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The State of the Art in Colorectal Cancer Molecular Biomarker Testing

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Abstract

The number of molecular biomarkers to inform treatment decisions in patients with metastatic colorectal cancer (mCRC) continues to expand and with it the methodologies that can be employed to evaluate these biomarkers. Beyond standard diagnostic and prognostic biomarkers, such as those used for Lynch Syndrome, mutations in *KRAS* exon 2 are well established as predictive for lack of response to the anti-epidermal growth factor receptor therapies panitumumab and cetuximab. Recent studies have extended these findings by demonstrating that mutations in *KRAS* exons 3 and 4 and in *NRAS* exons 2, 3, and 4 (with all *KRAS* and *NRAS* mutations collectively referred to as *RAS*) are also predictive for treatment outcomes among patients with mCRC receiving panitumumab and cetuximab in combination with chemotherapy or as monotherapy. Consequently, evaluation of these additional loci has been incorporated into current clinical guidelines, and pathologists will need to develop testing procedures and algorithms to reliably and rapidly evaluate *RAS* status. With the increased number of mutations that must be examined to evaluate the status of *RAS* and other emerging biomarkers, next-generation sequencing technologies are likely to become increasingly important in mCRC testing. This review describes new considerations for pathologists that have arisen as a consequence of the incorporation of additional biomarker testing into clinical practice for mCRC.

INTRODUCTION

New data derived from molecular biomarker analyses of pathology specimens from clinical trials are enabling the delivery of more personalized medicine for patients with metastatic colorectal cancer (mCRC). This article describes the currently used diagnostic molecular biomarkers (including testing for the identification of Lynch syndrome versus sporadic CRC) and then focus on new testing guidelines for predictive biomarkers in all patients with

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mCRC. These guidelines evolved from a hypothesis that mutations in *KRAS* exon 2 are predictive for outcomes with the anti-epidermal growth factor receptor (EGFR) therapies panitumumab (a recombinant fully human anti-EGFR monoclonal antibody¹) and cetuximab (a recombinant human/mouse chimeric anti-EGFR monoclonal antibody²). Confirmation of the *KRAS* biomarker hypothesis led to additional studies that resulted in the expanded *RAS* biomarker hypothesis – which postulated that any activating mutation in *KRAS* or *NRAS* exons 2, 3, and 4—not just mutations in *KRAS* exon 2—could predict lack of response to anti-EGFR monoclonal antibody therapy. Current practice guidelines from the National Comprehensive Cancer Network (NCCN) and the European Society of Pathology (ESP) strongly recommend biomarker analysis in the form of *KRAS/NRAS* genotyping of tumor tissue in patients with mCRC.^{3,4} The Association of Clinical Pathologists (ACP) Molecular Pathology and Diagnostics Group has provided guidance for *RAS* testing in the United Kingdom,⁵ and the American Society for Clinical Pathology (ASCP), College of American Pathologists (CAP), Association for Molecular Pathology (AMP), and the American Society of Clinical Oncology (ASCO) are developing guidelines under the working title “Guideline on the Evaluation of Molecular Markers for Colorectal Cancer.”

The objectives of this article are to discuss molecular tests that distinguish Lynch syndrome from sporadic CRC, to review existing biomarker testing in mCRC and discuss the importance of early *RAS* testing and appropriate procedures for validation, quality control and proficiency testing. The evidence supporting the importance of these new biomarkers will be presented as a review of key clinical data from trials that have investigated the *RAS* biomarker hypothesis, in particular the PRIME (Panitumumab Randomized Trial in Combination With Chemotherapy for Metastatic Colorectal Cancer to Determine Efficacy) Study, which evaluated first-line panitumumab therapy in patients with mCRC.⁶ In addition, other practical considerations for pathologists such as the role of next generation sequencing (NGS) and new biomarkers in mCRC and their growing applications in patient selection for genomically guided clinical trials will be reviewed.

MOLECULAR TESTING DIFFERENTIATES LYNCH SYNDROME FROM SPORADIC COLORECTAL CANCER

An important recent advance is universal testing, in most major institutions, of all colorectal cancer specimens to identify Lynch Syndrome (also known as hereditary non-polyposis colorectal cancer [HNPCC]).⁷ Lynch syndrome, which accounts for 2% to 4% of all colon cancers, is transmitted as an autosomal dominant disorder that is caused by a germline mutation in one of several DNA mismatch repair (MMR) genes.³ Inactivation of one of the MMR genes by germline mutations combined with inactivation of the second allele by mutation, loss of heterozygosity or promoter hypermethylation results in defective MMR.⁸ Screening for defective MMR genes is performed by immunohistochemistry (for MSH2, MSH6, MLH1, and PMS2) and/or identification of the genetic changes associated with a loss of the mismatch repair machinery (ie, microsatellite instability).³ Microsatellite instability can be evaluated by multiplex PCR and capillary electrophoresis using nearly monomorphic mononucleotide repeat markers (BAT-25, BAT-26, MONO-27, NR-21, and NR-24) and two highly polymorphic pentanucleotide repeat markers (Penta C and Penta D)

that are used as specimen identifiers.⁹ For the five nucleotide markers tested in this assay, the results are interpreted as MSI-high (MSI-H) if 2 markers are unstable and MSI low (MSI-L) if one marker is unstable, and microsatellite stable (MSS) if no markers are unstable.¹⁰ MSI-H status is observed in Lynch syndrome but is also detected in up to 15% of sporadic CRC, typically due to hypermethylation of *MLH1* gene.⁸ In *MLH1* and *PMS2* protein negative CRC, most sporadic CRC can be differentiated from Lynch syndrome by the presence of the *BRAF*V600E mutation and/or hypermethylation of the *MLH1* promoter. *BRAF*V600E and *MLH1* hypermethylation are highly correlated and rarely found in Lynch syndrome.^{8,11} In the *BRAF*V600E-negative (wild-type), *MLH1* promoter unmethylated subset, evaluation of MMR germline mutation analysis for *MLH1* and *PMS2* are useful. Non-Lynch syndrome CRC are *MLH1* hypermethylated and Lynch syndrome CRC display a germline mutation in *MLH1* or *PMS2*. Note that constitutional *MLH1* germline hypermethylation has rarely been reported in early onset CRC with normal germline testing.

Before germline testing, appropriate pretest counseling should be completed by an individual with expertise in hereditary clinical genetics.¹² Identification of a germline mutation in an MMR gene based on loss of the corresponding MMR protein varies depending on which protein is absent. In a series of unselected colorectal cancer cases, if *MLH1* and/or *PMS2* were absent, causative *MLH1* germline mutations were found in 6.1% of cases, reflecting that most of these cancers are sporadic due to *MLH1* promoter hypermethylation.¹³ If *MLH1* was absent and there was no *MLH1* promoter methylation, 33.3% were found to have a germline *MLH1* mutation.¹³ If *PMS2* alone was absent on IHC, 55.6% were found to have a *PMS2* germline mutation.¹³ If *MSH2* and/or *MSH6* proteins were absent, 66.7% were found to have a germline mutation.¹³ If *MSH2* alone was absent, *EPCAM* germline deletion testing was useful in distinguishing *MSH2* mutated versus *EPCAM*-mutated Lynch syndrome (LS-*EPCAM*). If *MSH6* alone was absent, only 23.5% were found to have a germline *MSH6* mutation.¹³ The low detection rate of *MSH6* germline mutations may indicate there are other unknown somatic mechanisms contributing to *MSH6* loss.

Recently, there has been interest in a rare subgroup of patients with CRC with loss of MMR protein expression and/or MSI-H, absence of hypermethylation of *MLH1*, yet undetectable germline MMR mutations or *EPCAM* deletions. In a recent study, 97% of MMR deficient-germline testing negative cases (31/32), were explained by either two somatic MMR mutations (22/32), loss of heterozygosity of one allele with a somatic MMR mutation likely on the other allele (3/32), or false positive initial tumor testing (6/32).¹⁴ Somatic MMR testing on tumor is clinically available.

The therapeutic implication of detecting MSI-H CRC patients is important as they are not as responsive to 5FU chemotherapy as are sporadic CRC. In addition, all CRC should be tested for UGT1A1 sensitivity for irinotecan, as described in the irinotecan prescribing information.¹⁵ UGT1A1 mutation patients heterozygous or homozygous for *28 polymorphism have increased sensitivity to Irinotecan and may develop irreversible and fatal neutropenias.

Testing for Lynch syndrome is summarized in Figure 1. Other algorithms for such testing have been described elsewhere.^{12,16} In situations where an MMR germline mutation is likely or found, consultation with a genetics professional or genetic counselor knowledgeable about Lynch syndrome can help implement an appropriate surveillance program for the proband and identify other potential carriers in the family appropriate for counseling and testing.

