

Molecular Biomarkers for the Evaluation of Colorectal Cancer

Guideline From the American Society for Clinical Pathology, College of American Pathologists, Association for Molecular Pathology, and American Society of Clinical Oncology

Antonia R. Sepulveda, MD, PhD,¹ Stanley R. Hamilton, MD,² Carmen J. Allegra, MD,⁵ Wayne Grody, MD, PhD,⁶ Allison M. Cushman-Vokoun, MD, PhD,⁷ William K. Funkhouser, MD, PhD,⁸ Scott E. Kopetz, MD, PhD,³ Christopher Lieu, MD,⁹ Noralane M. Lindor, MD,¹⁰ Bruce D. Minsky, MD,⁴ Federico A. Monzon, MD,¹¹ Daniel J. Sargent, PhD,¹² Veena M. Singh, MD,¹³ Joseph Willis, MD,¹⁴ Jennifer Clark, SCT, MB(ASCP)^{CM, 15} Carol Colasacco, MLIS,¹⁶ R. Bryan Rumble, MSc,¹⁷ Robyn Temple-Smolkin, PhD,¹⁸ Christina B. Ventura, MT(ASCP),¹⁶ and Jan A. Nowak, MD, PhD¹⁹

From the ¹Department of Pathology and Cell Biology, Columbia University, New York, NY; Departments of ²Pathology, ³Gastrointestinal (GI) Medical Oncology, and ⁴Radiation Oncology, University of Texas MD Anderson Cancer Center, Houston; ⁵Division of Hematology and Oncology, University of Florida Medical Center, Gainesville; ⁶Departments of Pathology and Laboratory Medicine, Pediatrics, and Human Genetics, UCLA Medical Center, Los Angeles, CA; ⁷Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha; ⁸Department of Pathology and Laboratory Medicine, University of North Carolina School of Medicine, Chapel Hill; ⁹Division of Medical Oncology, University of Colorado Denver School of Medicine, Denver; ¹⁰Department of Medical Genetics, Mayo Clinic, Scottsdale, AZ; ¹¹Castle Biosciences, Friendswood, TX; ¹²Department of Health Sciences Research, Mayo Clinic, Rochester, MN; ¹³Biocept, San Diego, CA; ¹⁴Department of Pathology, Case Western Reserve University, Cleveland, OH; ¹⁵ASCP Institute for Science, Technology, and Policy, American Society for Clinical Pathology, Washington, DC; ¹⁶Laboratory and Pathology Quality Center, College of American Pathologists, Northfield, IL; ¹⁷American Society of Clinical Oncology, Alexandria, VA; ¹⁸Association for Molecular Pathology, Bethesda, MD; and ¹⁹Department of Pathology and Laboratory Medicine, Roswell Park Cancer Institute, Buffalo, NY.

Key Words: Molecular diagnostics; Gastrointestinal; Histology; Genetics; Oncology

Am J Clin Pathol March 2017;147:221-260

DOI: 10.1093/AJCP/AQW209

ABSTRACT

Objectives: To develop evidence-based guideline recommendations through a systematic review of the literature to establish standard molecular biomarker testing of colorectal cancer (CRC) tissues to guide epidermal growth factor receptor (EGFR) therapies and conventional chemotherapy regimens.

Methods: The American Society for Clinical Pathology, College of American Pathologists, Association for Molecular Pathology, and American Society of Clinical Oncology convened an expert panel to develop an evidence-based guideline to establish standard molecular biomarker testing and guide therapies for patients with CRC. A comprehensive literature search that included more than 4,000 articles was conducted.

Results: Twenty-one guideline statements were established.

Conclusions: Evidence supports mutational testing for EGFR signaling pathway genes, since they provide clinically actionable information as negative predictors of benefit to anti-EGFR monoclonal antibody therapies for targeted therapy of CRC. Mutations in several of the biomarkers have clear prognostic value. Laboratory approaches to operationalize CRC molecular testing are presented.

Molecular testing to select targeted and conventional therapies for patients with colorectal cancer (CRC) has been the focus of a number of recent studies and is becoming standard practice for management of patients with CRC. Molecular markers that predict response to a specific therapy or treatment regimen are known as predictive biomarkers.¹ Monoclonal antibody therapies that target the epidermal growth factor receptor (EGFR) bind the EGFR extracellular domain, blocking EGFR signaling pathways. Anti-EGFR monoclonal antibodies have been the main targeted therapies for CRC that require knowledge of the mutational status of genes in the pathway as predictive biomarkers of response to these therapies.²⁻⁴ Initial clinical trial data demonstrated that patients with CRC carrying activating mutations of *KRAS* affecting exon 2 codons 12 and 13 did not benefit from anti-EGFR monoclonal antibody therapy.²⁻⁴ Subsequent studies described other mutations in genes of the EGFR signaling pathways involving other exons of *KRAS* and in *NRAS*, *BRAF*, *PIK3CA*, and *PTEN* that may affect response of CRC to anti-EGFR antibody therapies. Guidelines addressing the molecular testing of EGFR pathway genes beyond *KRAS* have not been established and are needed in clinical practice.

The DNA mismatch repair (MMR) status of CRC may have predictive value in some clinical settings. While testing of CRC for MMR has been recommended for all patients with CRC as a workup test to evaluate for possible Lynch syndrome,⁵ guidelines for the use of MMR as a predictive biomarker of response to therapy have not been reported. Recent molecular biomarker data have shown the importance of microsatellite instability (MSI) testing, a marker of deficient mismatch repair (dMMR), for the selection of patients for immunotherapy (see section on emerging biomarkers below).

Alterations of a number of critical genes in CRC development and progression such as dMMR and *BRAF* activating mutations have been shown to affect prognosis, as measured by several metrics of tumor progression or survival.⁶⁻⁸ The utility of incorporating prognostic biomarkers in the management of patients with CRC has not been well defined in clinical practice. Defining the utility of information gathered from prognostic molecular biomarkers for clinical management of patients with CRC is warranted.

The postgenome era and the emphasis on precision genomic-based medicine are providing enormous amounts of new data and many promising new molecular cancer biomarkers that may emerge as molecular diagnostic tools that can be used to enhance successful treatment of patients with CRC and other cancers. Laboratories and regulatory agencies are faced with challenges to rapidly and efficiently provide new test results for the management of patients with cancer. Laboratory testing of molecular biomarkers involves

the selection of assays, type of specimens to be tested, timing of ordering of tests, and turnaround time for testing results. Recent years have shown that a plethora of technical approaches can effectively be used as long as test specificity and sensitivity meet the clinical needs. While earlier testing approaches were focused on one or a few testing targets, the current need for multiple molecular markers from potentially minute tumor samples is leading to greater use of gene panels such as targeted next-generation sequencing (NGS) cancer panels, which can assay from a few to hundreds of genes and amplicons with known mutational hotspots in cancer.

There is a need for current evidence-based recommendations for the molecular testing of CRC tissues to guide EGFR-targeted therapies and conventional chemotherapy regimens. Therefore, the current recommendations were developed through collaboration of four societies: American Society for Clinical Pathology (ASCP), College of American Pathologists (CAP), Association for Molecular Pathology (AMP), and American Society of Clinical Oncology (ASCO). This guideline follows well-established methods used in their development as well as for regular updates, such that new advances in the molecular testing for clinical management of CRC can be integrated in future updates of the guideline in a timely manner.

Panel Composition

The ASCP, the CAP Pathology and Laboratory Quality Center (the Center), the AMP, and the ASCO convened an expert panel consisting of practicing pathologists, oncologists, geneticists, and a biostatistician with expertise and experience in molecular biomarker testing and targeted therapies for CRC. The ASCP, CAP, AMP, and ASCO jointly approved the appointment of the project, coauthors, and expert panel members. In addition, a methodologist experienced in systematic review and guideline development consulted with the panel throughout the project.

Conflict of Interest Policy

Prior to acceptance on the expert or advisory panel, potential members completed a joint guideline conflict of interest (COI) disclosure process, whose policy and form (in effect July 2011) require disclosure of material financial interest in, or potential for benefit of significant value from, the guideline's development or its recommendations 12 months prior through the time of publication. The potential members completed the COI disclosure form, listing any relationship that could be interpreted as constituting an actual, potential, or apparent conflict. All project participants were required to disclose conflicts prior to beginning and continuously throughout the project's timeline.

Disclosed conflicts of the expert panel members are listed in **Appendix 1** and **Appendix 2**.

The ASCP, CAP, AMP, and ASCO provided funding for the administration of the project; no industry funds were used in the development of the guideline. All panel members volunteered their time and were not compensated for their involvement, except for the contracted methodologist. Please see the Supplemental Digital Content (SDC) at *American Journal of Clinical Pathology* online for full details on the COI policy.

Objective

The scope of the project was to develop an evidence-based guideline to help establish standard molecular biomarker testing, guide targeted therapies, and advance personalized care for patients with CRC. The panel addressed the following key questions:

1. What biomarkers are useful to select patients with CRC for targeted and conventional therapies?
2. How should tissue specimens be processed for biomarker testing for CRC management?
3. How should biomarker testing for CRC management be performed?
4. How should molecular testing of CRC be implemented and operationalized?
5. Are there emerging genes/biomarkers that should be routinely tested in CRC?

Materials and Methods

This evidence-based guideline was developed following standards as endorsed by the Institute of Medicine.⁹ A detailed description of the methods and systematic review (including the quality assessment and complete analysis of the evidence) can be found in the SDC.

Literature Search and Selection

A comprehensive search for literature was performed in MEDLINE using the OvidSP (August 1, 2013) and PubMed (September 17, 2013) interfaces. The initial MEDLINE search encompassed the publication dates of January 1, 2008, through August 1, 2013 (OvidSP), and January 1, 2008, through September 17, 2013 (PubMed). A supplemental literature search was performed using Scopus (September 25, 2013) to identify relevant articles published between January 1, 2008, and September 25, 2013, in journals not indexed in MEDLINE. The literature search of the electronic databases involved two separate searches in each database, the first using Medical Subject Headings (MeSH) terms and keywords for the concepts “colorectal cancer,”

“biomarkers,” “treatment,” and “treatment outcomes” and the second using terms for the concepts “colorectal cancer,” “biomarkers,” and “laboratory methods.” Limits were set for human studies published in English, and a publication filter was applied to exclude lower levels of evidence such as letters, commentaries, editorials, and case reports. The Ovid search was rerun on February 12, 2015, to identify articles published since August 1, 2013.

