|---------------------------|---------------------|---------------------------|
| | Standard CET (n=23) | Dose escalated CET (n=31) | Standard CET (n=20) | Dose escalated CET (n=12) |
| ORR(%) | 30.4% | 41.9% | 0 | 0 |
| Median PFS | 5.8 months | | 2.8 months | |

CET=cetuximab; ORR=overall response rate; PFS=progression-free survival

Table 4. K-Ras Status and Best Support Care With/Without Panitumumab

| Efficacy measure | Wild-type K-Ras (n=243) | | Mutant K-Ras (n=184) | |
|--------------------|-------------------------|-------------------|----------------------|------------------|
| | BSC (n=119) | BSC + PAN (n=124) | BSC (n=100) | BSC + PAN (n=84) |
| Median PFS (weeks) | 7.3 | 12.3 | 7.3 | 7.4 |
| | HR .00 (P<.0001) | | HR 0.99 | |
| Median OS (months) | 7.6 | 8.1 | 4.4 | 4.9 |
| | NR | | NR | |

BSC=best supportive care; HR=hazard ratio; NR=Not reported; OS=overall survival; PAN=panitumumab; PFS=progression-free survival

mutation did not benefit from either standard or dose-escalated cetuximab treatment, and their median PFS was shorter (2.8 months) than those with wild-type K-Ras (5.8 months). This study further confirms that patients with irinotecan-refractory disease, who have mutant K-Ras, do not benefit from cetuximab therapy.

Panitumumab and K-Ras A phase III trial compared panitumumab, a fully human monoclonal antibody directed against the EGFR, plus best supportive care (BSC) to that of BSC alone in patients with mCRC who progressed after standard chemotherapy.¹³ A total of 463 patients with mCRC were randomized to panitumumab

(6 mg/kg every 2 weeks) plus BSC (n=231) or BSC alone (n=232). Patients were allowed to cross over to the panitumumab arm if their disease progressed. The mean PFS was significantly prolonged with panitumumab (8 vs 7.3 weeks; P<.0001; Table 4).

Amado and colleagues examined the effect of panitumumab on PFS by K-Ras status.¹⁴ K-Ras status was ascertained in 427 of 463 (92%) patients (208 panitumumab, 219 BSC). K-Ras mutations were found in 43% of patients. In the group of patients receiving panitumumab, responses were only seen in patients with wild-type K-Ras (P<.0001; Table 4). They also had a longer median time to progression (TTP). When

patients in the panitumumab group were analyzed together with crossover patients, a longer OS was seen in wild-type tumors than mutant K-Ras tumors (Hazard ratio [HR], 0.67; confidence interval [CI], 0.55–0.82).

This study revealed that the efficacy of panitumumab monotherapy in mCRC is limited to patients with normal K-Ras. Hence, K-Ras status determination should be considered prior to this treatment.

Another study by Hecht and coauthors investigated the interaction of K-Ras status and efficacy of panitumumab in chemorefractory mCRC patients with low (1–9%) or negative (<1%) EGFR tumor cell expression by immunohistochemistry.¹⁵ The patients received panitumumab (6 mg/kg every 2 weeks) until their disease progressed or they developed unacceptable toxicity.

Of the patients evaluable for K-Ras (n=171), 55% had wild-type and 45% had mutant K-Ras. EGFR expression level did not correlate with response rates. Response to panitumumab was significantly higher in patients with wild-type K-Ras. The median PFS doubled in panitumumab patients with wild-type compared to mutant K-Ras (15 vs 7.1 months). ORR was determined by wild-type K-Ras (12% vs 0%) and median OS nearly doubled (54.0 vs 29.1 months). Wild-type K-Ras patients had a higher incidence of grade 3 skin-related toxicities (17% vs 8%) and hypomagnesaemia (20% vs 8%).

The cumulative results of these studies strongly support the notion that patients with mCRC should be assessed for K-Ras mutation prior to starting anti-EGFR treatment. This would help in predicting clinical response and avoiding unwarranted financial burden, as well as side-effects, some of which can be fatal. Patients with wild-type K-Ras have improved PFS and ORR compared to patients with mutant K-Ras.