Studies using IHC of MSH2, MLH1, MSH6, and PMS2 have shown it has a predictive value virtually equivalent to MSI testing. IHC has the added value of PMS2 and MSH6 detection (which may be missed by PCR testing for MSI), is readily available, inexpensive, and identifies the affected gene.¹³ Consequently, IHC has evolved as a preferred first-line screening tool for identifying Lynch syndrome in many laboratories.

KRAS EXON 2 STATUS AS A BIOMARKER FOR ANTI-EGFR THERAPY

Testing for *KRAS* exon 2 mutations, presence of which has been shown to limit response to anti-EGFR therapy, has been used in routine clinical practice since 2008, and has been the cornerstone of personalized medicine approaches that guide appropriate use of panitumumab and cetuximab therapy in stage IV CRC.¹⁷ Members of the *RAS* family of small GTPases play an important signal transduction role in the EGFR signaling pathway. Importantly, mutations in these enzymes (commonly occurring at specific codons in exons 2, 3, and 4¹⁸) result in constitutive activation of the enzyme, which results in persistent signaling in the absence of ligand binding to the EGFR (Figure 2).¹⁹ Notably, different *RAS* enzymes appear to play different roles in oncogenic signaling. Whereas *KRAS* mutations result in hyperproliferation of CRC cells, *NRAS* mutations appear to promote CRC by suppressing inflammatory apoptotic signals.²⁰ The signaling pathways of the *RAS* family members also appear to differ: *NRAS* binds and signals through RAF-1 and STAT3 whereas *KRAS* and *HRAS* do not.²⁰

The role of *RAS* proteins in EGFR signaling suggested that mutations in these enzymes might result in diminished response to EGFR inhibition. In 2008, published data from prospective-retrospective analyses of two large, randomized, controlled phase 3 studies (one evaluating panitumumab²¹ and one evaluating cetuximab,²² each versus best supportive care [BSC] in patients who were refractory to chemotherapy) confirmed the hypothesis that the presence of one of the seven most frequently occurring mutations in *KRAS* exon 2, codons 12 or 13, was predictive of lack of response to anti-EGFR monoclonal antibody monotherapy. Some early studies suggested that there might be differences in the predictive value of some *KRAS* exon 2 mutations. Specifically, there was a suggestion in some retrospective studies that *KRAS*G13D mutations might be associated with improved outcomes during anti-EGFR therapy compared with other *KRAS* codon 12/13 mutations.²³ However, a subsequent meta-analysis of results from studies evaluating panitumumab did not support this hypothesis, demonstrating a lack of benefit for panitumumab therapy when combined with FOLFOX and FOLFIRI chemotherapy or as monotherapy in patients with any mutation in *KRAS* exon 2.²⁴

Based upon the lack of benefit of anti-EGFR monoclonal antibody therapies in patients with mCRC with *KRAS* mutations in exon 2 identified in these studies, the NCCN issued updated clinical practice guidelines for colon cancer,²⁵ and the American Society of Clinical Oncology (ASCO) published a provisional clinical opinion recommending *KRAS* exon 2 testing for patients with mCRC who were candidates for anti-EGFR antibody therapy and not recommending anti-EGFR antibody therapy if a mutation was detected.²⁶ CAP issued a Perspectives on Emerging Technology report describing acceptable sample and assay types that could be employed to conduct such testing,²⁷ and in July 2009, the US FDA implemented class labeling changes for cetuximab and panitumumab to include the results of the clinical trial analyses and statements that retrospective subset analyses of mCRC trials had not shown a treatment benefit for patients with *KRAS* mutations in exon 2, that use was not recommended for the treatment of colon cancer with these mutations, and that mutant *KRAS* activity appears independent of EGFR regulation.²⁸ Numerous pathology review articles were published in response to these changes in the recommended approach to therapy.^{17,29-32}

A companion diagnostic—the *therascreen*[®] *KRAS* RGQ PCR Kit (QIAGEN N.V., Venlo, the Netherlands)—was also approved to identify appropriate patients for treatment. This validated, allele-specific PCR assay determines the presence of seven mutations in the *KRAS* oncogene exon 2 (codons 12 and 13) in CRC patients.³³ If mutations are absent, treatment with panitumumab or cetuximab is indicated.^{34,35}

EXTENDED RAS ANALYSIS AS A BIOMARKER FOR ANTI-EGFR THERAPY

Although *KRAS* exon 2 testing better defined the patient population most likely to benefit from anti-EGFR therapy, it was apparent that room for improvement remained in methods of patient selection, because not all patients with *KRAS* exon 2 wild-type tumors respond to panitumumab and cetuximab, which suggested the possible presence of other mutations prohibiting response.³⁶ To further define the responding population among patients with *KRAS* exon 2 wild-type tumors, a multigene sequencing project was undertaken with the remaining banked tumor specimens from the panitumumab monotherapy study to evaluate mutations within genes that encoded components of the EGFR signaling pathway. Initially, mutations in nine genes—*KRAS*, *NRAS*, *AKT*, *BRAF*, *EGFR*, *PIK3CA*, *PTEN*, *CTNNB1* and *TP53*—were sequenced using 454 pyrosequencing technology (a technique that couples bioluminescence and enzyme reactions to monitor pyrophosphate release resulting from nucleotide incorporation in real-time³⁷; Roche 454 Life Sciences).^{36,37} Despite the small numbers of patients with many of the individual mutations, the results from this analysis were consistent with the hypothesis that activating *KRAS* and *NRAS* mutations are negative predictors of response to panitumumab treatment, but the other genes interrogated were not.³⁶ Panitumumab treatment was associated with significantly longer PFS among *KRAS* wild-type (codons 12, 13, or 61) patients (HR, 0.39 [95% CI, 0.28–0.56]), as well as *NRAS* wild-type patients (HR, 0.39 [95% CI, 0.27–0.56]), but not among *NRAS* mutant patients (HR, 1.94 [95% CI, 0.44–8.44]).³⁶ Similar results were obtained when the analysis was updated to interrogate mutations in *KRAS* exon 4 and *NRAS* exon 4.³⁸

To further test the hypothesis derived from the multigene sequence analysis study that activating *KRAS* and *NRAS* mutations are negative predictors of response to panitumumab, wild-type *KRAS* exon 2 tumor specimens from PRIME were tested for mutations in *KRAS* exons 3 and 4; *NRAS* exons 2, 3, and 4; and *BRAF* exon 15, and select analyses were repeated using the newly defined subsets based on the additional *RAS* and *BRAF* testing results.⁶ Mutation analysis was conducted using the reference method of bidirectional Sanger sequencing on DNA extracted from formalin-fixed, paraffin embedded tumor specimens with at least 50% neoplastic cell content (or macrodissected if not). Results from this prospective-retrospective analysis using the primary analysis data suggested that additional activating mutations in *RAS* beyond *KRAS* exon 2 were negatively predictive for outcomes to panitumumab + FOLFOX-4 compared with FOLFOX-4 alone (Table 1). Improvements were observed in median PFS (HR, 0.72; 95% CI, 0.58–0.90) and in median overall survival (OS; HR, 0.78; 95% CI, 0.62–0.99) among *RAS* wild-type patients, but not among *RAS* mutant patients (PFS, HR, 1.31; 95% CI, 1.07–1.60; OS, HR, 1.25; 95% CI, 1.02–1.55), including those with newly identified *RAS* mutations beyond *KRAS* exon 2 (Figure 3).

As summarized in Table 1, a number of further studies have provided supporting data for the *RAS* hypothesis in patients with mCRC.^{39–46} In general, these studies have shown improvements in outcomes with both panitumumab and cetuximab as first-line therapy and second-line therapy when patients are selected using extended *RAS* analysis (*KRAS* exons 2, 3, and 4; *NRAS* exons 2, 3, and 4) compared with *KRAS* exon 2 analysis.

The predictive value of *RAS* mutations beyond *KRAS* exon 2 has also been supported by a recent meta-analysis which evaluated *RAS* analysis in randomized clinical trials (5948 patients overall).¹⁸ The meta-analysis found that PFS and OS were both significantly improved among wild-type *RAS* patients receiving anti-EGFR therapy compared with outcomes of those patients with any *RAS* mutation.

The studies described above employed a variety of different techniques to evaluate *RAS* mutational status. Somatic mutation testing to determine *RAS* mutation status was evaluated using bidirectional Sanger dideoxynucleotide sequencing and WAVE[®] methods (which combine a proprietary nuclease and electrophoresis) in the PRIME⁶ and PEAK³⁹ clinical trials with panitumumab, and using pyrosequencing in the FIRE-3 trial⁴⁰ or via BEAMing (an allele-specific hybridization method for detection of defined mutations amplified by PCR^{47,48}) in the OPUS⁴² and CRYSTAL⁴⁵ trials with cetuximab. Other techniques being developed for *RAS* biomarker profiling to detect genetic variants, single-nucleotide polymorphisms, and genetic mutations include allele-specific polymerase chain reaction (AS-PCR) and the use of biomarker panels (which simultaneously evaluate multiple genes).