In addition to the searches of electronic databases, an Internet search of international health organizations, the National Guidelines Clearinghouse, and Guidelines International Network was conducted for existing relevant guidelines or protocols. Guidelines were included if they were published since 2008 in English. The proceedings of the meetings of the ASCO and ASCO-Gastrointestinal Cancers Symposium, European Society for Medical Oncology, and the American Association for Cancer Research from 2012 and 2013 were also searched for relevant abstracts.

A focused examination of all systematic reviews retrieved by the initial literature search and retained after full-text review was performed to identify primary research studies not already included. In addition, recommendations from the expert panel were reviewed, and the reference lists of all articles deemed eligible for inclusion were scanned for relevant reports. The results of all searches were combined and deduplicated.

Detailed information regarding the literature search strategy can be found in the SDC.

Eligible Study Designs

Practice guidelines, consensus documents, systematic reviews, meta-analyses, randomized controlled trials, comparative studies, reviews, and evaluation studies were eligible for inclusion. In addition to journal articles, the search identified meeting abstracts.

Inclusion Criteria

Published studies were selected for full-text review if they met each of the following criteria:

1. Patients with colorectal or rectal cancer with a pathology diagnosis of adenocarcinoma or adenocarcinoma with neuroendocrine differentiation, either primary or metastatic
2. Patients of all ages
3. Patients with cancer of any invasive stage (T1-T4)
4. Biomarker testing such as *KRAS* (Kirsten rat sarcoma viral oncogene homolog), DNA MMR/MSI, *BRAF* (V-raf murine sarcoma viral oncogene homolog B1), *NRAS* (neuroblastoma RAS viral [v-ras] oncogene homolog), *PIK3CA* (phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha), *PTEN* (phosphatase and tensin

- homolog), *MLH1* (MutL homolog 1) methylation, or gene expression profiles
5. Comparative studies
 6. Human studies
 7. Studies published in English

Exclusion Criteria

1. All other tumor primaries and types (ie, noncolorectal or nonrectal cancers, tumor types other than adenocarcinoma or adenocarcinoma with neuroendocrine differentiation)
2. Patients with noninvasive tumors (ie, intraepithelial, dysplasia, in situ, polyps without carcinoma)
3. Studies of colorectal cancers without biomarker testing, novel biomarkers—for example, VEG-F (vascular endothelial growth factor), XRCC1 (X-ray repair complementing defective repair in Chinese hamster cells 1), IGF (insulin-like growth factor), ERCC (excision repair cross-complementing rodent repair deficiency, complementation group 1), micro-RNA, TYMS (thymidylate synthetase), GCC (guanylyl cyclase C), LINE (long interspersed nucleotide element) methylation, CIMP (CpG island methylator phenotype), HER2 (V-erb-b2 erythroblastic leukemia viral oncogene homolog 2), CIN (chromosomal instability) status LOH (loss of heterozygosity), and germline (genetics only) testing
4. Non-English-language articles
5. Animal studies
6. Studies published prior to 2002
7. Noncomparative studies, letters, commentaries, or editorials
8. Studies that did not address at least one of the defined inclusion criteria
9. Studies with fewer than 50 patients per comparison arm

Outcomes of Interest

The primary outcomes of interest included survival outcomes and performance characteristics of laboratory testing assays. Survival outcomes included overall survival (OS), disease-free survival (DFS), progression-free survival (PFS), recurrence-free survival, time to recurrence, response to therapy (eg, complete and partial response). Laboratory data and test performance characteristics included percent mutation, concordance of testing methods, sensitivity of testing methods, specificity of testing methods, concordance of detected mutations between primary and metastatic mutations (number [%] of cases with mutations vs number of cases with no mutations in the gene of interest), and concordance of mutations (synchronous primary vs metastatic,

Table 1
Levels of Evidence^a

Level	Description
Level I	Evidence derived from systematic reviews of appropriate level II studies and/or clinical practice guidelines
Level II	Evidence derived from randomized controlled trials
Level III	Evidence derived from comparative studies (eg, prospective cohort studies, retrospective cohort studies)
Level IV	Evidence without a comparator (eg, case reports, case series, narrative reviews)

^aData derived from National Health and Medical Research Council.¹⁰

metachronous primary vs metastatic, between synchronous metastases, between metachronous metastases).

Quality Assessment

An assessment of the quality of the evidence was performed for all retained studies following application of the inclusion and exclusion criteria by the methodologist. Using this method, studies deemed to be of low quality would not be excluded from the systematic review but would be retained and their methodologic strengths and weaknesses discussed where relevant. Studies would be assessed by confirming the presence of items related to both internal and external validity, which are all associated with methodologic rigor and a decrease in the risk of bias. The quality assessment of the studies was performed by determining the risk of bias by assessing key indicators, based on study design, against known criteria. (Refer to the SDC for detailed discussion of the quality assessment.)

For strength of the evidence, the panel considered the level of evidence, as well as its quantity and quality of included studies. The level of evidence was based on the study design as described in **Table 1**.¹⁰ In general, level I and II evidence is considered most appropriate to answer clinical questions, but in the absence of such high-quality evidence, the panel considered data from lower quality studies. The quantity of evidence refers to the number of studies and number of cases included for each outcome in the recommendation. The quality of studies reflects how well the studies were designed to eliminate bias and threats to validity.

The appropriateness of the study design and data collected, relevance and clarity of findings, and adequacy of conclusions were evaluated. Each study was assessed individually (refer to the SDC for individual assessments and results) and then summarized by study type. Components such as generalizability and applicability were also considered when determining the strength of evidence. A summary of the overall quality of the evidence was given considering the evidence in totality. Ultimately, the designation (ie, rating or grade) of the strength of evidence is a judgment by

Table 2
Grades for Strength of Evidence^a

Designation	Description	Quality of Evidence
Convincing	High confidence that available evidence reflects true effect. Further research is very unlikely to change the confidence in the estimate of effect.	High/intermediate quality of evidence
Adequate	Moderate confidence that available evidence reflects true effect. Further research is likely to have an important impact on the confidence in estimate of effect and may change the estimate.	Intermediate/low quality of evidence
Inadequate	Little confidence that available evidence reflects true effect. Further research is very likely to have an important impact on the confidence in the estimate of effect and is likely to change the estimate.	Low/insufficient quality of evidence and expert panel uses formal consensus process to reach recommendation
Insufficient	Evidence is insufficient to discern net effect. Any estimate of effect is very uncertain.	Insufficient evidence and expert panel uses formal consensus process to reach recommendation

^aAdapted from Guyatt et al,¹¹ by permission of BMJ Publishing Group Limited.

Table 3
Grades for Strength of Recommendation^a

Designation	Recommendation	Rationale
Strong recommendation	Recommend for or against a particular molecular testing practice for colorectal cancer (can include <i>must</i> or <i>should</i>)	Supported by convincing or adequate strength of evidence, high or intermediate quality of evidence, and clear benefit that outweighs any harms
Recommendation	Recommend for or against a particular molecular testing practice for colorectal cancer (can include <i>should</i> or <i>may</i>)	Some limitations in strength of evidence (adequate or inadequate) and quality of evidence (intermediate or low), balance of benefits and harms, values, or costs, but panel concludes that there is sufficient evidence and/or benefit to inform a recommendation
Expert consensus opinion	Recommend for or against a particular molecular testing practice for colorectal cancer (can include <i>should</i> or <i>may</i>)	Serious limitations in strength of evidence (inadequate or insufficient), quality of evidence (intermediate or low), balance of benefits and harms, values, or costs, but panel consensus is that a statement is necessary
No recommendation	No recommendation for or against a particular molecular testing practice for colorectal cancer	Insufficient evidence or agreement of the balance of benefits and harms, values, or costs to provide a recommendation

^aData derived from Guyatt et al.¹¹

the expert panel of its level of confidence that the evidence from the studies informing the recommendations reflects true effect. **Table 2** describes the grades for strength of evidence.¹¹

Assessing the Strength of Recommendations

Development of recommendations requires that the panel review the identified evidence and make a series of key judgments (using procedures described in the SDC). Grades for strength of recommendations were developed by the CAP Pathology and Laboratory Quality Center and are described in **Table 3**.¹¹

Guideline Revision

This guideline will be reviewed every 4 years or earlier in the event of publication of substantive and high-quality

evidence that could potentially alter the original guideline recommendations. If necessary, the entire panel will reconvene to discuss potential changes. When appropriate, the panel will recommend revision of the guideline to the ASCP, CAP, AMP, and ASCO for review and approval.

Disclaimer

Practice guidelines and consensus statements reflect the best available evidence and expert consensus supported in practice. They are intended to assist physicians and patients in clinical decision making and to identify questions and settings for further research. With the rapid flow of scientific information, new evidence may emerge between the time a practice guideline or consensus statement is developed and when it is published or read. Guidelines and statements are not continually updated and may not reflect the most recent

evidence. Guidelines and statements address only the topics specifically identified therein and are not applicable to other interventions, diseases, or stages of diseases. Furthermore, guidelines and consensus statements cannot account for individual variation among patients and cannot be considered inclusive of all proper methods of care or exclusive of other treatments. It is the responsibility of the treating physician or other health care provider, relying on independent experience and knowledge, to determine the best course of treatment for the patient. Accordingly, adherence to any practice guideline or consensus statement is voluntary, with the ultimate determination regarding its application to be made by the physician in light of each patient's individual circumstances and preferences. The ASCP, CAP, AMP, and ASCO make no warranty, express or implied, regarding guidelines and statements and specifically exclude any warranties of merchantability and fitness for a particular use or purpose. The ASCP, CAP, AMP, and ASCO assume no responsibility for any injury or damage to persons or property arising out of or related to any use of this statement or for any errors or omissions.