Gefitinib and K-Ras Gefitinib is a potent small-molecule inhibitor of the tyrosine kinase domain of EGFR. It had been studied in patients with lung and head and neck cancers, but studies in patients with CRC are limited. Ogino and colleagues looked into 30 tumors evaluable for K-Ras status and found that 33% of patients (10/30) had mutations.¹⁶ However, no significant association between K-Ras status and response to gefitinib was found. The relationship between EGFR overexpression and response to gefitinib is not well understood.¹⁷ Further studies are needed to evaluate the molecular alterations of the EGFR gene and response to gefitinib in CRC.

VEGF-Inhibitor

Bevacizumab is a monoclonal antibody to VEGF. Bevacizumab in combination with chemotherapy (irinotecan, fluorouracil and leucovorin) has been shown to prolong

both PFS and OS in the first- and second-line treatment of mCRC.¹⁸

A retrospective analysis was conducted to describe the clinical benefit of bevacizumab according to K-Ras mutation status in patients with mCRC. Formalin-fixed, paraffin-embedded (FFPE) colorectal cancer tissue blocks and corresponding pathology reports were obtained for 295 of the 813 patients who participated in the bevacizumab trial from multiple centers. Patients included in these subset analyses had demographic and pathologic characteristics that were representative of the total patient population in the original trial. Tissue samples were analyzed by DNA sequence analysis. Mutations in K-Ras were found in 88 of 255 evaluable samples (35%) and all the mutations were in codon 12.

Addition of bevacizumab significantly improved the median PFS irrespective of the K-Ras status. Also, an increase in the OS was seen across the wild-type and mutant K-Ras groups (Table 5). ORR was increased with bevacizumab treatment in the wild-type K-Ras group; no difference was observed in the mutant K-Ras group. These findings suggest the independence of the VEGF and RAS signaling pathways regarding the therapeutic effect of bevacizumab. Thus, it can be concluded that K-Ras testing is not warranted in patient selection for treatment of mCRC patients with bevacizumab.

Dual Biologic Therapy (Anti-EGFR and Anti-VEGF)

A large phase III trial, known as the Panitumumab Advanced Colorectal Cancer Evaluation Study (PACCE), investigated the role of panitumumab in the first-line therapy of mCRC.¹⁹ Data from the PACCE trial is presented in Table 6. There were 2 separate study groups, depending on the investigator's choice: the first group (n=800) received oxaliplatin-based therapy (FOLFOX) plus bevacizumab with or without panitumumab. The second cohort (n=200) received irinotecan-based therapy (FOLFIRI) plus bevacizumab with or without panitumumab.

In the first cohort, the median PFS and ORR were 9.6 months and 45%, respectively, in the panitumumab; they were 11.1 months and 46%, respectively, in the bevacizumab only arm. Serious adverse events were higher in the panitumumab arm, affecting 60% of the patients, compared with 38% in the control arm. In the panitumumab arm, 27% of patients discontinued therapy due to adverse events, and in the bevacizumab arm, 24% discontinued. Hence, the addition of panitumumab to FOLFOX in combination with bevacizumab worsened PFS and increased toxicity.

In the second cohort, the median PFS and ORR were 10.1 months and 43%, respectively, in the panitumumab arm; they were 11.7 months and 39%, respectively, in the

Table 5. K-Ras and Bevacizumab

| Efficacy measure | Wild-type K-Ras (n=152) | | Mutant K-Ras (n=78) | |
|------------------|-------------------------|----------------|---------------------|----------------|
| | IFL+placebo (n=67) | IFL+BEV (n=85) | IFL+placebo (n=34) | IFL+BEV (n=44) |
| PFS (months) | 7.4 | 13.5 | 5.5 | 9.3 |
| | <i>P</i> <.0001 | | <i>P</i> =.0008 | |
| OS (months) | 17.6 | 27.7 | 13.6 | 19.9 |
| | <i>P</i> =.04 | | <i>P</i> =.26 | |
| ORR (%) | 37.3 | 60 | 41.2 | 43.2 |
| | <i>P</i> =.006 | | <i>P</i> =.86 | |

