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Hot Topic

Extended RAS analysis for anti-epidermal growth factor therapy in patients with metastatic colorectal cancer

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ABSTRACT

RAS family proteins (including *KRAS* and *NRAS*) play important roles in the epidermal growth factor receptor (EGFR) signaling pathway. Mutations in *RAS* genes (occurring at loci in exons 2, 3, and 4) often result in constitutive activation of RAS proteins and persistent downstream signaling. Mutations in *KRAS* exon 2 (codon 12/13) are an established predictor of lack of response to the anti-EGFR monoclonal antibodies cetuximab and panitumumab in patients with metastatic colorectal cancer (mCRC), and have been used routinely in clinical practice to identify patients unlikely to derive benefit from these therapies. However, a meaningful proportion of patients with mCRC have tumors bearing other mutations in *RAS* genes. Recent studies have demonstrated that evaluation of an extended panel of *RAS* mutations—including mutations in *KRAS* exon 2, 3, and 4 and *NRAS* exons 2, 3, and 4—can better define the patient population that is unlikely to benefit from anti-EGFR therapy, with concomitant improvements in outcomes in the more highly selected *RAS* wild-type group. This discovery has changed the practice of oncology and has the potential to spare patients from exposure to ineffective therapy. In the near future, it is important for the oncology community to validate extended *RAS* analysis assays and make certain that patients who are candidates for anti-EGFR therapy undergo appropriate testing and treatment.

Introduction

The epidermal growth factor receptor (EGFR), a transmembrane receptor tyrosine kinase overexpressed in a number of human cancers, has become an important therapeutic target in colorectal cancer [1]. The EGFR signaling pathway is involved in a range of cellular functions, including regulation of cell proliferation, migration, differentiation, and survival [1]. There is strong evidence that activation of the EGFR signaling pathway has a critical role in the malignant transformation of normal cells, and overexpression and activation of members of the EGFR family of membrane receptors is characteristic of a number of human cancers, including colorectal cancer [2]. Panitumumab and cetuximab are anti-EGFR monoclonal antibodies that have been shown to improve outcomes in patients with metastatic colorectal cancer (mCRC) [3–6]. Panitumumab, a recombinant, fully human immunoglobulin G2 anti-EGFR monoclonal antibody [7], and cetuximab, a recombinant human/mouse chimeric immunoglobulin G1 anti-EGFR monoclonal antibody [8], both have a high affinity for the extracellular domain of EGFR, competitively inhibiting the binding of EGFR ligands leading to inhibition of EGFR-mediated signaling, with resulting antitumor activity [7,8]. The activity of these agents in mCRC was first demonstrated in phase 3 studies in patients with chemotherapy-refractory disease [4,6]. They have subsequently been shown to have similar efficacy to one another in mCRC: an open-label, phase 3, randomized, noninferiority study (ASPECCT) in patients with chemotherapy-refractory wild-type *KRAS* exon 2 mCRC showed that panitumumab was noninferior to cetuximab, with similar hazard ratios for OS and PFS [9].

Initially, cetuximab and panitumumab were evaluated in unselected mCRC patients. Subsequent investigation has evaluated and

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validated biomarkers that predict lack of response to anti-EGFR monoclonal antibodies, in particular mutations in the RAS protein family. Use of these biomarkers help identify patients most likely to benefit from treatment with anti-EGFR monoclonal antibodies and spare potential treatment-related toxicity in patients who are unlikely to respond. This review discusses the biology of RAS proteins and the clinical utility of *RAS* mutations as predictors of response and summarizes analyses of phase 3 studies that have evaluated the predictive value of *RAS* mutations in patients with mCRC.

Biology of RAS proteins

Members of the RAS family of small guanine nucleotide-binding proteins play an important role in intracellular signaling pathways regulated by a range of cell surface receptors, including EGFR [10,11]. By acting as guanosine diphosphate/guanosine triphosphate (GTP)-regulated signal switch molecules, the *RAS* proteins control a diverse range of cellular responses, including proliferation, differentiation, and survival [11,12]. The three *RAS* isoforms (*KRAS*, *NRAS*, and *HRAS*) are highly homologous to one another, sharing a common identity over 80% to 90% of the coding sequence [11,12]. The amino-terminal portion of the proteins consists of a hypervariable region, allowing for posttranslational protein modifications resulting in significant divergence in trafficking and activity [11,12].