Results

A total of 4,197 studies met the search term requirements. A total of 123 articles were included for data extraction. Excluded articles were available as discussion or background references. The panel convened 14 times (11 teleconference webinars and three face-to-face meetings) from July 27, 2013, through September 24, 2015, to develop the scope, draft recommendations, review and respond to solicited feedback, and assess the quality of evidence that supports the final recommendations. Additional work was completed via electronic mail. An open comment period was held from March 30, 2015, through April 22, 2015, during which draft recommendations were posted on the AMP website. Twenty-one guideline statements had an agreement ranging from 60% to 94% for each statement from the open-comment period participants (refer to Outcomes in the SDC for full details). The website received a total of 248 comments. Teams of three to four expert panel members were assigned three to five draft recommendations to review all comments received and provide an overall summary to the rest of the panel. Following panel discussion and the final quality of evidence assessment, the panel members determined whether to maintain the original draft recommendation as is, revise it with minor language change, or consider it as a major recommendation change. The expert panel modified eight draft statements based on the feedback during the open-comment period and the considered judgment process. Resolution of all changes was obtained by majority

consensus of the panel using nominal group technique (rounds of email discussion and multiple edited recommendations) among the panel members. The final recommendations were approved by the expert panel with a formal vote. The panel considered the risks and benefits throughout the whole process in their considered judgment process. Formal cost analysis or cost-effectiveness was not performed.

Each organization instituted a review process to approve the guideline. The ASCP assigned the review of the guideline to a Special Review Panel. For the CAP, an independent review panel (IRP) representing the Council on Scientific Affairs was assembled to review and approve the guideline. The IRP was masked to the expert panel and vetted through the COI process. The AMP approval process required the internal review of an independent panel led by the Publications and Communications Committee Chair and Executive Committee approval. The ASCO approval process required the review and approval of the Clinical Practice Guidelines Committee.

Guideline Statements

1. Recommendation: Patients with CRC being considered for anti-EGFR therapy must receive *RAS* mutational testing. Mutational analysis should include *KRAS* and *NRAS* codons 12 and 13 of exon 2, 59 and 61 of exon 3, and 117 and 146 of exon 4 (“expanded” or “extended” *RAS*) **Table 4**.

Aberrant activation of EGFR signaling pathways in CRC is primarily associated with activating mutations of genes in the mitogen-activated protein kinase and phosphatidylinositol-3-kinase (PI3K) pathways. Together, *KRAS*, *NRAS*, and *BRAF* mutations have been reported to occur in more than half of all CRC cases, and *KRAS* or *NRAS* and *BRAF* mutations are inversely associated, with a small proportion of individual CRCs showing co-occurrence of *RAS* and *RAF* mutations.^{3,12}

Cetuximab and panitumumab are antibodies that bind to the extracellular domain of EGFR, blocking the binding of EGF and other EGFR endogenous ligands, thereby blocking EGFR signaling. Earlier studies reported the effects of anti-EGFR antibody treatment independent of *KRAS* status.¹³⁻¹⁶ However, it was later reported that targeted EGFR therapies with cetuximab or panitumumab improve PFS and OS in patients with metastatic CRC with wild-type *KRAS* but not for patients with mutated *KRAS*.^{2,3,17} In these earlier studies, only mutations of *KRAS* exon 2 were considered. Based on the available clinical trial data in 2009, the ASCO recommended that patients with metastatic CRC who are candidates for anti-EGFR antibody therapy should have their tumors tested for *KRAS* mutations in a Clinical Laboratory Improvements Amendments '88 (CLIA)-accredited laboratory.²

Table 4
Guideline Statements and Strength of Recommendations

Guideline Statement	Strength of Recommendation
1. Patients with colorectal carcinoma being considered for anti-EGFR therapy must receive <i>RAS</i> mutational testing. Mutational analysis should include <i>KRAS</i> and <i>NRAS</i> codons 12 and 13 of exon 2, 59 and 61 of exon 3, and 117 and 146 of exon 4 ("expanded" or "extended" <i>RAS</i>).	Recommendation
2a. <i>BRAF</i> p.V600 (<i>BRAF</i> c.1799 [p.V600]) mutational analysis should be performed in colorectal cancer tissue in patients with colorectal carcinoma for prognostic stratification.	Recommendation
2b. <i>BRAF</i> p.V600 mutational analysis should be performed in deficient MMR tumors with loss of MLH1 to evaluate for Lynch syndrome risk. Presence of a <i>BRAF</i> mutation strongly favors a sporadic pathogenesis. The absence of a <i>BRAF</i> mutation does not exclude risk of Lynch syndrome.	Recommendation
3. Clinicians should order mismatch repair status testing in patients with colorectal cancers for the identification of patients at high risk for Lynch syndrome and/or prognostic stratification.	Recommendation
4. There is insufficient evidence to recommend <i>BRAF</i> c.1799 p.V600 mutational status as a predictive molecular biomarker for response to anti-EGFR inhibitors.	No recommendation
5. There is insufficient evidence to recommend <i>PIK3CA</i> mutational analysis of colorectal carcinoma tissue for therapy selection outside of a clinical trial. Note: Retrospective studies have suggested improved survival with postoperative aspirin use in patients whose colorectal carcinoma harbors a <i>PIK3CA</i> mutation.	No recommendation
6. There is insufficient evidence to recommend <i>PTEN</i> analysis (expression by immunohistochemistry or deletion by fluorescence in situ hybridization) in colorectal carcinoma tissue for patients who are being considered for therapy selection outside of a clinical trial.	No recommendation
7. Metastatic or recurrent colorectal carcinoma tissues are the preferred specimens for treatment predictive biomarker testing and should be used if such specimens are available and adequate. In their absence, primary tumor tissue is an acceptable alternative and should be used.	Expert consensus opinion
8. Formalin-fixed, paraffin-embedded tissue is an acceptable specimen for molecular biomarker mutational testing in colorectal carcinoma. Use of other specimens (eg, cytology specimens) will require additional adequate validation, as would any changes in tissue-processing protocols.	Expert consensus opinion
9. Laboratories must use validated colorectal carcinoma molecular biomarker testing methods with sufficient performance characteristics for the intended clinical use. Colorectal carcinoma molecular biomarker testing validation should follow accepted standards for clinical molecular diagnostics tests.	Strong recommendation
10. Performance of molecular biomarker testing for colorectal carcinoma must be validated in accordance with best laboratory practices.	Strong recommendation
11. Laboratories must validate the performance of IHC testing for colorectal carcinoma molecular biomarkers (currently IHC testing for MLH1, MSH2, MSH6, and PMS2) in accordance with best laboratory practices.	Strong recommendation
12. Laboratories must provide clinically appropriate turnaround times and optimal utilization of tissue specimens by using appropriate techniques (eg, multiplexed assays) for clinically relevant molecular and immunohistochemical biomarkers of colorectal cancer.	Expert consensus opinion
13. Molecular and IHC biomarker testing in colorectal carcinoma should be initiated in a timely fashion based on the clinical scenario and in accordance with institutionally accepted practices. Note: Test ordering can occur on a case-by-case basis or by policies established by the medical staff.	Expert consensus opinion
14. Laboratories should establish policies to ensure efficient allocation and utilization of tissue for molecular testing, particularly in small specimens.	Expert consensus opinion
15. Members of the patient's medical team, including pathologists, may initiate colorectal carcinoma molecular biomarker test orders in accordance with institutionally accepted practices.	Expert consensus opinion
16. Laboratories that require send-out of tests for treatment predictive biomarkers should process and send colorectal carcinoma specimens to reference molecular laboratories in a timely manner. Note: It is suggested that a benchmark of 90% of specimens should be sent out within 3 working days.	Expert consensus opinion
17. Pathologists must evaluate candidate specimens for biomarker testing to ensure specimen adequacy, taking into account tissue quality, quantity, and malignant tumor cell fraction. Specimen adequacy findings should be documented in the patient report.	Expert consensus opinion
18. Laboratories should use colorectal carcinoma molecular biomarker testing methods that are able to detect mutations in specimens with at least 5% mutant allele frequency, taking into account the analytical sensitivity of the assay (limit of detection or LOD) and tumor enrichment (eg, microdissection). Note: It is recommended that the operational minimal neoplastic carcinoma cell content tested should be set at least two times the assay's LOD.	Expert consensus opinion
19. Colorectal carcinoma molecular biomarker results should be made available as promptly as feasible to inform therapeutic decision making, both prognostic and predictive. Note: It is suggested that a benchmark of 90% of reports be available within 10 working days from date of receipt in the molecular diagnostics laboratory.	Expert consensus opinion
20. Colorectal carcinoma molecular biomarker testing reports should include a results and interpretation section readily understandable by oncologists and pathologists. Appropriate Human Genome Variation Society and Human Genome Organisation nomenclature must be used in conjunction with any historical genetic designations.	Expert consensus opinion

(continued)

Table 4 (cont)

Guideline Statement	Strength of Recommendation
21. Laboratories must incorporate colorectal carcinoma molecular biomarker testing methods into their overall laboratory quality improvement program, establishing appropriate quality improvement monitors as needed to ensure consistent performance in all steps of the testing and reporting process. In particular, laboratories performing colorectal carcinoma molecular biomarker testing must participate in formal proficiency testing programs, if available, or an alternative proficiency assurance activity.	Strong recommendation

EGFR, epidermal growth factor receptor; IHC, immunohistochemistry; MMR, mismatch repair; PTEN, phosphatase and tensin homolog.