BEV=bevacizumab; IFL=irinotecan/fluorouracil/leucovorin; ORR=overall response rate; OS=overall survival; PFS=progression-free survival.

bevacizumab only arm. Toxicity of FOLFIRI plus bevacizumab/panitumumab was considerable, with 37% of patients developing grade 3/4 skin toxicity, 17% developing grade 3/4 neutropenia, 2% developing grade 5 infections, and 1% developing grade 5 pulmonary embolism. More patients died in the panitumumab arm than in the control arm (23% vs 16%). Worse outcomes were noticed in older patients (>80 years) and in those who had worse ECOG status. Therefore, although clinical efficacy is not compromised in this second cohort, there is significant toxicity with the addition of panitumumab and bevacizumab to FOLFIRI.

A retrospective analysis of tumor samples available for K-Ras mutation analysis revealed that 57 of 103 patients (55%) treated in the panitumumab arm and 58 of 97 (60%) patients in the control arm had wild-type K-Ras.¹⁹ In patients with wild-type K-Ras, the response rate was higher in the panitumumab arm compared with the control arm (54% vs 47%). The response was lower in the panitumumab arm among the patients with a mutant K-Ras, relative to those in the control arm (30% vs 38%).

Hence, looking at the impact of K-Ras status on outcome, the results were consistent with what has been seen with panitumumab monotherapy.

CAIRO 2 was a randomized phase III trial investigating the efficacy of dual therapy (anti-EGFR + anti-VEGF) in the treatment of mCRC.²⁰ Efficacy results from the BOND 2 study (cetuximab + bevacizumab + irinotecan) showed a response rate of 20% and a median TTP of 5.6 months. These results appeared to be superior to those obtained with the use of bevacizumab or cetuximab as single agents.

In contrast, the PACCE trial demonstrated decreased efficacy and increased toxicity with the addition of dual biologic therapy to standard chemotherapy.

The CAIRO2 trial randomized patients into 2 groups: One group (n=368) received capecitabine (1,000 mg/m² twice daily on days 1–14 of a 3 week cycle) plus oxaliplatin

(130 mg/m² on day 1) plus bevacizumab (7.5 mg/kg on day 1) and the other group (n=368) received capecitabine plus oxaliplatin plus bevacizumab plus cetuximab (250 mg/m² after an initial loading dose of 400 mg/m²). Oxaliplatin was omitted after the sixth cycle and capecitabine was then increased to a dose of 1,250 mg/m². The primary endpoint of PFS was significantly lower in the cetuximab treatment arm (9.6 months) compared to the capecitabine plus bevacizumab plus oxaliplatin arm (10.7 months) with a HR of 1.21 (*P*=.018). However, the addition of cetuximab to the treatment arm did not alter the OS (20.3 vs 20.4 months; *P*=.21) or the ORR (44% vs 44%; *P*=.88; Table 7).

Regarding the safety profile, overall grade 3/4 events were significantly higher in patients in the cetuximab arm (72% vs 82%; *P*=.0013). The authors of the CAIRO 2 trial looked at skin toxicity caused by cetuximab. They found a higher incidence of grade 3 acneiform skin reactions (25% vs 0.5%, *P*<.001) and grade 3 nail changes (4% vs 0.3%, *P*<.001) in the cetuximab containing arm compared to the capecitabine/irinotecan/bevacizumab (COB) only arm. There was no significant increase in noncutaneous toxicity, with the exception of an increase in grade 3/4 diarrhea in the cetuximab arm (19% vs 26%; *P*=.026)

A subgroup analysis of treatment outcomes and relationship to K-Ras status was performed. A total of 501 patients were evaluable, of which 305 (60.8%) patients had a wild-type K-Ras and 196 (39.1%) patients had a mutant K-Ras. Patients with wild-type K-Ras showed no difference in response rates between the 2 treatment arms. However, patients with mutant K-Ras who received cetuximab experienced a shorter median PFS (8.6 months) than patients who received no cetuximab (12.5 months; *P*=.043). There was no difference in the OS (Table 7).²¹