Ligand binding to the EGFR receptor with subsequent receptor dimerization activates a number of downstream signaling pathways. Among the best characterized of the RAS effector pathways is the mitogen-activated protein kinase (MAPK) cascade, in which the serine/threonine kinase BRAF plays a key regulatory role [12]. Other RAS signaling pathways include the phosphatase and tensin homolog (PTEN)/phosphatidyl-inositol 3-kinase (PI3K)/AKT pathway and the signal transducer and activator of transcription (STAT) pathway, which induces transcriptional regulation of cell division, differentiation, migration, adhesion, and apoptosis [11,12]. Activating mutations in RAS proteins (occurring at specific loci in exons 2, 3, and 4) inhibit GTPase activity, modulate the guanine nucleotide exchange rate, or desensitize their activation by GTPase-activating proteins, resulting in constitutive activation of RAS downstream effector pathways and imparting resistance to inhibition of EGFR and other cell surface receptor kinases (Fig. 1) [12–14]. Such dysregulation of the EGFR signaling pathway can stimulate tumor growth, prolong tumor survival, and advance the spread of metastatic disease [15]. In preclinical models of mCRC, mutations in *KRAS* and *NRAS* have been shown to be constitutively activating, and are associated with an oncogenic phenotype (with differing characteristics depending on the specific mutation present) [16]. Activating mutations have also been described in other RAS pathway effectors (eg, BRAF), and these play a role in mCRC tumor development and progression [17].

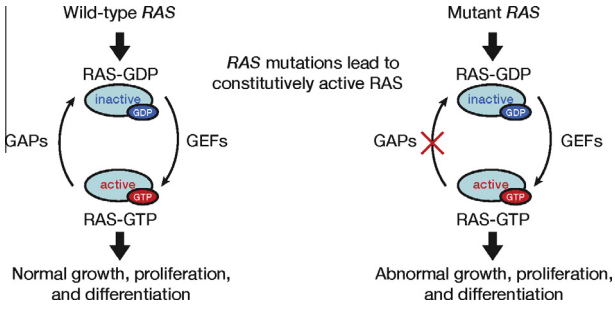

Fig. 1. Role of *KRAS* mutations in oncogenic activation and inactivation of normal *RAS* signaling pathways. GAP = guanosine triphosphatase-activating proteins; GDP = guanosine diphosphate; GEF = guanine exchange factors; GTP = guanosine triphosphate. Adapted with permission from van Krieken JH, Jung A, Kirchner T, et al. *KRAS* mutation testing for predicting response to anti-EGFR therapy for colorectal carcinoma: proposal for a European quality assurance program. Virchows Arch. 2008;453(5):417–431.

Activating mutations in *KRAS* are common in pancreatic cancer, non–small-cell lung cancer, and of particular relevance to this review, colorectal cancer [10]. Overall, mutations in *KRAS* may account for up to ~85% of *RAS* mutations, whereas activating mutations in *NRAS* and *HRAS* are less common, representing ~15% and less than 1%, respectively [10,11]. Amino acid substitutions at codons 12 and 13 in exon 2 are responsible for most mutations in *KRAS* (88% of the recurrent mutations across all tumor types), with the remainder present in exon 3 (codons 59, 61) and exon 4 (codons 117 and 146). In contrast, the most frequently occurring recurrent mutations in *NRAS* and *HRAS* are seen in codon 61 of exon 3 [10,18].

Mutations in *KRAS* exon 2 as a biomarker

It was hypothesized that triggering of EGFR-independent intracellular signal transduction activation of the *RAS* pathway by *KRAS* exon 2 mutations could impair response to anti-EGFR monoclonal antibodies in colorectal cancer [19,20]. Evidence supporting the hypothesis that mutations in *KRAS* exon 2 (codons 12 and 13) were associated with lack of response was provided by retrospective analyses of the pivotal phase 3 trials of single-agent panitumumab and cetuximab in patients with chemotherapy refractory disease [3,5]. These phase 3 studies had enrolled patients with chemotherapy-refractory disease without biomarker selection. Both cetuximab and panitumumab were shown to improve progression-free survival (PFS) compared with best supportive care (BSC) alone [4,6]. In the panitumumab study [6], the hazard ratio for PFS was 0.54 (95% CI, 0.44–0.66; $P < 0.0001$) whereas in the cetuximab study [4], the hazard ratio for PFS was 0.68 (95% CI, 0.57–0.80; $P < 0.001$). Cetuximab also improved median overall survival (OS; hazard ratio, 0.77; 0.64–0.92; $P = 0.005$). Although a significant improvement in OS was not shown for panitumumab, the crossover of 76% of BSC patients to panitumumab (which was allowed per the study protocol) likely confounded the evaluation of OS; crossover was not allowed in the cetuximab study.