A large body of evidence was available to guide the recommendation in the current guideline for *RAS* testing in colorectal cancers (Table 5 and Supplemental Table 14; all supplemental materials can be found at *American Journal of Clinical Pathology* online). From 2008 to 2015, there were 311 primary studies that included 74,546 patients and reported treatment outcomes for patients with *RAS* mutations compared with nonmutated/wild type.^{12-16,18-45} The most common comparison of anti-EGFR antibody treatment outcomes was between *KRAS* mutation vs *KRAS* nonmutated/wild type.^{18-20,22,24-26,28-31,33-42} Some studies also compared the effects of adding an anti-EGFR inhibitor to *KRAS* nonmutated/wild-type patients vs chemotherapy alone.^{18,22,24,26,28,36-38} A few studies reported anti-EGFR antibody treatment outcomes for the following comparisons: *KRAS* G13D vs codon 12 mutations,³² *KRAS* codon 13 mutations vs other mutations,²¹ and G13D vs other exon 2 mutations.²³

The reported anti-EGFR therapy outcomes in these studies were pooled survival,^{13-16,21-27,29,32-37,39,41} pooled PFS,^{13,15,16,18,21-27,29,31-36,39,41} and pooled objective response rate (ORR).^{13,15,16,18,21,22,25,26,30-36,41} Thirteen studies reported significant differences between comparators.^{15,21,23-27,32,33,35-37,39} The systematic review literature of data on anti-EGFR therapy outcomes is presented in Supplemental Table 14. Five of these studies detected a significant pooled survival advantage of anti-EGFR-treated patients for *KRAS* nonmutated/wild type compared with *KRAS* mutation.^{21,33,35,37,39} Three studies detected an advantage for patients with nonmutated tumors given anti-EGFR treatment compared with *KRAS* mutation-positive patients given chemotherapy alone.^{24,26,36} Twenty of the included studies pooled PFS,^{13,15,16,18,21-27,29,31-36,39,41} with 19 reporting significant differences between comparators.^{13,15,18,21-27,29,31-36,39,41} Fourteen papers detected a significant PFS advantage for adding an anti-EGFR inhibitor to chemotherapy for *KRAS* nonmutated/wild-type patients compared with chemotherapy alone.^{13,15,18,22,24-26,29,31,33,34,36,39,41} Sixteen of the included papers pooled ORR,^{13,15,16,18,21,22,25,26,30-36,41} with 14 reporting significant differences between comparators.^{15,18,21,22,25,26,30-36,41} Eight studies detected ORR advantages for adding an anti-EGFR inhibitor to

chemotherapy for patients with nonmutated/wild-type tumors compared with chemotherapy alone,^{18,25,26,30,33,34,36,41} and four detected an ORR advantage for *KRAS* nonmutated/wild-type patients over mutation patients.^{22,31,32,35} Survival advantages (OS and PFS, ORR) for G13D mutations over codon 12 and G13D over other mutations were reported in two studies^{23,32} and codon 13 over other *KRAS* mutations.²¹

Recent studies showed conclusive evidence that in addition to mutations in *KRAS* exon 2, other *RAS* mutations in *KRAS* exons 3 and 4 and *NRAS* exons 2, 3, and 4 were also associated with nonresponse of metastatic CRC to anti-EGFR monoclonal antibody therapy.^{12,44,46} Douillard et al⁴⁴ published a reanalysis of the Panitumumab Randomized Control Trial in Combination with Chemotherapy for Metastatic Colorectal Cancer to Determine Efficacy (PRIME) trial, reporting that patients with any *RAS* mutations were associated with inferior PFS and OS with panitumumab-FOLFOX4 treatment, which was consistent with the findings previously reported for patients with *KRAS* mutations in exon 2. Subsequently, a meta-analysis of nine randomized clinical trials provided further evidence that not all *KRAS* exon 2 nonmutated/wild-type tumors benefit from anti-EGFR monoclonal antibody treatment in metastatic CRC.¹² Patients with colorectal cancers that are *KRAS* exon 2 nonmutated/wild type but harbor *RAS* mutations in *KRAS* exons 3 and 4 or *NRAS* exons 2, 3, and 4 also have significantly inferior anti-EGFR treatment outcomes benefit compared with those without any *RAS* mutations (Table 5 and Table 6). *RAS* mutations occur mostly at exon 2, followed by mutations in exons 3 and 4 (Table 7). The results suggest that “extended” or “expanded” *RAS* mutation testing (*KRAS* exons 2, 3, and 4 and *NRAS* exons 2, 3, and 4) must be performed before the administration of an anti-EGFR monoclonal antibody therapy.¹² In summary, current evidence indicates that both cetuximab and panitumumab should only be prescribed for patients with metastatic CRCs that are nonmutated/wild type for all known *RAS*-activating mutations.¹²

This recommendation is supported by 34 studies,^{12-16,18-45,47} comprising 29 systematic studies,^{12,13,15,16,18-22,24-42,47} two meta-analyses,^{14,23} one randomized controlled trial,⁴⁴ one prospective cohort study,⁴⁵ and one retrospective cohort study.⁴³

Table 5
KRAS Clinical Practice Guidelines, Systematic Reviews, Meta-Analyses, Prospective Cohort Studies, and Retrospective Cohort Studies

Author, Year	No. of Studies (No. of Patients)	Comparison	Tests Used	Codons Studied	OS	PFS	ORR
CPGs, systematic reviews, and meta-analyses on <i>KRAS</i> mutation+ vs mutation- (n = 30)							
Petrelli et al, ³⁵ 2013	SR: 12 studies including 2,226 patients with mCRC treated with bevacizumab	Mut+ vs Mut-	NR	NR	Median, HR, 0.65; 95% CI, 0.46-0.92; <i>P</i> < .05, in favor of Mut-	Median PFS, HR, 0.85; 95% CI, 0.74-0.98; <i>P</i> < .05, in favor of Mut-	<i>KRAS</i> Mut+: 48.3% vs <i>KRAS</i> Mut-: 54.8% (OR, 1.42; 95% CI, 1.05-1.92; <i>P</i> < .05)
Mao et al, ³² 2013	SR: 10 studies including 1,487 patients with mCRC treated with cetuximab	p.G13D vs codon 12 Mut+	NR	G13D, 12	HR, 0.52; 95% CI, 0.33-0.80, <i>P</i> < .05, in favor of G13D	PFS, HR, 0.54; 95% CI, 0.36-0.81, <i>P</i> < .05, in favor of G13D	<i>KRAS</i> pG13D: 22% <i>KRAS</i> 12: 16% <i>KRAS</i> Mut-: 44% (pG13D vs 12: RR, 1.64; 95% CI, 1.13-2.38; <i>P</i> < .05) pG13D vs Mut-: RR, 0.54; CI, 0.38-0.77; <i>P</i> < .05)
Jiang et al, ²⁷ 2013	SR: 13 studies including 1,174 patients with mCRC treated with cetuximab or panitumumab	Increased vs not increased EGRF GCN	FISH, CISH, SISH, qPCR	NR	Increased GCN associated with improved OS among patients treated with anti-EGFR mAbs (HR, 0.62; 95% CI, 0.50-0.77; <i>P</i> < .05)	GCN associated with improved PFS (HR, 0.65; 95% CI, 0.47-0.89; <i>P</i> < .05)	NR
Hoyle et al, ²⁵ 2013	SR-HTA: 2 studies including EGFR-expressing mCRC patients total with cetuximab, bevacizumab, or panitumumab in the second-line and greater	Mut+ vs Mut-	NR	NR	Median, 9.5 months vs 4.8 months; HR, 0.55; 95% CI, 0.41-0.75, <i>P</i> < .05, in favor of cetuximab over BSC in Mut- Panitumumab + BSC compared with BSC alone in Mut-, <i>P</i> = ns	Median PFS, HR, 0.40; 95% CI, 0.30-0.54, <i>P</i> < .05, third-line cetuximab + BSC compared with BSC alone in Mut- Median PFS, HR, 0.45; 95% CI, 0.34-0.59, <i>P</i> < .05, panitumumab + BSC compared with BSC alone in Mut-	<i>KRAS</i> Mut-: 12.8% <i>KRAS</i> Mut+: 1.2%, <i>P</i> < .05, cetuximab + BSC compared with BSC alone in Mut- <i>KRAS</i> Mut-: 10% <i>KRAS</i> Mut+: 0, <i>P</i> < .05, panitumumab + BSC compared with BSC alone in Mut-
Chen et al, ²¹ 2013	SR: 7 studies including 2,802 patients with mCRC	Codon 13 Mut+ vs other mutations	PCR, direct sequencing	13, other Mut+, Mut-	Median OS: 14.6 months, codon 13 11.8 months (other mutation) 17.3 months, Mut-	Median PFS: 6.4 months, codon 13 4.1 months (other mutation) 6.6 months, Mut-	Codon 13 Mut+ vs other mutations: RR, 1.52 (95% CI, 1.10-2.09, <i>P</i> < .05) Codon 13 Mut+ vs Mut-: RR, 0.61 (95% CI, 0.45-0.83, <i>P</i> < .05)

(continued)

Table 5 (cont)