Therefore, the CAIRO 2 study did not reveal any added benefit of adding cetuximab to bevacizumab plus CAPOX (capecitabine + oxaliplatin). These results are

Table 6. PACCE Trial: K-Ras and Dual Biologic Therapy

| Efficacy measure | OX-CT +BEV (n=410) | OX-CT +BEV +PAN (n=413) | HR (95% CI) |
|------------------|---------------------|---------------------------|------------------|
| PFS (months) | 11.1 | 9.6 | 1.27 (1.05–1.53) |
| ORR (%) | 46% | 45% | |
| | IRI-CT +BEV (n=115) | IRI-CT +BEV + PAN (n=115) | HR |
| PFS (months) | 11.7 | 10.1 | 1.21 (0.80–1.82) |
| ORR (%) | 39% | 43% | 1.15 (OR) |

BEV=bevacizumab; HR=hazard ratio; IRI-CT= Irinotecan based chemotherapy; ORR=overall response rate; OX-CT=oxaliplatin-based chemotherapy; PAN=panitumumab; PFS=progression-free survival.

Table 7. CAIRO 2 Trial: Anti-EGFR + Anti-VEGF and K-Ras

| Efficacy measure | Wild-type K-Ras (n=305) | | Mutant K-Ras (n=195) | |
|--------------------|-------------------------|---------------|----------------------|--------------|
| | COB (n=152) | COB-C (n=153) | COB (n=103) | COB-C (n=93) |
| PFS (months) | 10.7 | 10.5 | 12.5 | 8.6 |
| | <i>P</i> =.10 | | <i>P</i> =.043 | |
| Median OS (months) | 23.0 | 22.2 | 24.9 | 19.1 |
| | <i>P</i> =.49 | | <i>P</i> =.39 | |

COB=capecitabine/irinotecan/bevacizumab; COB-C=Capecitabine/irinotecan/bevacizumab + cetuximab; OS=overall survival; PFS=progression-free survival.

consistent with the findings of the PACCE trial, which showed that the addition of panitumumab to bevacizumab plus FOLFOX or bevacizumab plus FOLFIRI did not benefit response rates, and might even be detrimental. Among patients with K-RAS mutant tumors, the addition of panitumumab was associated with a decrease in PFS, but did not significantly affect OS.

Based on the findings of these 2 studies, the use of dual agents with anti-EGFR and anti-VEGF antibodies is not indicated in the treatment of mCRC. Patients with mutant K-Ras do not react favorably to the addition of EGFR inhibitors. K-Ras status does not appear to play a major role in determining response to VEGF inhibitors.

Detection of K-Ras Gene Mutation

Tumor DNA is obtained from FFPE tissue specimens. Five unstained slides and 1 H&E-stained slide at 10 μM or a tissue block are collected. A minimum of 2 × 2 mm tumor area with more than 50% tumor cells should be available for accurate test interpretation.

This assay detects 7 K-Ras mutations in codons 12 and 13. Since preparation of DNA from tissue samples

is dependent on the quality of the specimen provided, inadequate DNA extraction may occur in a significant number of paraffin-embedded samples. The methods used in this assay are highly selective. They can detect approximately 1% of mutant DNA in a background of wild-type genomic DNA depending on the total amount of DNA present. The assay has a limit of detection of between 5 and 10 copies.²²

Different Methods of Identification of Mutation

Sequencing Methods Dideoxysequencing is the most widely used sequencing method. It is suitable for reading longer stretches of DNA and has readily available advanced software for analysis. However, its use is limited by the cost of sequencing equipment and time to prepare samples. It may not detect a minority of mutant sequences present in a background of abundant wild type sequence.²³