Subsequent analyses of these two phase 3 studies evaluated outcomes when patients were stratified by *KRAS* exon 2 mutation status. In both analyses, the seven most frequently occurring mutations in *KRAS* codons 12 and 13 (ie, exon 2) were evaluated using allele-specific PCR and were found to be predictive of lack of response to anti-EGFR monoclonal antibody monotherapy [3–6]. In the panitumumab study, in which *KRAS* status was ascertained for 92% of patients, median PFS was improved by the addition of panitumumab to BSC compared with BSC alone in patients with wild-type *KRAS* exon 2 (12.3 versus 7.3 weeks, respectively; hazard ratio, 0.45; 95% CI, 0.34–0.59), but not in patients with mutant *KRAS* (7.4 versus 7.3 weeks, respectively; hazard ratio, 0.99; 95% CI, 0.73–1.36) [3]. The quantitative interaction test for the relative treatment effect between the *KRAS* wild-type and mutant groups on PFS was $P < 0.0001$. Among patients in the *KRAS* wild-type group, the ORR was 17% for those receiving panitumumab and 0% for those receiving BSC. No patient with mutant *KRAS* had an objective response. Further analysis showed that the magnitude of the relative treatment effect of panitumumab on PFS was greater for the wild-type *KRAS* group ($P < 0.0001$); consistent results were obtained for propensity-score adjusted hazard ratios.

In the cetuximab study (*KRAS* ascertainment, 69%), 12.8% of patients with wild-type *KRAS* exon 2 tumors had an objective response, compared with only 1.2% of patients with mutant *KRAS*

exon 2, and benefits in OS and PFS were confined to patients with wild-type *KRAS* exon 2 tumors [5]. Median OS was 9.5 and 4.8 months ($P < 0.001$), respectively, in patients with wild-type *KRAS* codon 12/13 tumors treated with cetuximab plus BSC versus BSC only whereas median OS was 4.5 and 4.6 months, respectively, in patients with mutated *KRAS* tumors receiving cetuximab versus BSC only ($P = 0.89$). The addition of cetuximab to BSC prolonged median PFS in patients with wild-type *KRAS* compared with BSC alone (3.7 versus 1.9 months, respectively; hazard ratio, 0.40; 95% CI, 0.30–0.54; $P < 0.001$) but not in patients with mutant *KRAS* (1.8 months in both groups; hazard ratio, 0.99; 95% CI, 0.73–1.35; $P = 0.96$) [5]. The interaction between *KRAS* mutation status and treatment effect was $P = 0.01$ for OS and $P < 0.001$ for PFS.

Subsequently, a number of studies evaluating anti-EGFR monoclonal antibodies in mCRC were amended to focus on *KRAS* exon 2-selected populations, analyzed retrospectively to evaluate outcomes in *KRAS* exon 2 wild-type patients, or designed to prospectively enroll *KRAS* exon 2 wild-type patients [9,21–28]. These studies compared the effect of adding cetuximab or panitumumab to chemotherapy regimens with chemotherapy alone in first-line [21,22,24–27], second-line [23,28], or chemotherapy-refractory [9] settings in patients with mCRC. In brief, these studies confirmed the validity of selection of patients with mCRC by *KRAS* exon 2 mutation status. The addition of anti-EGFR monoclonal antibody therapy to FOLFOX (oxaliplatin, 5-fluorouracil, and leucovorin) or FOLFIRI (irinotecan, 5-fluorouracil, and leucovorin) was associated with improved outcomes in patients with wild-type *KRAS* exon 2 tumors, but not in patients with *KRAS* exon 2 mutant tumors.