Until an FDA approved test becomes available it is incumbent on individual laboratories to implement in-house laboratory developed tests (LDT) to address extended testing for mutations in *KRAS* exons 3 and 4 and *NRAS* exons 2, 3, and 4 beyond those detected by the FDA-approved *therascreen*[®] *KRAS* assay. Various methods can be used, such as multiplex PCR, AS-PCR, or Sanger sequencing. Alternatively, targeted NGS panels for *KRAS* and *NRAS* may also be considered clinically timely and cost-effective for high volume

laboratories. Sanger sensitivity is less than that for PCR (20% vs 5%, on average and therefore requires a higher neoplastic cell enrichment through microdissection to remove non-neoplastic tissue) but has the advantage of low cost and is still considered the reference method for molecular testing. Pathologists play a key role in ensuring the presence of tumor, its percentage relative to normal tissue, and enrichment (macrodissection) for optimal sensitivity and limit of detection. A limit of detection of at least 5% of mutant/wild-type *RAS* for PCR tests is usually acceptable.^{34,35}

CURRENT TREATMENT GUIDELINES AND PROFICIENCY TESTING

Among the broad array of potential mCRC biomarkers, there is only consensus for testing to guide anti-EGFR therapy for *KRAS* and *NRAS*. As with the confirmation of the predictive value of *KRAS* testing the generation of data to support extended *RAS* analysis to better guide anti-EGFR therapy has led to the development of new testing recommendations by various organizations. In 2013, the Evaluation of Genomic Applications in Practice and Prevention Working Group (EWG) published a recommendation statement regarding pharmacogenomic testing to inform anti-EGFR antibody therapy in mCRC, noting that “there is convincing evidence to recommend clinical use of *KRAS* mutation analysis to determine which patients are *KRAS* mutation positive and therefore unlikely to benefit from these agents before initiation of therapy.” Also in 2013, the European Medicines Agency stated that determination of wild-type *RAS* status (*KRAS* exon 2, 3, 4 and *NRAS* exon 2, 3, 4) is required before initiating treatment with panitumumab or cetuximab.⁴ Along with the 2015 NCCN recommendations for *KRAS/NRAS* genotyping for patients with mCRC, the NCCN Colon/Rectal Cancer Panel recommends that, whenever possible, non-exon 2 *KRAS* mutation status and *NRAS* mutation status should be determined, given that mutations in those areas are also predictive of lack of response.³

In addition, the EWG found “insufficient evidence to recommend for or against *BRAF* V600E testing” for making treatment decisions.⁴⁹ The 2015 NCCN guidelines also indicate that, although *BRAF* mutation appears to confer a poorer prognosis, there are insufficient data to guide the use of anti-EGFR therapy in the first-line setting with chemotherapy based on *BRAF* V600E mutation status. However, it is also mentioned that limited available data suggest lack of antitumor activity from anti-EGFR monoclonal antibodies in the presence of V600E mutation when used after a patient has progressed on first-line therapy.

Recent published guidelines from the ESP also indicate molecular testing of *RAS* status is a prerequisite for anti-EGFR treatment.⁵⁰ Additionally, ACP has provided guidance recommending *RAS* testing in the United Kingdom.⁵ Currently, the ASCP, CAP, AMP, and ASCO are collaborating to develop potential future guidance regarding molecular markers and colorectal cancer.

The ESP has also established an External Quality Assessment program requiring the testing of biomarker mutations in colorectal cancer patients. This initiative is necessary to ensure standardization of performance among laboratories using different methodologies, accuracy of results, and acceptable thresholds of limit of detection of *RAS* mutations to ensure optimal treatment efficacy. At Cedars-Sinai Medical Center, if a *RAS* mutation is detected, a

50-gene NGS cancer-associated panel may then be evaluated, at the discretion of the oncologist.

POTENTIAL FUTURE BIOMARKER TESTING: THE RAPIDLY GROWING ROLE OF NEXT-GENERATION SEQUENCING FOR GENOMICALLY GUIDED THERAPY IN METASTATIC COLORECTAL CANCER

Large-scale genomic studies such as The Cancer Genome Atlas project have led to many new discoveries regarding genetic alterations in colon cancers. In addition to *KRAS* and *NRAS*, recurrent mutations in many oncogenes and tumor suppressor genes such as *BRAF*, *EGFR*, *PIK3CA*, *AKT1*, *ERBB2*, *ERBB4*, *PTEN*, *MEK1*, *ALK*, *DDR2*, *CTNNB1*, *MET*, *TP53*, *SMAD4*, *FBXW7*, *NOTCH1*, *FGFR1*, *FGFR2*, *FGFR3*, and others have been described.⁵¹⁻⁶⁰ Studies of these oncogenes have also detected mutations that do not occur with high frequency, with these infrequently mutated genes constituting the “long tail” of driver genes, which reflects the heterogeneous nature of the mechanisms for pathway activation.^{61,62}

Notably, many of the oncogenes identified in mCRC are part of the EGFR signaling network (eg, *BRAF*, *PIK3CA*, *PTEN*; Figure 4).⁶³ However, at present none have been demonstrated to be predictive biomarkers for anti-EGFR therapy. For example, current NCCN guidelines recognize that a specific mutation in *BRAF* (*BRAFV600E*) is associated with poor prognoses in patients with wild-type *KRAS/NRAS* and non-MSI-H CRC; however, the guidelines do not require *BRAF* genotyping because of insufficient data regarding its value as a predictive biomarker of treatment benefit.³ In contrast to *RAS* analyses, when the influence of *BRAF* mutation status on outcomes with anti-EGFR therapies has been analyzed, results have been inconclusive. In PRIME, *BRAF* mutation status was prognostic for poor performance in *KRAS* exon 2 wild-type patients regardless of treatment, and it did not have additional predictive value for the effect of panitumumab therapy.⁶ In addition, a meta-analysis of 21 trials in mCRC concluded that *BRAF* mutations were associated with poor prognosis, particularly in *KRAS* WT patients.⁶⁴ Although studies may yet identify additional mutations that can predict response to therapy in mCRC, evaluation of such mutations should not be used to guide therapy at present.

Therapies are available that target some of these mutations or their signaling pathways and an exponentially growing number of other targeted therapies are in development and/or in clinical trials across the country and internationally.⁶⁵⁻⁶⁹ A paradigm shift is already happening in management of patients with cancer that may complement, or sometimes trump, conventional cancer therapies (ie, chemotherapy and radiation therapy) by identifying tumor-specific driver molecular mutations in an individual tumor and matching them with a companion therapy.⁷⁰ The potential success of such targeted therapies in clinical trial programs suggests that pathologists will need to rapidly develop more proactive testing algorithms in concert with the emergence of new targeted drugs and the increase in genomically guided clinical trials. In addition to single gene testing (*KRAS*, *NRAS*) by PCR or Sanger, NGS is likely to become increasingly important in mCRC testing as cost

continues to decrease, analysis softwares become more readily available and user-friendly, and more companion drugs to novel genetic targets enter the clinical/clinical trial arena.

NEXT-GENERATION SEQUENCING TEST CONSIDERATIONS AND CONTROL MATERIALS IN METASTATIC COLORECTAL CANCER

Next-generation sequencing is rapidly becoming a primary vehicle for biomarker testing. The availability of robust sequencing technology and the decrease in cost of sequencing in the last decade now enable sequencing of an array of genes, and even the whole exome/genome, at a reasonable cost. According to data collected from the National Human Genome Research Institute Genome Sequencing Program, the cost per megabase of procuring high-quality DNA sequencing has decreased from approximately \$1600 to \$0.05 in the last ten years.⁷¹ Indeed, the cost of sequencing an *entire genome* has decreased 5800-fold, from approximately \$28.8 million to just \$5000.⁷¹ Although advancements in NGS have been made, the challenge of finding the most cost-effective way to deliver clinically useful solutions remains. This challenge can partially be addressed by infrastructure sharing and outsourcing, but ultimately, adoption of NGS will require the clear demonstration of clinical utility.⁷²

The College of American Pathologist (CAP) developed 18 NGS-specific requirements included within CAP's molecular pathology checklist to facilitate the use of NGS technology for the clinical testing of inherited disorders.⁷³ The NGS-specified items approach the analytic "wet bench" process and the bioinformatics analyses ("dry bench") as separate entities requiring different considerations, and encompass new standards for documentation, validation, quality assurance, confirmatory testing, exception logs, monitoring of upgrades, variant interpretation and reporting, incidental findings, data storage, version traceability, and data transfer confidentiality. The goal of CAP when devising the checklist was to develop requirements for accreditation for NGS that could be used across multiple testing areas (eg, inherited diseases, molecular oncology, infectious diseases).⁷³ Similar standards were published in 2013 by the American College of Medical Genetics and Genomics, primarily as an educational resource for clinical laboratory geneticists.⁷⁴ These guidelines focus primarily on the three main levels of analysis (ie, disease-targeted gene panels, exome sequencing, and genome sequencing) and discuss the three main components of NGS: sample preparation, sequencing, and data analysis.⁷⁴ These guidelines were updated in 2015 to include standards for the classification and interpretation of sequence variants.⁷⁵ A third set of standards were released in December 2014 by EuroGentest that dictate procedures for NGS testing pertaining to diagnostic and/or clinical utility, informed consent to patients and clinicians, test validation, results reporting, and highlights the differences between research and diagnostic testing.⁷⁶

With the exception of the EuroGentest guidelines which state the basic principles may be applied to somatic testing for cancer (with the inclusion of additional quality parameters such as threshold for detection),⁷⁶ the aforementioned guidelines are intended for inherited disorders only. Further expansion on topics such as preanalytic sample assessment and the effect on quality (eg, specimen type, collection and handling), requirements for sample

processing and acceptability (eg, extraction methods, complexity and usability of DNA for amplification), metrics for sample types (eg, sensitivity controls), and interpretation are needed for somatic variant testing. These limitations are addressed in part by standards released by the New York State Department of Health;⁷⁷ however the field is evolving and other guidelines are in preparation.