Pyrosequencing is a relatively faster method with a real-time readout suitable for sequencing shorter stretches of nucleotides (up to 40–50). Since most of the K-Ras mutations are concentrated in codons 12 and 13, its shorter reading length does not pose a disadvantage. Pyrosequencing can accurately quantify the amount of each allele, which is useful in assay validation and assures

quality control. It is also more efficient in designing relatively small PCR products from degraded DNA samples. Since DNA derived from paraffin-embedded tissue is commonly degraded in short fragments, pyrosequencing is a more efficient technique.²⁴

Allele-specific Amplification Techniques Allele-specific PCR techniques include MASA (mutant allele specific amplification), PASA (PCR amplification of specific allele), MS-PCR (mutagenically separated PCR), MAMA (mutant allele-specific amplification), ARMS (amplification refractory mutation system), and ASO (allele specific oligonucleotide hybridization). These techniques cannot detect the full spectrum of K-Ras mutations. They are also more prone to false positive signals from either minute contamination or from introduction of point mutations by polymerase errors during PCR amplification.^{25,26}

Allele Discrimination There are 4 general mechanisms for sequence-specific detection for allelic discrimination: allele-specific hybridization, allele-specific nucleotide incorporation, allele-specific oligonucleotide ligation, and allele-specific invasive cleavage.²⁷ Allele discrimination assays are fast and sensitive, and allow for concurrent amplification and discrimination.

Multiplex Polymerase Chain Reaction/Ligase Detection Reaction (PCR/LDR) PCR/LDR separates the amplification and mutation discrimination and hence reduces the false positive rates. It allows detection of mutations in a high background of wild-type alleles and provides higher sensitivity and specificity.²⁵

PCR-restriction Fragment Length Polymorphism (RFLP) A point mutation in a certain DNA segment can create or destroy a restriction enzyme recognition site, resulting in RFLP. Mismatched primers are used to create (or destroy) a restriction enzyme site in PCR-amplified DNA. This is a sensitive, quick, and easy assay. Its reliability depends on the efficacy of the restriction enzyme digestion.

Other Sources of Clinical Samples for Detection of K-Ras Mutations

In order to diagnose and assess cancer susceptibility at an early stage, the type of collected sample and molecular genetic technique applied is a very important decision. Clinical samples suitable for molecular diagnosis include body secretions, lavage fluids, and cytology specimens, which represent the state of the entire organ. For the detection of colorectal cancer, various studies have found mutated K-Ras sequences as tumor markers in feces, lavage fluid (collected prior to colonoscopy), plasma, serum, and

urine. However, in the above clinical samples, the ratio of neoplastic or preneoplastic to normal cells is very low and has an inter-individual variability. Hence, extremely sensitive molecular methods are needed to detect the minute amount of neoplastic or preneoplastic cells with mutations.²⁸ Various studies have looked at different samples including stool, plasma/serum, lavage fluid, and urine.²⁹⁻⁴⁰ The modified sensitivity in these studies varies from 29–100%, in part due to the variability of the detection method used. The specificity ranged 81–100%. There appear to be very few false positives.

However, most of the patients with positive assays tended to have more extensive tumors, mainly metastatic tumors. More data are needed to evaluate the usefulness of detecting K-Ras mutations in body fluid samples in cases of lower stage cancers, before drawing any conclusions.⁴¹

Currently, the most commonly used test is the K-Ras mutational kit, based on a PCR developed by DxS Diagnostic Innovations.⁴² The kit is used on DNA samples and provides a qualitative assessment of mutation status. After DNA extraction, real time PCR assays are performed to detect the target molecule. By comparing control and mutant sample reactions, users can detect and estimate low levels of mutation. No further sample processing is necessary and the time to result is less than 3 hours. DxS has combined ARMS (allele specific PCR) with the Scorpions real-time PCR technology to develop this sensitive tool for tumor-borne K-RAS mutations. This particular kit was also used in the studies performed with cetuximab and panitumumab. This kit detects 7 K-Ras mutations in codons 12 and 13, presented below:

1. Gly12Asp (GGT>GAT)
2. Gly12Ala (GGT>GCT)
3. Gly12Val (GGT>GTT)
4. Gly12Ser (GGT>AGT)
5. Gly12Arg (GGT>CGT)
6. Gly12Cys (GGT>TGT)
7. Gly13Asp (GGC>GAC)

This kit is highly selective and, depending on the total amount of DNA present, can detect approximately 1% of mutant in a background of wild-type genomic DNA. The assays have limits of detection of between 5 and 10 copies. These selectivity and detection limits are superior to technologies such as dye terminator sequencing.