Based on these results, recommendations from the National Comprehensive Cancer Network (NCCN) and the European Society of Pathology strongly advised testing for *KRAS* gene mutations in patients with mCRC and specified that the use of anti-EGFR therapy in mCRC should be limited to patients with *KRAS* exon 2 wild-type tumors [29,30]. Moreover, prescribing information for panitumumab and cetuximab were modified to limit their use to patients with *KRAS* wild-type mCRC [31–34]. A companion diagnostic assay, the Therascreen® *KRAS* RGQ PCR Kit (QIAGEN N.V., Venlo, Netherlands) that detects the presence of the seven most frequent mutations in codons 12 and 13 was also approved by the FDA to identify patients suitable for treatment with panitumumab and cetuximab [35,36].

RAS mutations beyond *KRAS* exon 2 as predictors of response

Although it was clear that *KRAS* exon 2 (codon 12/13) testing helped identify a patient population unlikely to benefit from anti-EGFR therapy, not all patients with wild-type *KRAS* exon 2 tumors responded to treatment with anti-EGFR therapy. Further improvement in patient selection methods through the identification of additional predictive biomarkers could strengthen provision of patient-specific therapy and help avoid unnecessary treatment and treatment-related toxicity [37]. Given the evidence of constitutively activating mutations in a broad array of loci in *RAS* family genes (see above), it was suggested that *RAS* mutations beyond *KRAS* exon 2 might also be predictive of lack of clinical benefit with anti-EGFR therapy in patients with mCRC.

Mutations in *KRAS* with potential predictive value beyond those in codons 12/13 in exon 2 have been identified, particularly codon 61 in exon 3 and codon 146 in exon 4 [38]. The presence of these *KRAS* mutations in patients with *KRAS* codons 12 and 13 wild-type disease, although rarer than codon 12/13 (exon 2) mutations, was nevertheless associated with lack of response and shorter PFS duration compared to patients with *KRAS* codon 61/146 wild-type disease in small retrospective studies [38]. Mutations in *NRAS* exons 2, 3, and 4 (which are typically mutually exclusive from mutations in *KRAS*) also occur in colorectal cancer tumors [11]. The relative distribution of mutations in *KRAS* exon 3 and 4 and *NRAS* exons 2, 3, and 4 is shown in Fig. 2. Overall, the incidence of *RAS* mutations has been found to be approximately 20% in patients with wild-type *KRAS* exon 2 tumors, with a different distribution of mutations between exons in *KRAS* and *NRAS* [39,40]. This incidence of *RAS* mutations beyond *KRAS* exon 2 suggested that, if such mutations had predictive value, a clinically meaningful proportion of patients were exposed to potential toxicity of anti-EGFR agents without the potential for clinical benefit.

Extended *RAS* analysis in phase 2 and 3 studies

Early retrospective RAS analyses led to generation of the hypothesis that these mutations beyond *KRAS* exon 2 might have added additional predictive value for clinical outcomes [41–43]. In a retrospective analysis of the phase 3 study of single agent panitumumab in patients with chemotherapy refractory disease the hazard ratio for PFS improved to 0.38 (95% CI, 0.27–0.56) in the *RAS* wild-type group from 0.45 (95% CI, 0.34–0.59) in the *KRAS* exon 2 (codon 12/13) wild-type group [43]. The hazard ratio for PFS in the *RAS* mutant group was 0.98 (95% CI, 0.73–1.31), compared with a hazard ratio for PFS of 0.99 (95% CI, 0.73–1.36) in the original *KRAS* mutant group [3].

Results from the extended RAS analyses of randomized phase 3 studies in patients with mCRC treated with chemotherapy and anti-EGFR antibodies have provided the confirmatory evidence necessary to support refinement of the appropriate patient population by *RAS* mutation status. A summary of PFS and OS outcomes by *KRAS* exon 2 and *RAS* mutational status in key studies of panitumumab and cetuximab plus chemotherapy in mCRC is presented in Table 1.

The primary findings from a randomized study (PRIME) assessing the efficacy of first-line panitumumab plus FOLFOX versus FOLFOX alone showed, consistent with other studies, that *KRAS* exon 2 mutations were a predictor of lack of response to panitumumab in patients with mCRC [44]. In the study's primary analysis – which evaluated outcomes in the *KRAS* exon 2 wild-type population (*RAS* ascertainment, 90%), panitumumab significantly improved PFS (9.6 versus 8.0 months; hazard ratio, 0.80; 95% CI, 0.66–0.97; $P = 0.02$) and there was a trend toward improvement in overall survival (23.9 versus 19.7 months; hazard ratio, 0.83; 95% CI, 0.67–1.02; $P = 0.072$), although this was not significant. In a subsequent prospective-retrospective analysis (ie, the analysis plan was prespecified before the *RAS* testing but was performed after the primary analysis by *KRAS*), the treatment effect of panitumumab was evaluated by RAS mutations which include those from *KRAS* and *NRAS* exons 2, 3, and 4. In these patients