Author, Year	No. of Studies (No. of Patients)	Comparison	Tests Used	Codons Studied	OS	PFS	ORR
Zhou et al, ¹⁶ 2012	SR: 4 RCTs including 1,270 first-line patients with mCRC (all Mut-)	Oxaliplatin CT ± anti-EGFR mAbs + CT vs CT		Mut- only	HR, 1.00; 95% CI, 0.88-1.13, <i>P</i> = ns	HR, 0.86; 95% CI, 0.71-1.04, <i>P</i> = ns	RR, 1.08; 95% CI, 0.86-1.36, <i>P</i> = ns
Zhang et al, ⁴¹ 2011	SR: 4 studies including 2,912 patients with mCRC	Mut+ vs Mut-	NR	NR	Cetuximab + CT vs CT alone, Mut-: HR, 0.84; 95% CI, 0.64-1.11, <i>P</i> = ns Cetuximab + CT vs CT alone, mutation: HR, 1.03; 95% CI, 0.74-1.44, <i>P</i> = ns	Cetuximab + CT vs CT alone, Mut-: HR, 0.64; 95% CI, 0.50-0.84, <i>P</i> < .05, favors +cetuximab Cetuximab + CT vs CT alone, mutation: HR, 1.37; 95% CI, 0.81-2.31, <i>P</i> = ns	Cetuximab + CT vs CT alone: RR, 1.93; 95% CI, 1.14-3.26, <i>P</i> < .05, favors +cetuximab Cetuximab+CT vs CT alone, Mut-: RR, 1.44; 95% CI, 1.20-1.73, <i>P</i> < .05, favors +cetuximab
Yang et al, ⁴⁰ 2012	SR: 19 studies including 1,077 patients with mCRC	Mut+ vs Mut- GCN+ vs GCN-	FISH, qPCR, CISH	Exon 20	No pooling due to statistical heterogeneity	No pooling due to statistical heterogeneity	No pooling due to statistical heterogeneity
Vale et al, ³⁹ 2012	SR: 10 RCTs including 5,996 patients with advanced CRC	Mut+ vs Mut-	NR	NR	NR Third line, HR, 0.76; 95% CI, 0.62-0.92, <i>P</i> < .05	First/second line, PFS, HR, 0.83; 95% CI, 0.76-0.90, <i>P</i> < .05 Third line, PFS, HR, 0.43; 95% CI, 0.35-0.52, <i>P</i> < .05, in favor of anti-EGFR mAbs for Mut- only	NR
Tsoukalas et al, ³⁸ 2012	SR: 13 studies including 1,394 patients with CRC	Mut+ vs Mut- Response to cetuximab vs no response	NR	NR	NR	NR	NR
Ross et al, ⁴² 2012	SR: Six studies including 2,526 patients with mCRC	Mut+ vs Mut- Antibody vs control	Sanger, pyrosequencing, PCR, ARMS, Scorpion	NR	NR	NR	NR
Ren et al, ³⁷ 2012	SR: 23 studies including 1,362 patients with mutations (~100% at codons 12 and 13, n = 1 at codon 61)	Mut+ vs Mut-	— ^a	12, 13, 61	HR, 1.61; 95% CI, 1.19-2.18, <i>P</i> < .05, in favor of treatment in Mut- vs Mut+ patients	NR	NR
Petrelli et al, ³⁴ 2012	SR: 4 RCTs including 484 Mut- patients with mCRC	Mut+ vs Mut- Cetuximab and/or panitumumab + CT vs CT alone	NR	NR	<i>P</i> = ns	PFS, HR, 0.68, <i>P</i> < .05, in favor of adding cetuximab and/or panitumumab to CT in Mut- patients	RR, 1.67, <i>P</i> < .05, in favor of adding cetuximab and/or panitumumab to CT in Mut- patients

(continued)

Downloaded from https://academic.oup.com/ajcp/article-abstract/147/3/221/2967889 by Univ of North Carolina at Chapel Hill Health Sci Lib user on 12 August 2019

Table 5 (cont)

Author, Year	No. of Studies (No. of Patients)	Comparison	Tests Used	Codons Studied	OS	PFS	ORR
Modest et al, ¹⁴ 2012	M-A: 3 trials including 119 patients with mCRC with codon 12 mutations vs other mutations	Cetuximab ± CT	NR	12	<i>P</i> = ns	NR	NR
Loupakis et al, ³¹ 2012	SR: 8 trials including 6,609 patients with mCRC	Mut+ vs Mut-	NR	NR	NR	PFS, HR, 0.91; 95% CI, 0.84-0.99; <i>P</i> < .05, in favor of adding anti-EGFR mAbs to CT in Mut- patients (irinotecan favoring CT, <i>P</i> < .05)	RR, 1.17; 95% CI, 1.04-1.33; <i>P</i> < .05, in favor of <i>KRAS</i> Mut-
Ku et al, ²⁸ 2012	SR: 2 RCTs including 261 patients with mCRC	Cetuximab + 5FU with oxaliplatin vs irinotecan Mut+ vs Mut-	NR	NR	No pooling performed in this comparison AIO trial, <i>P</i> = ns CECOG trial, <i>P</i> < .05 in favor of cetuximab + FOLFOX in Mut- patients	No pooling performed in this comparison AIO trial, <i>P</i> = ns CECOG trial, <i>P</i> = ns in favor of cetuximab + FOLFOX in Mut- patients	NR
Petrelli et al, ¹⁵ 2011	SR: 7 trials including 5,212 patients with advanced CRC, <i>KRAS</i> Mut- only	Cetuximab or panitumumab + CT vs BSC	NR	NR	HR, 0.84; 95% CI, 0.73-0.98, <i>P</i> < .05, in favor of anti-EGFR mAbs vs no mAbs in Mut- patients	PFS, HR, 0.65; 95% CI 0.51-0.83, <i>P</i> < .05, in favor of anti-EGFR mAbs vs no mAbs in Mut- patients	RR, 1.69; 95% CI, 1.20-2.38; <i>P</i> < .05, in favor of anti-EGFR
Mao et al, ³³ 2012	SR: 13 studies including 576 patients with mCRC, all <i>KRAS</i> Mut- treated with anti-EGFR mAbs	Mut+ vs Mut-	Direct sequencing, survey analysis, allelic discrimination, Sanger	<i>PIK3CA</i> exon 9, 20	HR, 3.29; 95% CI, 1.60-6.74; <i>P</i> < .05	PFS, HR, 2.52; 95% CI, 1.33-4.78, <i>P</i> < .05, <i>PIK3CA</i> exon 20 mutations associated with significantly shorter PFS duration	RR, 0.25; 95% CI, 0.05-1.19; <i>P</i> < .05, <i>PIK3CA</i> exon 20 mutations associated with lower ORR
Lin et al, ²⁹ 2011	SR: 8 studies including 5,325 patients with advanced CRC	Mut+ vs Mut-	NR	NR	<i>P</i> = ns	PFS, HR, 0.66; 95% CI, 0.53-0.82, <i>P</i> < .05, in favor of adding anti-EGFR to CT in Mut- patients	NR
Ibrahim et al, ¹³ 2011	SR: 4 studies including 2,115 patients with mCRC with Mut- <i>KRAS</i>	Panitumumab-based treatment vs control	NR	NR	<i>P</i> = ns	PFS, HR, 0.58; 95% CI, 0.36-0.93; <i>P</i> < .05, in favor of adding panitumumab to CT in Mut- patients	OR, 1.08; 95% CI, 0.75-1.58; <i>P</i> = ns

(continued)

Table 5 (cont)

Author, Year	No. of Studies (No. of Patients)	Comparison	Tests Used	Codons Studied	OS	PFS	ORR
Dahabreh et al, ²² 2011	SR: 29 poolable studies including 5,032 patients with mCRC treated with anti-EGFR mAbs Mut+ vs Mut-	Mut+ vs Mut- Cetuximab or panitumumab + CT vs CT alone	NR	NR	HR, 1.30; 95% CI, 0.95-1.78, <i>P</i> = ns in Mut- patients	PFS, HR, 2.22; 95% CI, 1.74-2.84, <i>P</i> < .05, in favor of anti-EGFR + CT in Mut- patients only	Positive likelihood ratio, 7.35 (95% CI, 3.72-14.50) Negative likelihood ratio, 0.55 (95% CI, 0.49-0.61) <i>KRAS</i> mutations associated with higher likelihood of response failure
Baas et al, ²⁰ 2011	SR: 21 studies including ~1,213 patients with mCRC (one study, N = NR)	Concordance between <i>KRAS</i> Mut+/Mut- between primary and metastases	Sequencing, pyrosequencing, PCR-RFLP, SSCP, AS-PCR, ASO	<i>KRAS</i> , <i>PIK3CA</i> , <i>BRAF</i> , or of loss of PTEN	NR	NR	NR
Adelstein et al, ¹⁸ 2011	SR: 11 studies including 8,924 patients with mCRC treated with anti-EGFR mAbs	Mut+ vs Mut- Cetuximab or panitumumab + CT vs CT alone	NR	12, 13, 61	NR	PFS, HR, 0.80; 95% CI, 0.64-0.99, <i>P</i> < .05, in favor of anti-EGFR mAbs in Mut- patients	RD, 15%; 95% CI, 8%-22%, <i>P</i> < .05, in favor of <i>KRAS</i> Mut+ + anti-EGFR treatment
Qiu et al, ³⁶ 2010	SR: 22 studies including 2,188 patients with mCRC	Mut+ vs Mut- Cetuximab + CT vs CT alone	DS, surveyor analysis, qPCR, AD, melting curve analysis	Exon 1, 2	Median OS, 6.9 vs 13.5 months, HR, 2.17; 95% CI, 1.72-2.74, <i>P</i> < .05, longer median survival shown in Mut- patients who received anti-EGFR mAbs + CT	Median PFS, 3.0 vs 5.8 months, HR, 1.94; 95% CI, 1.62-2.33; <i>P</i> < .05, longer median PFS shown in Mut- patients who received anti-EGFR mAbs + CT	<i>KRAS</i> Mut-: 39% <i>KRAS</i> Mut+: 14% RR, 0.24; 95% CI, 0.16-0.38, <i>P</i> < .05
Health Quality Ontario, ²⁴ 2010	SR: 14 observational studies in patients with advanced CRC	Mut+ vs Mut- Cetuximab or panitumumab + CT vs CT alone	NR	NR	Mean OS, MD, -4.11; 95% CI, -5.60 to -2.62, <i>P</i> < .05, longer survival detected in Mut- patients treated with cetuximab + irinotecan	Mean PFS, MD, = -3.32; 95% CI, -4.86 to -1.78, <i>P</i> < .05, longer duration detected in Mut- patients treated with cetuximab + irinotecan	NR
Ibrahim et al, ²⁶ 2010	SR: 10 studies including 2,703 patients with mCRC	Mut+ vs Mut- Cetuximab + CT vs CT alone	NR	NR	<i>P</i> < .05, in favor of treatment with cetuximab + CT in Mut- patients	PFS, <i>P</i> < .05, in favor of treatment with cetuximab + CT in Mut- patients	OR, 2.10; 95% CI, 1.42-3.10, <i>P</i> < .05

(continued)

Downloaded from https://academic.oup.com/ajcp/article-abstract/147/3/221/2967889 by Univ of North Carolina at Chapel Hill Health Sci Lib user on 12 August 2019

Table 5 (cont)