Future Directions

Mutations in genes such as K-Ras and p53 cause major clonal expansion of colorectal tumors. Metastases from colorectal cancer patients are also clonal, and whether they carry the same K-Ras and p53 mutations as the primary tumor is controversial. Few studies have described the presence of de novo mutations in metastases derived

from colorectal carcinomas or the absence in the distant recurrence of mutations present in the primary tumor. Whether K-Ras mutations are stable throughout the progression of disease or whether they undergo clonal evolution during tumor progression is still a matter of debate.⁴³⁻⁴⁵ This may have implications in the treatment of metastatic colorectal cancer.

Conclusions

We are entering an era of personalized medicine in cancer therapy where treatment is tailored to the individual. The predictive value of K-Ras mutations in the treatment of mCRC is very useful to clinicians and patients in terms of decision making, avoiding toxicities, and decreasing financial burden. The results of recent studies are highly encouraging for assessment of K-Ras status prior to starting anti-EGFR therapy. Because there is no effect of K-Ras on the treatment response with anti-VEGF drugs, a pre-treatment mutation assessment is not warranted in those patients. The studies also highlight the need to use sensitive molecular methods of mutation detection.

References

- Forbes S, Clements J, Dawson E, COSMIC 2005. *Br J Cancer*. 2006;94:318-322.
- Poehlmann A, Kuester D, Meyer F, Lippert H, Roessner A, Schneider-Stock R. K-Ras mutation detection in colorectal cancer using the Pyrosequencing technique. *Pathol Res Pract*. 2007;203:489-497.
- Levi S, Urbano-Ispizua A, Gill R. Multiple K-Ras codon 12 mutations in cholangiocarcinomas demonstrated with a sensitive polymerase chain reaction technique. *Cancer Res*. 1991;51:3497-3502.
- Ji Z, Mei FC, Xie J, Cheng X. Oncogenic K-RAS activates hedgehog signaling pathway in pancreatic cancer cells. *J Biol Chem*. 2007;282:14048-14055.
- Raponi M, Winkler H, Dracopoli NC. K-RAS mutations predict response to EGFR inhibitors. *Curr Opin Pharmacol*. 2007;8:413-418.
- Fang JY, Richardson BC. The MAPK signaling pathways and colorectal cancer. *Lancet Oncol*. 2005;6:322-327.
- Ferrara N, Davis-Smyth T. The biology of vascular endothelial growth factor. *Endocr Rev*. 1997;18:4-25.
- Rak J, Mitsuhashi Y, Bayko L. Mutant ras oncogenes upregulate VEGF/VPF expression: implications for induction and inhibition of tumor angiogenesis. *Cancer Res*. 1995;55:4575-4580.
- Van Cutsem E, Lang I, D'haens G, et al. K-RAS status and efficacy in the first-line treatment of patients with metastatic colorectal cancer (mCRC) treated with FOLFIRI with or without cetuximab: The CRYSTAL experience. *J Clin Oncol*. (ASCO Annual Meeting Abstracts). 2008;26:2
- Bokemeyer C, Bondarenko I, Hartmann JT, et al. K-RAS status and efficacy of first-line treatment of patients with metastatic colorectal cancer (mCRC) with FOLFOX with or without cetuximab: The OPUS experience. *J Clin Oncol*. (ASCO Annual Meeting Abstracts). 2008;26:4000.
- Bokemeyer C, Bondarenko I, Makhson A, et al. Cetuximab plus 5-FU/FA/oxaliplatin (FOLFOX-4) versus FOLFOX-4 in the first-line treatment of metastatic colorectal cancer (mCRC): OPUS, a randomized phase II study. *J Clin Oncol*. (ASCO Annual Meeting Abstracts). 2007;25(18S):4035.
- Tejpar S, Peeters M, Humblet Y, et al. Relationship of efficacy with K-RAS status (wild type versus mutant) in patients with irinotecan-refractory metastatic colorectal cancer (mCRC), treated with irinotecan (q2w) and escalating doses of cetuximab (q1w): The EVEREST experience (preliminary data). *J Clin Oncol*. (ASCO Annual Meeting Abstracts). 2008;26:4001.
- Van Cutsem E, Peeters M, Siena S, et al. Open-label phase III trial of panitumumab plus best supportive care compared with best supportive care alone in patients with chemotherapy-refractory metastatic colorectal cancer. *J Clin Oncol*. 2007;25:1658-1664.
- Amado RG, Wolf M, Peeters M, et al. Wild-type K-RAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. *J Clin Oncol*. 2008;26:1626-1634.