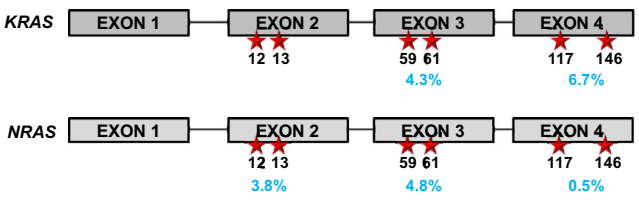

Fig. 2. Relative distribution of *KRAS* exon 3 and 4 and *NRAS* exon 2, 3, and 4 in patients with wild-type KRAS exon 2 tumors. Approximately 58% of patients have KRAS exon 2 wild-type tumors. Incidence of mutations is from Sorich MJ, Wiese MD, Rowland A, et al. Extended RAS mutations and anti-EGFR monoclonal antibody survival benefit in metastatic colorectal cancer: a meta-analysis of randomized controlled trials. Ann Oncol. 2015;26(1):13–21.

Table 1
Summary of PFS and OS outcomes by *KRAS* exon 2 and *RAS* mutational status in key studies of panitumumab and cetuximab in patients with metastatic colorectal cancer.

	KRAS exon 2 wild-type	Extended RAS ascertainment	*RAS* wild-type
PRIME [44] *(Panitumumab + FOLFOX4 vs FOLFOX4)*			
PFS, HR (95% CI)	0.80 (0.66–0.97)	90%	0.72 (0.58–0.90)
P	0.02		0.004
OS*, HR (95% CI)	0.83 (0.67–1.02)		0.78 (0.62–0.99)
P	0.07		0.04
Study 20050181 [23,50] *(Panitumumab + FOLFIRI vs FOLFIRI)*			
PFS, HR (95% CI)	0.73 (0.59–0.90)	85%	0.70 (0.54–0.91)
P	0.004		0.007
OS, HR (95% CI)	0.85 (0.70–1.04)		0.81 (0.63–1.03)
P	0.12		0.08
PEAK [45] *(Panitumumab + mFOLFOX6 vs Bevacizumab + mFOLFOX6)*			
PFS, HR (95% CI)	0.87 (0.65–1.17)	82%	0.65 (0.44–0.96)
P	0.353		0.029
OS, HR (95% CI)	0.62 (0.44–0.89)		0.63 (0.39–1.02)
P	0.009		0.058
OPUS [26,47] *(Cetuximab + FOLFOX4 vs FOLFOX4)*			
PFS, HR (95% CI)	0.57 (0.38–0.86)	75%	0.53 (0.27–1.04)
P	0.0064		0.062
OS, HR (95% CI)	0.86 (0.60–1.22)		0.94 (0.56–1.56)
P	0.39		0.80
CRYSTAL [46] *(Cetuximab + FOLFIRI vs FOLFIRI)*			
PFS, HR (95% CI)	0.70 (0.56–0.87)	69%	0.56 (0.41–0.76)
P	0.0012		0.0002
OS, HR (95% CI)	0.80 (0.67–0.95)		0.69 (0.54–0.88)
P	0.0093		0.0024
FIRE-3 [27] *(Cetuximab + FOLFIRI vs Bevacizumab + FOLFIRI)*			
PFS, HR (95% CI)	1.06 (0.88–1.26)	69%	0.93 (0.74–1.17)
P	0.55		0.54
OS, HR (95% CI)	0.77 (0.62–0.96)		0.70 (0.53–0.92)
P	0.017		0.01
CALGB/SWOG 80405 [49] *(Cetuximab + FOLFIRI or FOLFOX vs Bevacizumab + FOLFIRI or FOLFOX)*			
PFS, HR (95% CI)	1.04 (0.91–1.17)	55%	1.1 (0.9–1.3)
P	0.55		0.31
OS, HR (95% CI)	0.92 (0.78–1.09)		0.9 (0.7–1.1)
P	0.34		0.40