Author, Year	No. of Studies (No. of Patients)	Comparison	Tests Used	Codons Studied	OS	PFS	ORR
De Roock et al, ²³ 2010	MA: 7 studies including 774 patients with mCRC who received cetuximab-based treatment ± CT	pG13D vs other mutation	NR	pG13D, 13	Median (95% CI): pG13D: 7.6 months (5.7-20.5) Other mutations: 5.7 months (4.9-6.8) Mut-: 10.1 months (9.4-11.3) <i>P</i> < .05, pG13D superior to other mutations	Median (95% CI) PFS: pG13D: 4.0 months (1.9-6.2) Other mutations: 1.9 months (1.8-2.8) Mut-: 4.2 months (3.9-5.4) <i>P</i> < .05, pG13D superior to other mutations	NR
Allegra et al, ¹⁹ 2009	SR: 5 RCTs including 627 patients with mCRC and 5 single-arm studies including 247 patients	Mut+ vs Mut-	PCR, direct sequencing	12, 13	No pooling was performed	No pooling was performed	No pooling was performed
Linardou et al, ³⁰ 2008	SR: 8 studies including 817 patients with mCRC (306 with <i>KRAS</i> mutations)	Mut+ vs Mut-	NR	12, 13, 61	NR	NR	
Sorich et al, ¹² 2015	SR: 9 RCTs including 5,948 patients with mCRC	Mut+ vs Mut- Anti-EGFR mAb treatment effect size between <i>RAS</i> subgroups, including Mut+ vs Mut-	Bidirectional Sanger sequencing, pyrosequencing, MALDI-TOF analysis, and WAVE-based Surveyor analysis	<i>KRAS</i> / <i>NRAS</i> 12, 13, 59, 61, 117, 146	<i>RAS</i> Mut- vs <i>RAS</i> Mut+: HR, 0.72 (95% CI, 0.56-0.92; <i>P</i> < .01) <i>RAS</i> Mut- superior <i>KRAS</i> exon 2 mutant vs new <i>RAS</i> mutant: <i>P</i> = ns <i>RAS</i> Mut-, anti-EGFR vs no anti-EGFR: HR, 0.87 (95% CI, 0.77-0.99; <i>P</i> < .04) <i>KRAS</i> exon 2 Mut-, anti-EGFR vs no anti-EGFR: HR, 0.90 (95% CI, 0.83-0.98; <i>P</i> = ns) Any <i>RAS</i> mutant, anti-EGFR vs no anti-EGFR: HR, 1.08 (95% CI, 0.97-1.21; <i>P</i> = ns) <i>KRAS</i> exon 2 mutant, anti-EGFR vs no anti-EGFR: HR, 1.05 (95% CI, 0.95-1.17; <i>P</i> = ns)	<i>RAS</i> Mut- vs <i>RAS</i> Mut+: HR, 0.60 (95% CI, 0.48-0.76; <i>P</i> < .001) <i>RAS</i> Mut- superior <i>KRAS</i> exon 2 mutant vs new <i>RAS</i> mutant: <i>P</i> = ns <i>RAS</i> Mut-, anti-EGFR vs no anti-EGFR: HR, 0.62 (95% CI, 0.50-0.76; <i>P</i> < .001) <i>KRAS</i> exon 2 Mut-, anti-EGFR vs no anti-EGFR: HR, 0.68 (95% CI, 0.58-0.80; <i>P</i> < .001) Any <i>RAS</i> mutant, anti-EGFR vs no anti-EGFR: HR, 1.12 (95% CI, 0.94-1.34; <i>P</i> = ns) <i>KRAS</i> exon 2 mutant, anti-EGFR vs no anti-EGFR: HR, 1.14 (95% CI, 0.95-1.36; <i>P</i> = ns)	NR

(continued)

Table 51 (cont)

Author, Year	No. of Studies (No. of Patients)	Comparison	Tests Used	Codons Studied	OS	PFS	ORR
Randomized controlled trials (n = 1)							
Douillard et al, ⁴⁴ 2013	RCT: reanalysis of PRIME trial (NCT: 00364013) data, including 1,060 patients	RAS Mut± and FOLFOX4 ± anti-EGFR mAb	PCR, Sanger, Surveyor	KRAS/NRAS 12, 13, 61, 117, 146	Mut± and anti-EGFR mAb±: 26 months vs 20.2 months HR, 0.78 (95% CI, 0.62-0.99; P < .05) in favor of Mut- and + anti-EGFR mAb	Mut± and anti-EGFR mAb±: 10.1 months vs 7.9 months HR, 0.72 (95% CI, 0.58-0.90; P < .05) in favor of Mut- and + anti-EGFR mAb	NR
Prospective cohort studies (n = 1)							
Etienne-Grimaldi et al, ⁴⁵ 2014	251 patients	KRAS Mut+ vs KRAS Mut-	NR	KRAS 12, 13	NR	RR, 2.40 (95% CI, 1.27-4.55; P < .05), RFS shorter in KRAS Mut+ patients with stage III tumors	NR
Retrospective cohort studies (n = 1)							
Bando et al, ⁴³ 2013	82 samples from 376 patients	All Mut- vs KRAS 12, 13 vs KRAS 61, 146	Luminex xMAP vs DS (concordance rate 100%)	KRAS 12, 13, 61, 146	All Mut-: 13.8 months (9.2-18.4) vs KRAS Mut+: 8.2 months (5.7-10.7; P < .05)	All Mut-: 6.1 months (3.1-9.2) vs KRAS Mut+: 2.7 months (1.2-4.2; P < .05)	All Mut-: 38.8% vs KRAS Mut+: 4.8%, P < .05

AD, allelic discrimination; AIO, German AIO colorectal study group; ARMS, amplification refractory mutation system; AS-PCR, allele-specific polymerase chain reaction; ASO, allele-specific oligonucleotide; BRAF, proto-oncogene B-Raf/v-Raf murine sarcoma viral oncogene homolog B; BSC, best supportive care; CECOG, Central European Cooperative Oncology Group; CI, confidence interval; CISH, chromogenic in situ hybridization; CPG, clinical practice guideline; CRC, colorectal cancer; CT, chemotherapy; DS, direct sequencing; EGFR, epidermal growth factor receptor; FOLFOX4, folacin, 4-fluorouracil, oxaliplatin; FISH, fluorescence in situ hybridization; FOLFOX, folinic acid (leucovorin calcium), 5-fluorouracil, and oxaliplatin; 5FU, fluorouracil; GCN, gene copy number; HR, hazard ratio; HTA, health technology assessment; KRAS, Kirsten rat sarcoma viral oncogene homolog; M-A, meta-analysis; mAbs, monoclonal antibodies; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; mCRC, metastatic colorectal cancer; MD, mean difference; Mut-, mutation negative or wild type; Mut+, mutation positive; NR, not reported; NRAS, neuroblastoma RAS viral (v-ras) oncogene homolog; ns, nonsignificant; OR, odds ratio; ORR, objective response rate; OS, overall survival; PCR, polymerase chain reaction; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism; PFS, progression-free survival; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; PRIME, Panitumumab Randomized Control Trial in Combination with Chemotherapy for Metastatic Colorectal Cancer to Determine Efficacy; PTEN, phosphatase and tensin homolog; qPCR, quantitative polymerase chain reaction; RCT, randomized controlled trial; RAS, rat sarcoma viral oncogene homolog; RD, risk difference; RFS, recurrence-free survival; RR, response rate; SISH, silver in situ hybridization; SR, systematic review; SSCP, single-strand conformation polymorphism; xMAP, multiplex assay.

*Tests used by Ren et al³⁷: hybridization, PCR, direct sequencing, topographic genotyping, AS-PCR, tissue transglutaminase enzyme, high-performance liquid chromatography, pyrosequencing, capillary sequencing.

Of the 29 systematic reviews,^{12,13,15,16,18-22,24-42,47} only three reported using a multidisciplinary panel,^{19,25,30} and only one reported taking patient preferences into account,³⁷ although 13 examined important patient subtypes.^{12,15,16,18,21,22,24,27,30,33,37,39,40} All but one had well-described and reported methods sections.⁴² Seven did not report on conflict of interest.^{13,15,16,34,38,41,42} Only nine rated the quality of the included evidence, and these same nine were the only ones that reported on the strength of the included evidence.^{16,18,21,22,24,25,32,37,39} None of the studies included a plan for updating. None of the systematic reviews reported industry funding, two reported no funding,^{16,31} and 11 did not report on the source of funding, if any.^{13,15,26,32,34-36,38,41,42,47} Two of these systematic reviews were deemed to have a low risk of bias,^{24,37} 14

were deemed to have a low to moderate risk of bias,^{12,16,18,19,21,22,25,27,29,30,32,35,39,47} 12 were deemed to have a moderate risk of bias,^{13,15,20,26,28,31,33,34,36,38,40,41} and one was deemed to have a high risk of bias.⁴²

Of the two meta-analyses obtained,^{14,23} both had well-reported and reproducible methods sections, both described the planned pooling a priori, and both discussed the limitations of their analyses. Neither was based on a systematic review of the literature, and neither did a quality assessment of the included studies. One reported nonindustry funding,²³ and the other reported industry funding.¹⁴ One was deemed to have a low to moderate risk of bias,²³ and the other was deemed to have a moderate risk of bias.¹⁴

The single randomized controlled trial did not report on any details of the randomization, including blinding, the

Table 6
Outcomes of RAS Mutations and Anti-EGFR Therapy¹²

Characteristic	Overall Survival		Progression-Free Survival	
	HR (95% CI)	P Value	HR (95% CI)	P Value
RAS nm vs RAS mutation, RAS nm superior	0.72 (0.56-0.92)	<.01	0.60 (0.48-0.76)	<.001
KRAS exon 2 mutant vs new RAS mutant		ns		ns
KRAS nm exon 2, anti-EGFR vs no anti-EGFR	0.90 (0.83-0.98)	ns	0.68 (0.58-0.80)	<.001
KRAS exon 2 mutant, anti-EGFR vs no anti-EGFR	1.05 (0.95-1.17)	ns	1.14 (0.95-1.36)	ns
RAS nm, anti-EGFR vs no anti-EGFR	0.87 (0.77-0.99)	<.04	0.62 (0.50-0.76)	<.001
Any RAS mutant, anti-EGFR vs no anti-EGFR	1.08 (0.97-1.21)	ns	1.12 (0.94-1.34)	ns

CI, confidence interval; EGFR, epidermal growth factor receptor; HR, hazard ratio; KRAS, Kirsten rat sarcoma viral oncogene homolog; nm, nonmutated; ns, nonsignificant; RAS, rat sarcoma viral oncogene homolog.