In addition to selecting target genes, NGS (and other methods) testing and interpretation needs to consider several important factors. The variant allele frequency, which represents the mutant/wild-type ratio or the sensitive/resistant fraction of the tumor, is affected by the ratio of neoplastic/tumor DNA to normal DNA (which can be enriched by microdissection), technical factors related to NGS testing, and the heterogeneity of the tumor. Most NGS strategies involve a comparison of mutations to determine variant detection concordance, followed by confirmation, if required per laboratory policy, via another orthogonal method (most commonly PCR or Sanger sequencing). However, the degree of neoplastic cells to normal cells and/or tumor heterogeneity can result in mutant allelic burdens below 10% to 15%, which makes confirmation testing difficult with Sanger sequencing.⁷⁸ Sensitivity can be further reduced if the overall quality of the DNA is less than expected. Many manufacturers are introducing methods to determine the ability of a DNA sample to be amplified in order to reduce false results. For example, KapaBiosystems has created a hybrid quantification and quality analysis kit for generating NGS libraries, and Asuragen has developed a novel quality control assay to determine sample DNA quality.^{79,80}

The determination of a cutoff value for the analytical sensitivity of the primary NGS platform should be taken into account, in addition to secondary confirmation techniques and the correlation of clinical efficacy of targeted therapies with variant allele frequency (ie, mutant allele frequency). Currently, the neoplastic fraction of a sample is estimated from a pathologist's examination of an adjacent hematoxylin-eosin stained section. This information, together with the analytical sensitivity of the platform ultimately defines the cut-off value at the clinical decision point. Assuming all tumor cells are heterozygous for a mutation of interest in a sample with a mutant allele fraction of 5%, a theoretically tumor cell content of at least 10% would be necessary for detection, underscoring the importance of tissue assessment, tissue macro- and microdissection, and pathologist review. For comparison, with a minimum sensitivity of 10% to 15% (compared with 5% for NGS), the tumor neoplastic fraction would need to be at least 20% to 30% for Sanger sequencing. In the PRIME and 20050181 studies that used Sanger sequencing to evaluate tumor *RAS* status, a 50% minimum tumor neoplastic cell fraction was employed.^{6,44} Therefore, the analytical sensitivity of a method alone is insufficient to describe the cut-off. Tissue-based analyses such as IHC and fluorescence in situ hybridization allow for estimation of the fraction of neoplastic cells within a tumor that express the biomarker of interest as part of the analytical method, albeit the section analyzed is through an excised portion of the tumor thought to represent the tumor existing in the patient to be treated.^{81,82} This caveat applies to sections from which DNA is extracted for NGS analysis.

Metastases are important to consider because they cause clinical morbidity and are often responsible for mortality, it is appropriate to test the metastatic lesions. Although discordances may occur in rare instances, studies have demonstrated a high concordance rate

in *KRAS* gene status between primary tumors and metastases, suggesting that analysis of any available tumor tissue, primary or metastatic, for *KRAS* status can be considered adequate in most patients.^{83,84} However, when testing large gene panels, the differences in mutational profiles may potentially be significantly different between primary tumor and distant metastases (DM). A recent pilot study performed on 32 CRC samples at Cedars-Sinai revealed that genetic differences may occur between tumors that metastasize synchronously versus metachronously to distant sites. About half of the synchronous DM demonstrated completely divergent mutations from the primary tumor; similar results have been reported in the literature.⁸⁵ This was in contrast to metachronous DM in which all had the same or overlapping mutations as the primary tumor.⁸⁶ Although the number of cases was relatively small, these preliminary results suggest that selection of appropriate tissue for sequencing is important, particularly with respect to testing of synchronous metastatic lesions, and may help design genomically-guided therapy in advanced or refractory CRC patients. Clearly, further studies on a larger number of patients are needed to further ascertain the genomic diversity of mCRC vis-a-vis their primary site.

Control materials are also required to enable demonstration of the ability to correctly assign the patient at the clinical decision point. Cell line mixtures that allow defined levels of mutant alleles in a background of wild-type alleles are becoming available as formalin-fixed, paraffin-embedded sections (AcroMetrix, Life Technologies, Grand Island, NY; Horizon Diagnostics, Cambridge, UK). The National Institute of Standards and Technology has developed the Genome in a Bottle, a well-characterized whole-genome reference that is publicly available for sequencing and assessing variant-call accuracy and understanding biases.⁸⁷ Other control materials are available for concurrent processing with samples to ensure accuracy of NGS in addition to providing a genetic reference sample (AcroMetrix). The question remains as to whether these contrived materials can be used as an adjunct to external quality assessment/proficiency testing programs, internal quality assessment, and/or used for validation of new diagnostic tests. If such materials are used during the development of companion diagnostics, they can be employed to demonstrate the ability of the laboratory to correctly assign patients to treatment at the clinical decision point.

In addition to ensuring the accuracy of mutational analysis, pathologists must also ensure that results of such testing are available promptly to allow physicians and patients to make appropriate decisions regarding therapy. Sequencing platforms using bidirectional methods can sequence many kilobases in a few hours.⁸⁸ Pyrosequencing has a low error rate (0.4%). However, these reactions can take from 27 hours to 11 days to complete.^{37,88} BEAMing has low error rate (1.78%) and can complete a mutational analysis in approximately 2 hours.⁸⁸ However, achievement of this low error rate requires careful evaluation of the amount of input DNA. Notably, although turnaround times reported for NGS sequencing methods are improving, these times do not include upfront quality control requirements and the subsequent quantification that can elongate the time between when samples are acquired and patients obtain results,⁸⁸ nor do they account for the time spent obtaining, reviewing and preparing banked tumor specimens for analysis. Given the importance of availability of *RAS* analysis before initiation of treatment, every effort should be made to ensure that these results are made available in a timely manner to guide the time-sensitive selection of matched molecular-targeted therapies and/or non-matched chemotherapies. Hence, targeted

NGS at selected genes (20 to 30) most commonly associated with mCRC is a clinically highly informative assay of potential actionable (“druggable”) mutations in real-time. In our experience (unpublished data on 12 CRC samples) at our institution, a significant proportion of CRC submitted for NGS were found to have mutations for which a targeted therapy exists (although whether such targeted therapy might provide a clinically meaningful benefit remains to be determined in many instances). Similarly, a study conducted at the Dana Farber Cancer Institute found that in 40 CRC tumor samples, 52.5% (n=21) contained at least one mutation that had been linked to a potential clinical treatment option.⁸⁹

LIQUID BIOPSIES – THE FUTURE?

As the search for, and characterization of biomarkers continues, the importance of circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA), grows. CTCs and ctDNA, regarded as measures of tumor burden or minimal residual disease, are signs of systemic disease progression and may be associated with poor patient survival.⁹⁰ CTCs and ctDNA can be isolated quickly and efficiently from patients through a simple blood draw; however, owing to low numbers, CTC yields are often minimal. Despite discrepancies in isolation, recent studies have utilized these circulating cells as “liquid biopsies” in the development and identification of potential biomarkers. Genomic analyses have identified genetic heterogeneity between CTCs and the tumor, indicating the possibility for identifying low-level mutational differences that were previously undetectable by other methods.^{91,92} CTCs may also allow clinicians to follow the genetic evolution of the tumor noninvasively and may aid in predicting treatment resistance or nonresponse. The utility of ctDNA analysis as a potential measure of the average somatic mutation burden of a patient at the time of sampling is being investigated in a number of studies to determine whether the patient has a tumor(s) carrying a specific mutation(s), at what level that mutation is observed, and whether that level varies or additional mutations emerge over time.⁹³⁻⁹⁶ This method was recently validated in a patient with NSCLC who developed resistance to gefitinib; blood samples were drawn over time and analyzed using the Qiagen® QIAamp Circulating Nucleic Acid kit, and the development of mutations in *KRAS*, *BRAF*, and *EGFR* were identified.⁹⁷

CONCLUSIONS

In summary, with the discovery of genomic mutations that affect therapeutic efficacy, there is a growing need for genetic screening in conjunction with treatment. Pathologists play key roles in the implementation and reporting of molecular testing in mCRC (Figure 5). For patients with mCRC, evaluation of *RAS* mutational status (*KRAS* exon 2, 3, and 4; *NRAS* exons 2, 3, and 4) should be performed at diagnosis to guide decision-making for anti-EGFR treatment. With the rapid development of new and faster screening procedures and clinically actionable targeted NGS in real-time, the dichotomy between technological capability and clinical utility is decreasing. The data derived from these biomarker studies and the research conducted on new or improved methods for genomic analysis will enable the delivery of more personalized therapy to patients with mCRC.