CRYSTAL = cetuximab combined with irinotecan in first-line therapy for metastatic colorectal cancer; FOLFIRI = fluorouracil, folinic acid, and irinotecan; FOLFOX4 = fluorouracil, leucovorin, and oxaliplatin; *KRAS* = the Kirsten rat sarcoma-2 virus oncogene; HR = hazard ratio; mCRC = metastatic colorectal cancer; mFOLFOX6 = modified fluorouracil, leucovorin, and oxaliplatin; MT = mutant type; NA = not available; *NRAS* = neuroblastoma *RAS* viral oncogene homolog; OS = overall survival; PEAK = panitumumab efficacy in combination with modified fluorouracil, leucovorin, and oxaliplatin (mFOLFOX6) against bevacizumab plus mFOLFOX6 in mCRC subjects with wild-type *KRAS* tumors; PFS = progression-free survival; PRIME = panitumumab randomized trial in combination with chemotherapy for metastatic colorectal cancer to determine efficacy; *RAS* = rat sarcoma-2 virus oncogene; WT = wild type.

* Primary analysis. With the exception of Study 20050181 (second-line treatment), all studies were first-line treatment.

without *RAS* mutations in their tumor, panitumumab plus FOLFOX4 versus FOLFOX4 alone was associated with significant improvements in PFS (10.1 versus 7.9 months; hazard ratio, 0.72; 95% CI, 0.58–0.90; $P = 0.004$) and OS (26.0 versus 20.2 months; hazard ratio, 0.78; 95% CI, 0.62–0.99; $P = 0.04$) [44]. In patients with wild-type *KRAS* exon 2 tumors but with other *RAS* mutations, panitumumab plus FOLFOX4 did not improve PFS (hazard ratio, 1.28; 95% CI, 0.79–2.07; $P = 0.33$) or OS (hazard ratio, 1.29; 95% CI, 0.79–2.10; $P = 0.31$). The results of interaction testing for the comparison of outcomes in these patients with those with *RAS* wild-type tumors was $P = 0.04$ for PFS and $P = 0.07$ for OS. These data represented the first demonstration in a phase 3 study (albeit a prospective-retrospective analysis) of the value of extended *RAS* analysis for anti-EGFR therapy.

RAS status was prospectively evaluated in PEAK, a randomized phase 2 study of panitumumab plus modified (m)FOLFOX6 versus bevacizumab plus mFOLFOX6 [45]. Eighty-two percent of patients who underwent extended *RAS* analysis had *KRAS* or *RAS* status [45]. In a prespecified extended *RAS* analysis, *RAS* mutations beyond *KRAS* exon 2 appeared to be predictive for the treatment effect of panitumumab on OS.

Results from retrospective analyses of studies evaluating treatment outcome with cetuximab in the first-line setting according to *RAS* mutational status have provided further data supporting validity of extended *RAS* analysis. The CRYSTAL study randomly assigned 1198 patients with EGFR-positive mCRC to cetuximab plus FOLFIRI or FOLFIRI alone [24]. When treatment outcomes were analyzed according to *RAS* mutation status, *KRAS* and *NRAS* mutational status was confirmed to be strongly predictive for the efficacy of cetuximab plus FOLFIRI: mutations in *KRAS* and *NRAS* were associated with lack of clinical benefit with the addition of cetuximab to FOLFIRI [46].

Similarly, in the OPUS study of cetuximab plus FOLFOX4 versus FOLFOX4 alone, when *KRAS* exon 2 wild-type tumors were screened for additional *RAS* mutations, the presence of any activating *RAS* mutation was predictive of lack of benefit from the addition of cetuximab [26,47]. When the effects of mutations within *KRAS and NRAS;* exon 2, 3, and 4; *BRAF* V600E; *PIK3CA;* exon 9 and 20; and *AKT* were examined in patients in the FIRE-3 study of cetuximab plus FOLFIRI versus FOLFIRI alone, the exclusion of patients with any *RAS* mutation was predictive of improved benefit from cetuximab [48].