Table 7
Prevalence of New RAS Mutations Across Studies^a

Study	New RAS Total, ^b %	KRAS Exon 3, ^b %	KRAS Exon 4, ^b %	NRAS Exon 2, ^b %	NRAS Exon 3, ^b %	NRAS Exon 4, ^b %
		Codons 59, 61	Codons 117, 146	Codons 12, 13	Codons 59, 61	Codons 117, 146
OPUS	26.3	5.9	9.3	6.8	5.1	0.8
PICCOLO	9.8	NR ^c	3.7 ^d	6.3 ^e	NR ^c	NE
20020408	17.6	4.8 ^c	5.0	4.2	3.0 ^c	1.1
20050181	20.5	4.6	7.9	2.3	5.8	0.0
PRIME	17.4	3.7 ^c	5.6	3.4	4.1 ^c	0.0
FIRE-3	16.0	4.3 ^c	4.9 ^d	3.8	2.0 ^c	0.0
PEAK	20.1	4.1	7.7	5.4	5.9	0.0
COIN	8.4	2.1 ^c	NE	0.9 ^f	3.0 ^c	NE
CRYSTAL	14.7	3.3	5.6	3.5	2.8	0.9
Summary (95% CI) ^g	19.9 (16.7-23.4)	4.3 (3.3-5.5)	6.7 (5.7-7.9)	3.8 (3.0-4.8)	4.8 (3.4-6.8)	0.5 (0.2-1.2)

CI, confidence interval; COIN, Combination Chemotherapy With or Without Cetuximab as First-Line Therapy in Treating Patients With Metastatic Colorectal Cancer Trial; CRYSTAL, Cetuximab Combined with Irinotecan in First-Line Therapy for Metastatic Colorectal Cancer Trial; FIRE-3, FIRE-3, Folinic Acid and Irinotecan (FOLFIRI) Plus Cetuximab vs FOLFIRI Plus Bevacizumab in First-Line Treatment Colorectal Cancer (CRC) Trial; NA, not applicable; NE, not evaluated; NR, evaluated but not reported; OPUS, Effect of Roflumilast on Exacerbation Rate in Patients With Chronic Obstructive Pulmonary Disease (BY217/M2-111) Trial; PEAK, Panitumumab Plus mFOLFOX6 vs Bevacizumab Plus mFOLFOX6 for First-Line Treatment of Metastatic Colorectal Cancer (mCRC) Patients With Wild-Type Kirsten Rat Sarcoma-2 Virus (KRAS) Tumors Trial; PICCOLO, Panitumumab and Irinotecan vs Irinotecan Alone for Patients With KRAS Wild-Type, Fluorouracil-Resistant Advanced Colorectal Cancer Trial; PRIME, Panitumumab Randomized Trial in Combination With Chemotherapy for Metastatic Colorectal Cancer to Determine Efficacy Trial.

^aModified from Sorich et al¹² by permission of Oxford University Press on behalf of the European Society for Medical Oncology.

^bNew RAS mutations are reported as a proportion of the KRAS exon 2 nonmutated/wild-type group.

^cKRAS and NRAS codon 59 mutation was not evaluated.

^dKRAS codon 117 mutation was not evaluated.

^eExon 3 codon 61 mutations in addition to the exon 2 mutations.

^fOnly NRAS mutation G12C evaluated.

^gRandom-effects meta-analysis summary estimates.

expected effect size and power calculation, and the length of follow-up.⁴⁴ It did report on differences in baseline patient characteristics. This trial did report at least partial industry funding and was deemed to have a low to moderate risk of bias.⁴⁴

The single prospective cohort study reported a balance between treatment and assessment groups, reported on baseline characteristics, and made adjustments in the analysis when differences were found.⁴⁵ It reported non-industry funding and was deemed to have a low risk of bias.⁴⁵

The single retrospective cohort study reported that the treatment and assessment groups were in balance and also reported on baseline patient characteristics.⁴³ It did not report that adjustments were made in the analysis to account for differences, where differences were found. This study

reported nonindustry funding and was deemed to have a low risk of bias.⁴³

All of the evidence that supported this recommendation was assessed, and none was found to have methodologic flaws that would raise concerns about their findings.

2a. Recommendation: BRAF p.V600 (BRAF c.1799 [p.V600]) position mutational analysis should be performed in CRC tissue in selected patients with colorectal carcinoma for prognostic stratification.

BRAF activating mutations occur in about 8% of advanced disease patients with CRC^{47,48} and in approximately 14% of patients with localized stage II and III CRC.^{8,49} As such, mutations in BRAF constitute a substantial subset of patients with CRC. The key questions related to BRAF mutations are whether patients whose cancers

Table 8
BRAF Clinical Practice Guidelines, Systematic Reviews, Meta-Analyses, Prospective Cohort Studies, and Retrospective Cohort Studies

Author, Year	Study Type and Evidence	Comparison	Tests Used	Codons Studied	OS	PFS	ORR
CPGs, systematic reviews, and meta-analyses (n = 8)							
Parsons et al, ⁵² 2012	SR: 36 studies including 4,562 CRC tumors (<i>BRAF</i>), 43 studies including 2,975 CRC tumors (<i>MLH1</i>)	Correlation study	NR	<i>BRAF</i> p.V600E, <i>MLH1</i>	NR	NR	NR
Mao et al, ⁵¹ 2011	SR: 11 studies including 1,046 patients with mCRC	Mut+ vs Mut-	NR	V600E	NR	NR	<i>BRAF</i> Mut+: 0 <i>BRAF</i> Mut-: 36.3%; <i>P</i> < .05; RR, 0.14; 95% CI, 0.04-0.53
Lin et al, ⁵⁰ 2011	SR: 1 study of 649 patients with mCRC, all <i>KRAS</i> Mut-; 6.5% were <i>BRAF</i> Mut+	Mut+ vs Mut-	NR	V600E	Shorter duration in <i>BRAF</i> Mut+ patients, difference 28 weeks, <i>P</i> < .05	PFS, shorter duration in <i>BRAF</i> Mut+ patients, difference 18 weeks, <i>P</i> < .05	NR
Baas et al, ²⁰ 2011	SR: 7 studies including 538 patients with mCRC	Mut+ vs Mut-	Sequencing, pyrosequencing	V600E	NR	NR	NR
Cui et al, ⁵³ 2014	SR: 4 studies including 1,245 patients	Mut+ vs Mut- CT± anti-EGFR mAbs	PCR	V600E	NR	NR	Mut+ vs Mut- (all <i>KRAS</i> Mut-): RR, 0.43 (95% CI, 0.16-0.75; <i>P</i> < .05) in favor of Mut- Mut± vs CT± anti-EGFR mAbs (all <i>KRAS</i> Mut-): RR, 0.38 (95% CI, 0.20-0.73; <i>P</i> < .05) in favor of Mut- Mut+ and CT± anti-EGFR mAbs; <i>P</i> = ns Mut- and <i>KRAS</i> Mut- and CT± anti-EGFR mAbs: RR, 1.48 (95% CI, 1.28-1.71; <i>P</i> < .05) in favor of <i>BRAF</i> Mut- with CT+ anti-EGFR mAbs
Yang et al, ⁷¹ 2013	SR: 17 studies (patients, n = NR)	Mut+ vs Mut-	— ^a	V600E, 599, 466, 469	(7 studies) <i>BRAF</i> Mut±: HR, 2.74 (95% CI, 1.79-4.19; <i>P</i> < .05) in favor of <i>BRAF</i> Mut-	(8 studies) <i>BRAF</i> Mut±: HR, 2.59 (1.67, 4.03; <i>P</i> < .05) in favor of <i>BRAF</i> Mut-	<i>BRAF</i> Mut-: 46.4% <i>BRAF</i> Mut: 18.5% <i>P</i> < .05 in favor of <i>BRAF</i> Mut-

(continued)

Downloaded from https://academic.oup.com/ajcp/article-abstract/147/3/221/2967889 by Univ of North Carolina at Chapel Hill Health Sci Lib user on 12 August 2019

Table 8 (cont)

Author, Year	Study Type and Evidence	Comparison	Tests Used	Codons Studied	OS	PFS	ORR
Yuan et al, ⁴⁸ 2013	SR: 21 studies including 5,229 patients	Mut+ vs Mut-	NR	V600E	HR, 0.35 (95% CI, 0.29-0.42; <i>P</i> < .05) in favor of <i>BRAF</i> Mut-	HR, 0.38 (95% CI, 0.29-0.51; <i>P</i> < .05) in favor of <i>BRAF</i> Mut-	RR, 0.31 (95% CI, 0.18-0.53; <i>P</i> < .05) in favor of <i>BRAF</i> and <i>KRAS</i> Mut-
Xu et al, ⁴⁷ 2013	SR: 19 studies including 2,875 patients	Mut+ vs Mut-	NR	V600E, K601E (1 study), D549C (1 study)	HR, 2.85 (95% CI, 2.31-3.52; <i>P</i> < .05) in favor of <i>BRAF</i> Mut-	HR, 2.98 (95% CI, 2.07-4.27; <i>P</i> < .05) in favor of <i>BRAF</i> Mut-	ORR, 0.58 (95% CI, 0.35-0.94; <i>P</i> < .05) in favor of <i>BRAF</i> Mut-
Prospective cohort studies (n = 1)							
Etienne-Grimaldi et al, ⁴⁵ 2014	251 patients	Mut+ vs Mut-	NR	<i>BRAF</i> p.V600E	NR	Shorter RFS in <i>KRAS</i> Mut- and <i>BRAF</i> Mut- patients with stage III tumors (<i>P</i> < .05)	
Retrospective cohort studies (n = 1)							
Bando et al, ⁴³ 2013	82 samples from 376 patients	All Mut- vs <i>BRAF</i> Mut+ and <i>PIK3CA</i> Mut-	Luminex xMAP vs DS (concordance rate 100%)	600	All Mut-: 13.8 months (95% CI, 9.2-18.4) vs <i>BRAF/PIK3CA</i> Mut: 6.3 months (95% CI, 1.3-11.3; <i>P</i> < .05)	All Mut-: 6.1 months (95% CI, 3.1-9.2) vs <i>BRAF/PIK3CA</i> Mut+: 1.6 months (95% CI, 1.5-1.7; <i>P</i> < .05)	All Mut-: 38.8% vs <i>BRAF/PIK3CA</i> Mut+: 0%, <i>P</i> < .05

BRAF, proto-oncogene B-Raf/v-Raf murine sarcoma viral oncogene homolog B; CI, confidence interval; CPG, clinical practice guideline; CRC, colorectal cancer; CT, chemotherapy; DS, direct sequencing; EGFR, epidermal growth factor receptor; HR, hazard ratio; *KRAS*, Kirsten rat sarcoma viral oncogene homolog; mAbs, monoclonal antibodies; mCRC, metastatic colorectal cancer; *MLH1*, mutL homolog 1; Mut-, mutation negative or wild type; Mut+, mutation positive; NR, not reported; ns, nonsignificant; ORR, objective response rate; OS, overall survival; PCR, polymerase chain reaction; PFS, progression-free survival; *PIK3CA*, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; RR, response rate; RFS, recurrence-free survival; SR, systematic review; xMAP, multiplex assay.