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References

1. Vectibix® (panitumumab). Full Prescribing Information. Amgen Inc.; Thousand Oaks, CA: 2014.
2. Erbitux® (cetuximab). Full Prescribing Information. ImClone Systems Incorporated and Bristol-Myers Squibb Company; Princeton, NJ: 2013.
3. NCCN clinical practice guidelines in oncology: colon cancer v.3.2014. National Comprehensive Cancer Network; Available at: http://www.nccn.org/professionals/physician_gls/pdf/colon.pdf [February 11, 2014]
4. European Society of Pathology. [July 18, 2014] Colon External Quality Assessment Scheme. Available at: <http://kras.eqascheme.org>
5. Wong NA, Gonzalez D, Salto-Tellez M, et al. RAS testing of colorectal carcinoma-a guidance document from the Association of Clinical Pathologists Molecular Pathology and Diagnostics Group. *J Clin Pathol*. 2014; 67(9):751–757. [PubMed: 24996433]
6. Douillard JY, Oliner KS, Siena S, et al. Panitumumab-FOLFOX4 treatment and RAS mutations in colorectal cancer. *N Engl J Med*. 2013; 369(11):1023–1034. [PubMed: 24024839]
7. Evaluation of Genomic Applications in P, Prevention Working G. Recommendations from the EGAPP Working Group: genetic testing strategies in newly diagnosed individuals with colorectal cancer aimed at reducing morbidity and mortality from Lynch syndrome in relatives. *Genet Med*. 2009; 11(1):35–41. [PubMed: 19125126]
8. Shi C, Washington K. Molecular testing in colorectal cancer: diagnosis of Lynch syndrome and personalized cancer medicine. *Am J Clin Pathol*. 2012; 137(6):847–859. [PubMed: 22586043]
9. Bacher JW, Flanagan LA, Smalley RL, et al. Development of a fluorescent multiplex assay for detection of MSI-High tumors. *Dis Markers*. 2004; 20(4-5):237–250. [PubMed: 15528789]
10. Boland CR, Thibodeau SN, Hamilton SR, et al. A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. *Cancer Res*. 1998; 58(22):5248–5257. [PubMed: 9823339]
11. Bettstetter M, Dechant S, Ruemmele P, et al. Distinction of hereditary nonpolyposis colorectal cancer and sporadic microsatellite-unstable colorectal cancer through quantification of MLH1 methylation by real-time PCR. *Clin Cancer Res*. 2007; 13(11):3221–3228. [PubMed: 17545526]
12. National Comprehensive Cancer Network. [July 2, 2015] NCCN Clinical Practice Guidelines in Oncology: Genetic/Familial High-Risk Assessment: Colorectal v1.2015. Available at: http://www.nccn.org/professionals/physician_gls/pdf/genetics_colon.pdf
13. Hampel H, Frankel WL, Martin E, et al. Feasibility of screening for Lynch syndrome among patients with colorectal cancer. *J Clin Oncol*. 2008; 26(35):5783–5788. [PubMed: 18809606]
14. Haraldsdottir S, Hampel H, Tomsic J, et al. Colon and endometrial cancers with mismatch repair deficiency can arise from somatic, rather than germline, mutations. *Gastroenterology*. 2014; 147(6):1308–1316 e1301. [PubMed: 25194673]
15. Camptosar® (irinotecan). Full Prescribing Information. Pfizer Inc.; New York, NY: 2014.
16. Goodenberger M, Lindor NM. Lynch syndrome and MYH-associated polyposis: review and testing strategy. *J Clin Gastroenterol*. 2011; 45(6):488–500. [PubMed: 21325953]
17. Wang HL, Lopategui J, Amin MB, Patterson SD. KRAS mutation testing in human cancers: the pathologist's role in the era of personalized medicine. *Adv Anat Pathol*. 2010; 17(1):23–32. [PubMed: 20032635]
18. Soric 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*. 2014; 26(1):13–21. [PubMed: 25115304]

19. Siddiqui AD, Piperdi B. KRAS mutation in colon cancer: a marker of resistance to EGFR-I therapy. *Ann Surg Oncol*. 2010; 17(4):1168–1176. [PubMed: 19936839]
20. Wang Y, Velho S, Vakiani E, et al. Mutant N-RAS protects colorectal cancer cells from stress-induced apoptosis and contributes to cancer development and progression. *Cancer Discov*. 2013; 3(3):294–307. [PubMed: 23274911]
21. Amado RG, Wolf M, Peeters M, et al. Wild-type *KRAS* is required for panitumumab efficacy in patients with metastatic colorectal cancer. *J Clin Oncol*. 2008; 26(10):1626–1634. [PubMed: 18316791]
22. Karapetis CS, Khambata-Ford S, Jonker DJ, et al. K-ras mutations and benefit from cetuximab in advanced colorectal cancer. *N Engl J Med*. 2008; 359(17):1757–1765. [PubMed: 18946061]
23. De Roock W, Jonker DJ, Di Nicolantonio F, et al. Association of KRAS p.G13D mutation with outcome in patients with chemotherapy-refractory metastatic colorectal cancer treated with cetuximab. *JAMA*. 2010; 304(16):1812–1820. [PubMed: 20978259]
24. Peeters M, Douillard JY, Van Cutsem E, et al. Mutant KRAS codon 12 and 13 alleles in patients with metastatic colorectal cancer: assessment as prognostic and predictive biomarkers of response to panitumumab. *J Clin Oncol*. 2013; 31(6):759–765. [PubMed: 23182985]
25. NCCN Clinical Practice Guidelines in Oncology: Colon Cancer v.2.2009. Fort Washington, PA: National Comprehensive Cancer Network; Mar 3, 2009
26. Allegra CJ, Jessup JM, Somerfield MR, et al. American Society of Clinical Oncology provisional clinical opinion: testing for KRAS gene mutations in patients with metastatic colorectal carcinoma to predict response to anti-epidermal growth factor receptor monoclonal antibody therapy. *J Clin Oncol*. 2009; 27(12):2091–2096. [PubMed: 19188670]
27. Perspectives on Emerging Technology (POET) Report: KRAS Mutation Testing for Colorectal Cancer (CRC). Northfield, IL: College of American Pathologists; [June 26 2009]
28. US Food and Drug Administration. [July 15, 2014] Cetuximab (Erbix) and panitumumab (Vectibix): class labeling changes to anti-EGFR monoclonal antibodies, cetuximab (Erbix) and panitumumab (Vectibix): *KRAS* mutations. Available at: <http://www.fda.gov/AboutFDA/CentersOffices/OfficeofMedicalProductsandTobacco/CDER/ucm172905.htm>
29. van Krieken JH, Jung A, Kirchner T, et al. KRAS mutation testing for predicting response to anti-EGFR therapy for colorectal carcinoma: proposal for an European quality assurance program. *Virchows Arch*. 2008; 453(5):417–431. [PubMed: 18802721]
30. Garcia J, Riely GJ, Nafa K, Ladanyi M. KRAS mutational testing in the selection of patients for EGFR-targeted therapies. *Semin Diagn Pathol*. 2008; 25(4):288–294. [PubMed: 19013894]
31. Plesec TP, Hunt JL. KRAS mutation testing in colorectal cancer. *Adv Anat Pathol*. 2009; 16(4):196–203. [PubMed: 19546608]
32. Normanno N, Tejpar S, Morgillo F, et al. Implications for KRAS status and EGFR-targeted therapies in metastatic CRC. *Nat Rev Clin Oncol*. 2009; 6(9):519–527. [PubMed: 19636327]
33. Harbison CT, Horak CE, Ledezine JM, et al. Validation of companion diagnostic for detection of mutations in codons 12 and 13 of the KRAS gene in patients with metastatic colorectal cancer: analysis of the NCIC CTG CO.17 trial. *Arch Pathol Lab Med*. 2013; 137(6):820–827. [PubMed: 23030695]
34. US Food and Drug Administration. [July 15, 2014] Medical Devices. theascreen KRAS RGQ PCR Kit-P110030. Available at: <http://www.fda.gov/MedicalDevices/ProductsandMedicalProcedures/DeviceApprovalsandClearances/Recently-ApprovedDevices/ucm312055.htm>
35. US Food and Drug Administration. [July 15, 2014] Medical Devices. theascreen KRAS RGQ PCR Kit-P110027. Available at: <http://www.fda.gov/MedicalDevices/ProductsandMedicalProcedures/DeviceApprovalsandClearances/Recently-ApprovedDevices/ucm400569.htm>
36. Peeters M, Oliner KS, Parker A, et al. Massively parallel tumor multigene sequencing to evaluate response to panitumumab in a randomized phase III study of metastatic colorectal cancer. *Clin Cancer Res*. 2013; 19(7):1902–1912. [PubMed: 23325582]
37. Ronaghi M, Uhlen M, Nyren P. A sequencing method based on real-time pyrophosphate. *Science*. 1998; 281(5375):363–365. [PubMed: 9705713]