- Hecht JR, Mitchell EP, Baranda J, et al. Panitumumab efficacy in patients with metastatic colorectal cancer with low or undetectable levels of epidermal growth factor receptor: final efficacy and K-RAS analysis. Program and abstracts of the 2008 Gastrointestinal Cancers Symposium (GCS); January 25-27, 2008; Orlando, Florida. Abstract 343.
- Ogino S, Meyerhardt JA, Cantor M, et al. Molecular alterations in tumors and response to combination chemotherapy with gefitinib for advanced colorectal cancer. *Clin Cancer Res*. 2005;11:6650-6656.
- Cascinu S, Berardi R, Salvagni S, et al. A combination of gefitinib and FOLFOX-4 as first-line treatment in advanced colorectal cancer patients. A GIS-CAD multicentre phase II study including a biological analysis of EGFR overexpression, amplification and NF- κ B activation. *Br J Cancer*. 2008;98:71-76.
- Hurwitz H, Fehrenbacher L, Novotny W, et al. Bevacizumab plus Irinotecan, Fluorouracil, and Leucovorin for Metastatic Colorectal Cancer. *NEJM*. 2004;350:2335-2342.
- Hecht JR, Mitchell E, Chidiac T, et al. Interim results from PACCE: irinotecan (Iri)/bevacizumab (bev) +/- panitumumab (pmab) as first-line treatment (tx) for metastatic colorectal cancer (mCRC). Program and abstracts of the 2008 Gastrointestinal Cancers Symposium (GCS); January 25-27, 2008; Orlando, Florida. Abstract 279.
- Saltz L, Lenz H, Kindler HL, et al. Interim report of randomized phase II trial of cetuximab/bevacizumab/irinotecan (CBI) versus cetuximab/bevacizumab (CB) in irinotecan-refractory colorectal cancer, Program and abstracts of the American Society of Clinical Oncology 2005 Gastrointestinal Cancers Symposium; January 27-29, 2005; Abstract 169b.
- Tol J, Koopman M, Rodenburg CJ, et al. A randomized phase III study of capecitabine, oxaliplatin, and bevacizumab with or without cetuximab in first-line advanced colorectal cancer (ACC), the CAIRO2 study of the Dutch Colorectal Cancer Group (DCCG). *Ann Oncol*. 2008;19:734-738.
- Laboratory Corporation of America® Holdings and Lexi-Comp Inc. 2007.
- Gharizadeh B, Herman ZS, Eason RG, Jejelowo O, Pourmand N. Large-scale pyrosequencing of synthetic DNA: a comparison with results from Sanger dideoxy sequencing. *Electrophoresis*. 2006;27:3042-3047.
- Ogino S, Kawasaki T, Brahmandam M, et al. Sensitive sequencing method for K-RAS mutation detection by Pyrosequencing. *J Mol Diagn*. 2005;7:413-421.
- Khanna M, Park P, Zirvi M, et al. Multiplex PCR/LDR for detection of K-Ras mutations in primary colon tumors. *Oncogene*. 1999;18:27-38.
- van Mansfeld AD, Bos JL. PCR-based approaches for detection of mutated ras genes. *PCR Methods Appl*. 1992;1:211-216.
- Kwok PY. Methods for genotyping single nucleotide polymorphisms. *Annu Rev Genomics Hum Genet*. 2001;2:235-258.
- Minamoto T, Mai M, Ronai Z. K-Ras mutation: early detection in molecular diagnosis and risk assessment of colorectal, pancreas, and lung cancers—a review. *Cancer Detect Prev*. 2000;24:1-12.
- Sidransky D, Tokino T, Hamilton SR, et al. Identification of ras oncogene mutations in the stool of patients with curable colorectal tumors. *Science*. 1992;256:102-105.
- Smith-Ravin J, England J, Talbot IC, Bodmer W. Detection of c-Ki-ras mutations in faecal samples from sporadic colorectal cancer patients. *Gut*. 1995;36:81-86.
- Hasegawa Y, Takeda S, Ichii S, et al. Detection of K-Ras mutations in DNAs isolated from feces of patients with colorectal tumors by mutant-allele-specific amplification (MASA). *Oncogene*. 1995;10:1441-1445.
- Nollau P, Moser C, Weinland G, Wagener C. Detection of K-Ras mutations in stools of patients with colorectal cancer by mutant-enriched PCR. *Int J Cancer*. 1996;66:332-336.
- Villa E, Dugani A, Rebecchi AM, et al. Identification of subjects at risk for colorectal carcinoma through a test based on K-Ras determination in the stool. *Gastroenterology*. 1996;110:1346-1353.
- Potter MA, Morris GR, Ferguson A. Detection of mutations associated with colorectal cancer in DNA from whole-gut lavage fluid. *J Natl Cancer Inst*. 1998;90:623-626.