Results from a retrospective extended *RAS* analysis of the CALGB/SWOG 80405 study were recently reported [49]. The study randomized patients with wild-type *KRAS* exon 2 mCRC to receive either cetuximab or bevacizumab plus chemotherapy (FOLFOX or

FOLFIRI per physician's choice). In the primary analysis, the hazard ratio for OS was 0.92 (0.78–1.09; $P = 0.34$) for cetuximab versus bevacizumab. At the time of presentation, 55% of patients with *KRAS* exon 2 wild-type tumors were evaluable for additional mutations in *KRAS* and *NRAS* status and, among this *RAS*-evaluable group, the hazard ratio for OS was 0.9 (0.8–1.1; $P = 0.49$). Among patients with *RAS* wild-type tumors, the hazard ratio for OS was 0.9 (0.7–1.1; $P = 0.40$) and therefore, selection of patients using extended *RAS* analysis did not improve outcomes. Definitive conclusions from this study will require maturation of the data with increased sample ascertainment for extended *RAS* analysis, evaluation of subsequent treatments, and distribution of RAS mutations across arms.

In the second-line setting, improvements in predictive value were reported in a prospective-retrospective study evaluating *RAS* mutations in patients receiving panitumumab plus FOLFIRI versus FOLFIRI alone (the 20050181 study) [50]. A total of 18% of patients with wild-type *KRAS* exon 2 tumors had additional *RAS* mutations (*KRAS* exons 3, and 4; *NRAS* exons 2, 3, and 4) [50]. Patients with mutated *RAS* tumors were unlikely to benefit from panitumumab, similar to the findings in patients with *KRAS* exon 2 mutations. *BRAF* mutations in the absence of *RAS* mutations appeared to be associated with poorer OS regardless of treatment arm [50].

A systematic review and meta-analysis of nine randomized controlled trials of anti-EGFR therapies for mCRC that assessed the predictive value of *RAS* mutations beyond *KRAS* exon 2 has recently been published [39]. Collectively, the available data supported the value of extended *RAS* analysis to guide treatment decisions for anti-EGFR therapy and challenges the ability of *KRAS* exon 2 biomarker testing to adequately identify those patients with mCRC most likely to benefit from anti-EGFR therapy and to protect patients unlikely to respond from unnecessary treatment-related toxicity [39]. Approximately 20% of the 5948 patients with wild-type *KRAS* exon 2 tumors evaluated in the studies had at least one *RAS* mutation other than *KRAS* exon 2 (*KRAS* exon 3 or 4 or *NRAS* 2, 3, or 4). The meta-analysis found that panitumumab and cetuximab both significantly improved hazard ratios for PFS (0.60; 95% CI, 0.48–0.76; $P < 0.001$), OS (0.72; 95% CI, 0.56–0.92; $P = 0.008$), and treatment effect on response rate (odds ratio, 3.71; 95% CI, 2.16–6.36) in patients with *RAS* wild-type tumors compared with those patients with any new *RAS* mutation. Meanwhile, there was no difference in PFS or OS benefit between patients with tumors with *KRAS* exon 2 mutations and those with other *RAS* mutations. The results were found to be consistent independent of anti-EGFR agent, lines of therapy, and concomitant chemotherapy agent [39]. It is also important to note that a variety of different analytical techniques were used to evaluate *RAS* mutational status in these studies, including bidirectional Sanger sequencing/WAVE-based sequencing [44,45,50], BEAMing [46,47], and pyrosequencing [48], but that this did not influence the predictive value for *RAS* mutations.

Acquisition of RAS mutations as a mechanism of resistance

Some patients develop *RAS* mutations during therapy with anti-EGFR monoclonal antibodies [51–53]. For example, among 24 *KRAS* wild-type patients who received treatment with panitumumab, 9 developed *KRAS* mutations during therapy, typically within 6 months of initiation of therapy [52]. Development of such mutations during therapy may be a result of expansion of *KRAS*-mutant subclones present before initiation of therapy and may represent a potential mechanism of resistance to anti-EGFR therapy [52]. Preclinical studies have suggested that combined anti-EGFR and MEK inhibition might overcome such resistance [54], and this hypothesis is being evaluated in ongoing clinical studies (ClinicalTrials gov identifier, NCT01750918).