38. Patterson SD, Peeters M, Siena S, et al. Comprehensive analysis of KRAS and NRAS mutations as predictive biomarkers for single agent panitumumab (pmab) response in a randomized, phase III metastatic colorectal cancer (mCRC) study (20020408). *J Clin Oncol.* 2013; 31(suppl) abstract 3617.
39. Schwartzberg LS, Rivera F, Karthaus M, et al. PEAK: A randomized, multicenter phase II study of panitumumab plus modified fluorouracil, l, and oxaliplatin (mFOLFOX6) or bevacizumab plus mFOLFOX6 in patients with previously untreated, unresectable, wild-type KRAS exon 2 metastatic colorectal cancer. *J Clin Oncol.* 2014 Epub ahead of print.
40. Heinemann V, von Weikersthal LF, Decker T, et al. FOLFIRI plus cetuximab versus FOLFIRI plus bevacizumab as first-line treatment for patients with metastatic colorectal cancer (FIRE-3): a randomised, open-label, phase 3 trial. *Lancet Oncol.* 2014; 15(10):1065–1075. [PubMed: 25088940]
41. Lenz H, Niedzwiecki D, Innocenti F, et al. CALGB/SWOG 80405: Phase III trial of irinotecan/5-FU/leucovorin (FOLFIRI) or of oxaliplatin/5-FU/leucovorin (mFOLFOX6) with bevacizumab (BV) or cetuximab (CET) for patients (pts) with untreated metastatic adenocarcinoma of the colon or rectum (mCRC): expanded RAS analyses. *Ann Oncol.* 2014; 25(Suppl 5) Abstract 501O.
42. Bokemeyer C, Kohne C-H, Ciardiello F, et al. Treatment outcome according to tumor RAS mutation status in OPUS study patients with metastatic colorectal cancer (mCRC) randomized to FOLFOX4 with/without cetuximab. *J Clin Oncol.* 2014; 32(5s) abstract 3505.
43. Bokemeyer C, Bondarenko I, Hartmann JT, et al. Efficacy according to biomarker status of cetuximab plus FOLFOX-4 as first-line treatment for metastatic colorectal cancer: the OPUS study. *Ann Oncol.* 2011; 22(7):1535–1546. [PubMed: 21228335]
44. Peeters, M., Oliner, KS., Price, TJ., et al. Updated analysis of *KRAS/NRAS* and *BRAF* mutations in study 20050181 of panitumumab (pmab) + FOLFIRI for second-line treatment (tx) of metastatic colorectal cancer (mCRC). Presented at: American Society of Clinical Oncology Annual Meeting; May 30-June 3, 2014; Chicago, IL.
45. Ciardiello, F., Lenz, H-J., Kohne, C-H., et al. Treatment outcome according to tumor *RAS* mutation status in CRYSTAL study patients with metastatic colorectal cancer (mCRC) randomized to FOLFIRI with/without cetuximab. Presented at: American Society of Clinical Oncology Annual Meeting; May 30-June 3, 2014; Chicago, IL.
46. Peeters M, Price TJ, Cervantes A, et al. Randomized phase III study of panitumumab with fluorouracil, leucovorin, and irinotecan (FOLFIRI) compared with FOLFIRI alone as second-line treatment in patients with metastatic colorectal cancer. *J Clin Oncol.* 2010; 28(31):4706–4713. [PubMed: 20921462]
47. Dressman D, Yan H, Traverso G, Kinzler KW, Vogelstein B. Transforming single DNA molecules into fluorescent magnetic particles for detection and enumeration of genetic variations. *Proc Natl Acad Sci U S A.* 2003; 100(15):8817–8822. [PubMed: 12857956]
48. Diehl F, Li M, He Y, et al. BEAMing: single-molecule PCR on microparticles in water-in-oil emulsions. *Nat Methods.* 2006; 3(7):551–559. [PubMed: 16791214]
49. Evaluation of Genomic Applications in Practice Prevention (EGAPP) Working Group. Recommendations from the EGAPP Working Group: can testing of tumor tissue for mutations in EGFR pathway downstream effector genes in patients with metastatic colorectal cancer improve health outcomes by guiding decisions regarding anti-EGFR therapy? *Genet Med.* 2013; 15(7):517–527. [PubMed: 23429431]
50. Van Cutsem E, Cervantes A, Nordlinger B, Arnold D. on behalf of the EGWG. Metastatic colorectal cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol.* 2014
51. Cancer Genome Atlas N. Comprehensive molecular characterization of human colon and rectal cancer. *Nature.* 2012; 487(7407):330–337. [PubMed: 22810696]
52. Bavi P, Jehan Z, Bu R, et al. ALK gene amplification is associated with poor prognosis in colorectal carcinoma. *Br J Cancer.* 2013; 109(10):2735–2743. [PubMed: 24129244]
53. Wang H, Daouti S, Li WH, et al. Identification of the MEK1(F129L) activating mutation as a potential mechanism of acquired resistance to MEK inhibition in human cancers carrying the B-RafV600E mutation. *Cancer Res.* 2011; 71(16):5535–5545. [PubMed: 21705440]

54. Beauchemin N. The colorectal tumor microenvironment: the next decade. *Cancer Microenviron*. 2011; 4(2):181–185. [PubMed: 21735168]
55. Perkins G, Pilati C, Blons H, Laurent-Puig P. Beyond KRAS status and response to anti-EGFR therapy in metastatic colorectal cancer. *Pharmacogenomics*. 2014; 15(7):1043–1052. [PubMed: 24956256]
56. Wang Z, Liu P, Inuzuka H, Wei W. Roles of F-box proteins in cancer. *Nat Rev Cancer*. 2014; 14(4): 233–247. [PubMed: 24658274]
57. Soung YH, Lee JW, Kim SY, et al. Somatic mutations of the ERBB4 kinase domain in human cancers. *Int J Cancer*. 2006; 118(6):1426–1429. [PubMed: 16187281]
58. Lee SH, Jeong EG, Yoo NJ. Mutational analysis of NOTCH1, 2, 3 and 4 genes in common solid cancers and acute leukemias. *APMIS*. 2007; 115(12):1357–1363. [PubMed: 18184405]
59. Jang JH, Shin KH, Park JG. Mutations in fibroblast growth factor receptor 2 and fibroblast growth factor receptor 3 genes associated with human gastric and colorectal cancers. *Cancer Res*. 2001; 61(9):3541–3543. [PubMed: 11325814]
60. Beadling C, Heinrich MC, Warrick A, et al. Multiplex mutation screening by mass spectrometry evaluation of 820 cases from a personalized cancer medicine registry. *J Mol Diagn*. 2011; 13(5): 504–513. [PubMed: 21726664]
61. Ding L, Wendl MC, Koboldt DC, Mardis ER. Analysis of next-generation genomic data in cancer: accomplishments and challenges. *Hum Mol Genet*. 2010; 19(R2):R188–196. [PubMed: 20843826]
62. Wood LD, Parsons DW, Jones S, et al. The genomic landscapes of human breast and colorectal cancers. *Science*. 2007; 318(5853):1108–1113. [PubMed: 17932254]
63. Chong CR, Janne PA. The quest to overcome resistance to EGFR-targeted therapies in cancer. *Nat Med*. 2013; 19(11):1389–1400. [PubMed: 24202392]
64. Yuan ZX, Wang XY, Qin QY, et al. The prognostic role of BRAF mutation in metastatic colorectal cancer receiving anti-EGFR monoclonal antibodies: a meta-analysis. *PLoS ONE*. 2013; 8(6):e65995. [PubMed: 23776587]
65. Muhammad S, Jiang Z, Liu Z, Kaur K, Wang X. The role of EGFR monoclonal antibodies (MoABs) cetuximab/panitumab, and BRAF inhibitors in BRAF mutated colorectal cancer. *J Gastrointest Oncol*. 2013; 4(1):72–81. [PubMed: 23451330]
66. Connolly K, Brungs D, Szeto E, Epstein RJ. Anticancer activity of combination targeted therapy using cetuximab plus vemurafenib for refractory BRAF (V600E)-mutant metastatic colorectal carcinoma. *Curr Oncol*. 2014; 21(1):e151–154. [PubMed: 24523613]
67. Troiani T, Napolitano S, Vitagliano D, et al. Primary and acquired resistance of colorectal cancer cells to anti-EGFR antibodies converge on MEK/ERK pathway activation and can be overcome by combined MEK/EGFR inhibition. *Clin Cancer Res*. 2014; 20(14):3775–3786. [PubMed: 24812410]
68. Bertotti A, Migliardi G, Galimi F, et al. A molecularly annotated platform of patient-derived xenografts (“xenopatients”) identifies HER2 as an effective therapeutic target in cetuximab-resistant colorectal cancer. *Cancer Discov*. 2011; 1(6):508–523. [PubMed: 22586653]
69. Bardelli A, Corso S, Bertotti A, et al. Amplification of the MET receptor drives resistance to anti-EGFR therapies in colorectal cancer. *Cancer Discov*. 2013; 3(6):658–673. [PubMed: 23729478]
70. Tsimberidou AM, Wen S, Hong DS, et al. Personalized medicine for patients with advanced cancer in the phase I program at MD Anderson: validation and landmark analyses. *Clin Cancer Res*. 2014; 20(18):4827–4836. [PubMed: 24987059]
71. Wetterstrand, K. [August 27, 2014] DNA Sequencing Costs: Data from the NHGRI Genome Sequencing Program (GSP). Available at: www.genome.gov/sequencingcosts
72. Thomas F, Desmedt C, Aftimos P, Awada A. Impact of tumor sequencing on the use of anticancer drugs. *Curr Opin Oncol*. 2014; 26(3):347–356. [PubMed: 24709974]
73. Aziz N, Zhao Q, Bry L, et al. College of American Pathologists’ Laboratory Standards for Next-Generation Sequencing Clinical Tests. *Arch Pathol Lab Med*. 2014
74. Rehm HL, Bale SJ, Bayrak-Toydemir P, et al. ACMG clinical laboratory standards for next-generation sequencing. *Genet Med*. 2013; 15(9):733–747. [PubMed: 23887774]
75. Richards S, Aziz N, Bale S, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and