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35. Anker P, Lefort F, Vasioukhin V, et al. K-Ras mutations are found in DNA extracted from the plasma of patients with colorectal cancer. *Gastroenterology*. 1997;112:1114-1120.
36. Hibi K, Robinson CR, Booker S, et al. Molecular detection of genetic alterations in the serum of colorectal cancer patients. *Cancer Res*. 1998;58:1405-1407.
37. de Kok JB, van Solinge WW, Ruers TJ, et al. Detection of tumour DNA in serum of colorectal cancer patients. *Scand J Clin Lab Invest*. 1997;57:601-604.
38. Kopreski MS, Benko FA, Kwee C, et al. Detection of mutant K-Ras DNA in plasma or serum of patients with colorectal cancer. *Br J Cancer*. 1997;76:1293-1299.
39. Dillon DA, Salem R, Anderson D, et al. K-Ras mutations are DNA tumor markers in serum and plasma of patients with cancer of colon or pancreas. *Abstr N Engl Cancer Soc*. 1997:37.
40. Su YH, Wang M, Brenner DE, et al. Human urine contains small, 150 to 250 nucleotide-sized, soluble DNA derived from the circulation and may be useful in the detection of colorectal cancer. *J Mol Diagn*. 2004;6:101-107.
41. Sorenson GD. Detection of Mutated K-RAS2 Sequences as Tumor Markers in Plasma/Serum of Patients with Gastrointestinal Cancer. *Clin Cancer Res*. 2000;6:2129-2137.
42. Cancer Mutation Products-K-RAS Mutation Test Kit. Available at <http://www.dxs genotyping.com/Content/ K-RASMutationTestKit.aspx?Z=39>. Accessed on October 16, 2008.
43. Fahd Al-Mulla, Going JJ, Sowden ET. Heterogeneity of mutant versus wild-type Ki-ras in primary and metastatic colorectal carcinomas, and association of codon-12 valine with early mortality. *J Pathol*. 1998;185:130-138.
44. Andersen SN, Løvig T, Breivik J. K-Ras Mutations and Prognosis in Large-Bowel Carcinomas. *Scand J Gastroenterol*. 1997;32:62-69.
45. Törtola S, Steinert R, Hantschick M, et al. Discordance between K-Ras mutations in bone marrow micrometastases and the primary tumor in colorectal cancer. *J Clin Oncol*. 2001;19:2837-2843.