Prognostic/predictive value of *BRAF* mutations

It is clear that a *BRAF* mutation is prognostic of a poor outcome irrespective of treatment [44,50,55–57]. In the PRIME study, for instance, median OS among patients with *BRAF* mutations in the FOLFOX arm was 9.2 months compared with 20.9 months among patients without either *RAS* or *BRAF* mutations. Whether mutations in *BRAF* might also have predictive value, however, has been controversial. While some studies have suggested that patients with *BRAF* mutations may be less likely to respond to anti-EGFR therapy [41,58] others have not. In the open-label phase 3 study of panitumumab plus BSC compared to BSC alone, the hazard ratio for PFS was 0.34 in patients with *BRAF*-mutant tumors and 0.37 in patients with *BRAF* wild-type tumors [42]. In the PRIME study, the presence of *BRAF* mutations did not appear to be associated with lack of treatment effect to panitumumab [44]. Similarly, *BRAF* mutations were not predictive for the treatment effect of cetuximab in the CRYSTAL study, but were associated with poor prognosis [59]. Careful consideration should be given to enrollment of patients with *BRAF* mutant tumors into clinical trials to address this high unmet need. It is encouraging to note that early phase prospective studies are currently evaluating novel treatment combinations of anti-EGFR monoclonal antibodies and BRAF inhibitors in patients with *BRAF* mutant mCRC (ClinicalTrials gov identifiers, NCT02164916; NCT01750918).

Discussion

It has become clear from emerging data that *KRAS* exon 2 (codon 12/13) mutation status alone is not sufficient to fully explain heterogeneity of treatment response to anti-EGFR therapy in mCRC, and that molecular testing for biomarkers beyond *KRAS* codons 12 and 13 has additional predictive value. There is now strong evidence that extended *RAS* analysis (*KRAS* exons 2, 3, and 4 and *NRAS* exons 2, 3, and 4) is appropriate to enhance identification of patients most likely to benefit from anti-EGFR monoclonal antibody therapy and to avoid treatment unlikely to be of value to the patient and with the potential for treatment-related toxicity. Although there has been evaluation of other potential predictive biomarkers for anti-EGFR therapy in patients with mCRC (including EGFR ligands [60,61], *PTEN* [42,62,63], *PIK3CA* [42,62,64], and, most notably, *BRAF* [42,44,62]), *RAS* mutations represent the only clinically validated biomarkers.

These findings are now being incorporated into the latest clinical practice guidelines. The European Society for Medical Oncology (ESMO), NCCN, the European Society of Pathology, and the Association of Clinical Pathologists Molecular Pathology and Diagnostics Group in the United Kingdom now strongly recommend determination of *KRAS*/*NRAS* gene status prior to the initiation of treatment, including whenever possible non-*KRAS* exon 2 mutation status, of tumor tissue in patients with mCRC [29,30,65,66]; the American Society for Clinical Pathology, College of American Pathologists, and Association for Molecular Pathology are currently developing revised guidelines. The European Medicines Agency's summaries of product characteristics for cetuximab and panitumumab require evidence of wild-type *RAS* (*KRAS*/*NRAS*) status before treatment [67,68].

However, barriers to widespread clinical use remain and it is unclear how many eligible patient tumors are undergoing extended *RAS* analysis. The difference between *KRAS* exon 2 testing ("*KRAS*") and extended *RAS* analysis ("*RAS*") is not yet widely recognized by oncologists and pathologists. The FDA-approved

TheraScreen assay only evaluates mutations in KRAS exon 2 (codons 12/13) [35,36]. There is currently no standardized extended *RAS* testing and the large studies described above used a variety of methods including bidirectional Sanger sequencing and WAVE-based Surveyor Scan Kits (PRIME) [44], pyrosequencing (FIRE-3) [27], and BEAMing (CRYSTAL) [46]. Other groups have employed targeted multigene next-generation sequencing panels [42], and some are even performing whole exome analysis [69]. While the fraction of patients with non-exon 2 *KRAS* and *NRAS* mutations appears similar between studies, the sensitivity, neoplastic cell content and cut-offs for mutation calling vary. It is unknown whether these analytical methods yield clinically significant differences. Analyses performed to date have used relatively conservative neoplastic cell content and mutant DNA sensitivity levels of 5% to 10%, but some recent evidence has suggested that lower levels of *RAS* mutation might also predict poor response to anti-EGFR therapy [70]. Such analyses will require closer attention to microdissection of tissue to account for the high variability of stromal infiltrates, and harmonization of methods for meta-analyses.

Conclusion

The discovery that extended *RAS* analysis identifies a group of patients with advanced colorectal cancer who do not benefit from anti-EGFR therapy has changed the practice of oncology and has the potential to spare patients from exposure to ineffective therapy. In the near future, it is important for the oncology community to validate extended *RAS* analysis assays and make certain that patients who are candidates for anti-EGFR therapy undergo appropriate testing and treatment.

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All authors participated in literature research and drafting the manuscript. All authors have approved the final article.

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