- Genomics and the Association for Molecular Pathology. *Genet Med*. 2015; 17(5):405–424. [PubMed: 25741868]
76. EuroGentest. [July 2, 2015] Guidelines for diagnostic next generation sequencing. Available at: <http://www.eurogentest.org/index.php?id=958>
 77. New York State Department of Health. [July 2, 2015] “Next Generation” Sequencing (NGS) guidelines for somatic genetic variant detection. Available at: http://www.wadsworth.org/labcert/TestApproval/forms/NextGenSeq_ONCO_Guidelines.pdf
 78. Daber R, Sukhadia S, Morrisette JJ. Understanding the limitations of next generation sequencing informatics, an approach to clinical pipeline validation using artificial data sets. *Cancer Genet*. 2013; 206(12):441–448. [PubMed: 24528889]
 79. Niu G, Carter WB. Human epidermal growth factor receptor 2 regulates angiopoietin-2 expression in breast cancer via AKT and mitogen-activated protein kinase pathways. *Cancer Res*. 2007; 67(4): 1487–1493. [PubMed: 17308086]
 80. Carter WB, Ward MD. HER2 regulatory control of angiopoietin-2 in breast cancer. *Surgery*. 2000; 128(2):153–158. [PubMed: 10922985]
 81. Sajanti S, Sirnio P, Vayrynen JP, et al. VE1 immunohistochemistry accurately detects BRAF V600E mutations in colorectal carcinoma and can be utilized in the detection of poorly differentiated colorectal serrated adenocarcinoma. *Virchows Arch*. 2014; 464(6):637–643. [PubMed: 24722974]
 82. Hu L, Ru K, Zhang L, et al. Fluorescence in situ hybridization (FISH): an increasingly demanded tool for biomarker research and personalized medicine. *Biomark Res*. 2014; 2(1):3. [PubMed: 24499728]
 83. Santini D, Loupakis F, Vincenzi B, et al. High concordance of KRAS status between primary colorectal tumors and related metastatic sites: implications for clinical practice. *Oncologist*. 2008; 13(12):1270–1275. [PubMed: 19056857]
 84. Molinari F, Martin V, Bordoni A, et al. Analysis of Epidermal Growth Factor Receptor (EGFR) Gene Status and Protein Expression, and K-RAS Gene Mutations in Metastatic Colorectal Cancer Patients: Comparison Between Primary Tumor and Related Metastatic Sites [abstract O5.05]. *Ann Oncol*. 2008; 19(suppl 1):i10–i25.
 85. Balschun K, Haag J, Wenke AK, et al. KRAS, NRAS, PIK3CA exon 20, and BRAF genotypes in synchronous and metachronous primary colorectal cancers diagnostic and therapeutic implications. *J Mol Diagn*. 2011; 13(4):436–445. [PubMed: 21704278]
 86. Patel, S., Farahani, N., Chevarie-Davis, M., et al. Next-generation sequencing (NGS) identifies mutational distinction between synchronous and metachronous distant metastases of colorectal carcinoma (CRC). Presented at: USCAP Annual Meeting; March 21–27, 2015; Boston, MA.
 87. Zook JM, Chapman B, Wang J, et al. Integrating human sequence data sets provides a resource of benchmark SNP and indel genotype calls. *Nat Biotechnol*. 2014; 32(3):246–251. [PubMed: 24531798]
 88. Quail MA, Smith M, Coupland P, et al. A tale of three next generation sequencing platforms: comparison of Ion Torrent, Pacific Biosciences and Illumina MiSeq sequencers. *BMC Genomics*. 2012; 13:341. [PubMed: 22827831]
 89. Lipson D, Capelletti M, Yelensky R, et al. Identification of new ALK and RET gene fusions from colorectal and lung cancer biopsies. *Nat Med*. 2012; 18(3):382–384. [PubMed: 22327622]
 90. Bork U, Grutzmann R, Rahbari NN, et al. Prognostic relevance of minimal residual disease in colorectal cancer. *World J Gastroenterol*. 2014; 20(30):10296–10304. [PubMed: 25132746]
 91. Gasch C, Bauernhofer T, Pichler M, et al. Heterogeneity of epidermal growth factor receptor status and mutations of KRAS/PIK3CA in circulating tumor cells of patients with colorectal cancer. *Clin Chem*. 2013; 59(1):252–260. [PubMed: 23136247]
 92. Heitzer E, Auer M, Gasch C, et al. Complex tumor genomes inferred from single circulating tumor cells by array-CGH and next-generation sequencing. *Cancer Res*. 2013; 73(10):2965–2975. [PubMed: 23471846]
 93. Bettegowda C, Sausen M, Leary RJ, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. *Sci Transl Med*. 2014; 6(224):224ra224.

94. Diaz LA Jr, Williams RT, Wu J, et al. The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. *Nature*. 2012; 486(7404):537–540. [PubMed: 22722843]
95. Misale S, Yaeger R, Hobor S, et al. Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. *Nature*. 2012; 486(7404):532–536. [PubMed: 22722830]
96. Thierry AR, Mouliere F, El Messaoudi S, et al. Clinical validation of the detection of KRAS and BRAF mutations from circulating tumor DNA. *Nat Med*. 2014; 20(4):430–435. [PubMed: 24658074]
97. Spada D, Del Re M, Citi V, et al. Liquid Biopsy to Monitor the Evolution of NSCLC EGFR+ During Treatment with Gefitinib. *Ann Oncol*. 2014; 25(supplement 4):iv546–iv563.

Figure 1.

IHC and MSI initial tumor screening strategies vary by institution; IHC and/or MSI is acceptable. Similarly, *BRAF*V600E and *MLH1* promoter hypermethylation testing strategies vary by institution; *BRAF*V600E and/or *MLH1* promoter hypermethylation is acceptable. *Further work-up for hereditary conditions may still be appropriate if other personal or family history features are present (ie, early age of onset, polyposis, hamartomas, strong family history of cancer, etc). †In the case of IHC protein loss of only PMS2, MSH2, or MSH6, germline testing could first be completed for the specific protein lost, and then if negative, proceed to the protein partner pair or full MMR panel testing. ‡Prior to germline testing (blood or saliva), appropriate pretest counseling should be completed by an individual with hereditary clinical genetics expertise. §Somatic MMR sequencing has shown that tumors can have biallelic loss of MMR genes, loss of heterozygosity; false positive MMR tumor testing may also be causative (see text).

Figure 2.

Role of *RAS* enzymes in the EGFR signaling pathway. *RAS*=*KRAS/NRAS*. Reprinted with permission from Siddiqui AD, Piperdi B. *KRAS* mutation in colon cancer: a marker of resistance to EGFR-I therapy. *Ann Surg Oncol* 2010;17:1168-1176.

Figure 3.

Hazard ratios for (A) disease progression or (B) death by *KRAS* and *RAS* mutation status in patients with mCRC treated with panitumumab + FOLFOX-4 and FOLFOX-4 alone in PRIME. FOLFOX-4=fluorouracil, leucovorin, and oxaliplatin; PRIME=Panitumumab Randomized Trial in Combination With Chemotherapy for Metastatic Colorectal Cancer to Determine Efficacy; *RAS*=rat sarcoma-2 virus oncogene. Reprinted with permission from Douillard JY, Oliner KS, Siena S, et al. Panitumumab-FOLFOX4 treatment and *RAS* mutations in colorectal cancer. *N Engl J Med*. 2013;369:1023-1034.

Figure 4.

The EGFR signaling network. Reprinted with permission from Chong CR, Jänne PA. Nature Med. 2013;19:1389-1400.

Figure 5.
Role of pathologists in conducting and reporting *RAS* analysis in patients with mCRC.

Table 1

Summary of PFS and OS Outcomes by *KRAS* and *RAS* Mutation Status

	<i>KRAS</i> Exon 2 WT	<i>KRAS</i> Exon 2 MT	<i>RAS</i> (<i>KRAS</i> + <i>NRAS</i>) WT	<i>KRAS</i> Exon 2 WT, New <i>RAS</i> MT	<i>RAS</i> (<i>KRAS</i> + <i>NRAS</i>) MT
<i>PRIME⁵</i> (<i>P</i> + <i>FOLFFOX-4</i> vs <i>FOLFFOX-4</i>)					
Median PFS, mo	9.6 vs. 8.0	7.3 vs. 8.8	10.1 vs. 7.9	7.3 vs. 8.0	7.3 vs. 8.7
HR (95% CI)	0.80 (0.66–0.97)	1.29 (1.04–1.62)	0.72 (0.58–0.90)	1.28 (0.79–2.07)	1.31 (1.07–1.60)
<i>P</i>	0.02	0.02	0.004	0.33	0.008
Median OS, * mo	23.9 vs. 19.7	15.5 vs. 19.3	26.0 vs. 20.2	17.1 vs. 18.3	15.6 vs. 19.2
HR (95% CI)	0.83 (0.67–1.02)	1.24 (0.98–1.57)	0.78 (0.62–0.99)	1.29 (0.79–2.10)	1.25 (1.02–1.55)
<i>P</i>	0.07	0.07	0.04	0.31	0.03
<i>OPUS^{42,43}</i> (<i>C</i> + <i>FOLFFOX-4</i> vs <i>FOLFFOX-4</i>)					
Median PFS, mo	8.3 vs. 7.2	5.5 vs. 8.6	12.0 vs. 5.8	7.5 vs. 7.4	5.6 vs. 7.8
HR (95% CI)	0.57 (0.38–0.86)	1.72 (1.10–2.68)	0.53 (0.27–1.04)	0.77 (0.28–2.08)	1.54 (1.04–2.29)
<i>P</i>	0.0064	0.0153	0.062	0.60	0.031
Median OS, mo	22.8 vs. 18.5	13.4 vs. 17.5	19.8 vs. 17.8	18.4 vs. 17.8	13.5 vs. 17.8
HR (95% CI)	0.86 (0.60–1.22)	1.29 (0.87–1.91)	0.94 (0.56–1.56)	1.09 (0.44–2.68)	1.29 (0.91–1.84)
<i>P</i>	0.39	0.20	0.80	0.86	0.157
<i>Study 2005018^{44,46}</i> (<i>P</i> + <i>FOLFIRI</i> vs <i>FOLFIRI</i>)					
Median PFS, mo	5.9 vs. 3.9	5.0 vs. 4.9	6.4 vs. 4.6	NA	4.8 vs. 4.0
HR (95% CI)	0.73 (0.59–0.90)	0.85 (0.68–1.06)	0.70 (0.54–0.91)	0.89 (0.56–1.42)	0.86 (0.71–1.05)
<i>P</i>	0.004	0.14	0.007	NA	0.14
Median OS, mo	14.5 vs. 12.5	11.8 vs. 11.1	16.2 vs. 13.9	NA	11.8 vs. 11.1
HR (95% CI)	0.85 (0.70–1.04)	0.94 (0.76–1.15)	0.81 (0.63–1.03)	0.83 (0.53–1.29)	0.91 (0.76–1.10)
<i>P</i>	0.12	NA	0.08	NA	0.34
<i>CRYSTAL⁴⁵</i> (<i>C</i> + <i>FOLFIRI</i> vs <i>FOLFIRI</i>)					
Median PFS, mo	9.9 vs. 8.4	NA	11.4 vs. 8.4	7.2 vs. 6.9	7.4 vs. 7.5
HR (95% CI)	0.70 (0.56–0.87)	NA	0.56 (0.41–0.76)	0.81 (0.39–1.67)	1.10 (0.85–1.42)
<i>P</i>	0.0012	NA	0.0002	0.56	0.47
Median OS, mo	23.5 vs. 20.0	NA	28.4 vs. 20.2	18.2 vs. 20.7	16.4 vs. 17.7
HR (95% CI)	0.80 (0.67–0.95)	NA	0.69 (0.54–0.88)	1.22 (0.69–2.16)	1.05 (0.86–1.28)
<i>P</i>	0.0093	NA	0.0024	0.50	0.64

	KRAS Exon 2 WT	KRAS Exon 2 MT	RAS (KRAS + NRAS) WT	KRAS Exon 2 WT, New RAS MT	RAS (KRAS + NRAS) MT
<i>PEAK⁸⁹ (P + mFOLFOX6 vs B + mFOLFOX6)</i>					
Median PFS, mo	10.9 vs. 10.1	NA	13.0 vs. 9.5	7.8 vs. 8.9	NA
HR (95% CI)	0.87 (0.65–1.17)	NA	0.65 (0.44–0.96)	1.39 (0.73–2.64)	NA
<i>P</i>	0.353	NA	0.029	0.318	NA
Median OS, mo	34.2 vs. 24.3	NA	41.3 vs. 28.9	27.0 vs. 16.6	NA
HR (95% CI)	0.62 (0.44–0.89)	NA	0.63 (0.39–1.02)	0.41 (0.19–0.87)	NA
<i>P</i>	0.009	NA	0.058	0.020	NA
<i>FIRE-3⁴⁰ (C + FOLFIRI vs B + FOLFIRI)</i>					
Median PFS, mo	10.0 vs. 10.3	NA	10.4 vs. 10.2	6.1 vs. 12.2	NA
HR (95% CI)	1.06 (0.88–1.26)	NA	0.93 (0.74–1.17)	2.22 (1.28–3.86)	NA
<i>P</i>	0.55	NA	0.54	0.004	NA
Median OS, mo	28.7 vs. 25.0	NA	33.1 vs. 25.6	16.4 vs. 20.6	NA
HR (95% CI)	0.77 (0.62–0.96)	NA	0.70 (0.53–0.92)	1.20 (0.64–2.28)	NA
<i>P</i>	0.017	NA	0.011	0.57	NA
<i>CALGB/SWOG 80405⁴¹ (C + FOLFIRI/mFOLFOX6 vs B + FOLFIRI/mFOLFOX6)</i>					
Median PFS, mo	10.8 vs. 10.4	NA	11.3 vs. 11.4	NA	NA
HR (95% CI)	1.04 (0.91–1.17)	NA	1.1 (0.9–1.3)	NA	NA
<i>P</i>	0.55	NA	0.31	NA	NA
Median OS, mo	29.0 vs. 29.9	NA	31.2 vs. 32.0	NA	NA
HR (95% CI)	0.92 (0.78–1.09)	NA	0.9 (0.7–1.1)	NA	NA
<i>P</i>	0.34	NA	0.40	NA	NA

* Primary analysis.

B=bevacizumab; C=ceutuximab; CRYSTAL=Cetuximab Combined With Irinotecan in First-Line Therapy for Metastatic Colorectal Cancer; FOL-FIR=5-fluorouracil, folinic acid, and irinotecan; FOLFOX-4=fluorouracil, leucovorin, and oxaliplatin; HR=hazard ratio; mCRC=metastatic colorectal cancer; mFOLFOX6=modified fluorouracil, leucovorin, and oxaliplatin; MT=mutant type; NA=not available; OS=overall survival; P=panitumumab; 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; PRIME=Panitumumab Randomized Trial in Combination With Chemotherapy for Metastatic Colorectal Cancer to Determine Efficacy; WT=wild type.