⁴³Yang et al⁷¹: adenovirus-PCR pyrosequencing, allele-specific PCR, DS, PCR amplification, quantitative PCR, Sanger, real-time PCR, genotyping + DS, PCR clamping, melting curve analysis, DNA sequencing, and Taqman single-nucleotide polymorphism assay.

carry a *BRAF* mutation have a poorer outcome compared with *BRAF* mutation-negative tumors and whether the presence of a mutation predicts benefit from or lack thereof to anti-EGFR therapy.

Four systematic reviews^{20,50-52} and three systematic reviews that included meta-analyses^{47,48,53} pertaining to the prognostic and predictive value of *BRAF* mutations in patients with CRC were identified through our systematic review process (Table 8 and Supplemental Table 14). These studies revealed that patients with advanced CRC who possess a *BRAF* mutation have significantly poorer outcomes as measured by PFS and OS and have a decreased response rate to anti-EGFR therapy relative to those with nonmutated *BRAF*. Poorer OS was also demonstrated for those patients with earlier stage II and III CRC having a *BRAF* mutation^{8,54}; however, the poorer outcome appears to be primarily the result of decreased OS after relapse in these patients rather than a harbinger of an increased rate of relapse. Finally, while outcomes in advanced disease patients with *BRAF* mutations were poorer relative to nonmutation patients, the data were consistent with a modest beneficial impact from the use of

anti-EGFR agents relative to those patients whose tumors contained a *RAS* mutation.⁵⁵ In summary, patients with CRC that contains a *BRAF* mutation have a worse outcome relative to nonmutation patients. Selected patients for *BRAF* mutation testing include patients with metastatic disease, since these patients have particularly poor outcomes. It is important to know the *BRAF* c.1799 (p.V600) mutation status of a patient's CRC since standard therapy is not adequate for patients with metastatic disease and *BRAF* mutation. For these patients, some studies suggest the use of FOLFIRINOX (folinic acid [leucovorin calcium], 5-fluorouracil, irinotecan hydrochloride, and oxaliplatin) as first-line therapy, followed by enrollment in a clinical trial.⁵⁶ Furthermore, early clinical trials data suggest that the combination of a *BRAF* plus EGFR inhibitor appears to be effective in this population.⁵⁷⁻⁵⁹ Data in support of molecular testing for *BRAF* c.1799 (p.V600) mutations in CRC continue to emerge from clinical trials. A recent publication of the PETACC-8 (oxaliplatin, fluorouracil, and leucovorin with or without cetuximab in patients with resected stage III colon cancer randomised phase 3) trial reported that trials in the adjuvant setting should

Table 9
Summary of Frequencies of Tumor V600E Mutation Status^a

Sample Group	No. of Studies	Positive p.V600E, No.	Negative <i>BRAF</i> p.V600E, No.	<i>BRAF</i> p.V600E, % (95% CI)
Known negative MMR mutation status				
MSI-H known mutation status	11	115	216	36.10 (20.95-52.84)
<i>MLH1</i> methylation or <i>MLH1</i> loss of expression (known or assumes MSI-H status)	9	191	141	63.50 (46.98-78.53)
MSS	11	85	1,538	5.00 (3.55-6.68)
Known positive MMR mutation status				
All mutation carriers	26	4	546	1.40 (0.06-2.25)

BRAF, proto-oncogene B-Raf/v-Raf murine sarcoma viral oncogene homolog B; CI, confidence interval; MMR, mismatch repair; MSI-H, microsatellite instability high; *MLH1*, mutL homolog 1; MSS, microsatellite stable.

^aAdapted from Parsons et al⁵² by permission from BMJ Publishing Group Limited.

consider mismatch repair, *BRAF*, and *KRAS* status for stratification, since *BRAF* p.V600 and *KRAS* mutations were associated with shorter DFS and OS in patients with microsatellite-stable colon cancer but not in those with tumors with MSI.^{60,61}

This recommendation is supported by seven systematic reviews,^{20,47,48,50-53} three of which included meta-analysis.^{47,48,53} None of the systematic reviews reported the composition of their panel, so multidisciplinary panel representation could not be confirmed, and none reported patient representation on the panel. All but the systematic review reported by Baas et al²⁰ reported examining important patient subgroups. All of the systematic reviews reported well-described and reproducible methods. Three did not report how conflicts of interest were managed and reported on.^{47,51,53} Only two reported on a quality assessment of the included literature,^{48,50} and only one rated the strength of the evidence.⁵⁰ None reported a plan for updating. While none of the systematic reviews reported industry funding, one study did not report any funding support.⁴⁷ Overall, the risk of bias assessment for this body of evidence ranged from low^{48,50} to moderate,^{20,51,53} and none were found to have methodologic flaws that would raise concerns about their findings.

2b. Recommendation: *BRAF* p.V600 mutational analysis should be performed in dMMR tumors with loss of *MLH1* to evaluate for Lynch syndrome risk. Presence of a *BRAF* mutation strongly favors a sporadic pathogenesis. The absence of *BRAF* mutation does not exclude risk of Lynch syndrome.

dMMR occurs via several mechanisms. In sporadic CRC, dMMR is most frequently caused by epigenetic silencing through CpG methylation primarily of *MLH1*, with few cases resulting from somatic mutation of one of the MMR genes. In Lynch syndrome CRC, the underlying

mechanism is usually a germline mutation of one of the four (*MLH1*, *MSH2*, *MSH6*, and *PMS2*) mismatch repair genes and, in rare patients, a deletion involving *EPCAM* (epithelial cell adhesion molecule), a gene adjacent to *MSH2*, that leads to epigenetic inactivation of the *MSH2* gene. dMMR occurs in 15% to 20% of all colorectal cancers, and of these, about three-fourths are due to *MLH1* epigenetic silencing.^{5,62} dMMR underlies widespread mutations in the genome and MSI. *BRAF* p.V600 mutations rarely occur in patients with germline-based dMMR but have been reported in up to three-fourths of those with epigenetic MMR gene silencing (Table 8 and **Table 9**). Thus, testing for *BRAF* mutations serves as a means for distinguishing germline from epigenetic dMMR, particularly in those cases where the dMMR is the result of epigenetic silencing of *MLH1*. For tumors with a mutation in *BRAF* and dMMR, it may be concluded that the basis for their dMMR is less likely to be germline.^{5,52,62} In contrast, tumors with dMMR in the absence of a *BRAF* mutation may have either germline or an epigenetic (*MLH1* gene promoter hypermethylation) basis for the dMMR, and specific testing for *MLH1* promoter hypermethylation may be used to further refine the risk of Lynch syndrome before initiating definitive genetic testing. Identification of those patients with germline-based dMMR has clear implications for the patient's family members.

3. Recommendation: Clinicians should order mismatch repair status testing in patients with colorectal cancers for the identification of patients at high risk for Lynch syndrome and/or prognostic stratification.

The molecular pathology underlying most MSI tumors is somatically acquired CpG methylation of the promoter of the gene, *MLH1*. About three-fourths of colorectal cancers with MSI due to *MLH1* promoter hypermethylation will have an acquired *BRAF* mutation as well. The reason for this is not understood. Less than one-third of individuals

Table 10
Mismatch Repair/Microsatellite Instability Systematic Reviews

Author, Year	Study Type and Evidence	Comparison	Tests Used	OS	PFS
Guastadisegni et al, ⁷ 2010	SR: 31 studies including 12,782 patients with CRC	MSI vs MSS	MSI by PCR in all and IHC in 6 studies	OR, 0.6; 95% CI, 0.53-0.69, $P < .0001$, MSI is associated with longer survival	DFS, OR, 0.58; 95% CI, 0.47-0.72, $P < .0001$, MSI is associated with a longer PFS duration
Des Guetz et al, ⁶ 2009	SR: 7 studies including 3,690 patients with CRC on effect of adjuvant chemotherapy 1,444 treated with 5-FU-based therapy and 1,518 not treated	MSI vs MSS	PCR in all and IHC in 2 studies	MSI-H: HR, 0.70; 95% CI, 0.44-1.09, $P = \text{ns}$; no significant benefit of chemotherapy in MSI-H patients	MSI-H: RFS, HR, 0.96; 95% CI, 0.62-1.49, $P = \text{ns}$; no significant difference if treated or not treated MSI-H vs MSS: RFS, HR, 0.77; 95% CI, 0.67-0.87, $P < .05$, MSI patients had no effect of treatment compared with beneficial effect in MSS patients

CI, confidence interval; CRC, colorectal cancer; DFS, disease-free survival; 5-FU, 5-fluorouracil; HR, hazard ratio; IHC, immunohistochemistry; MSI, microsatellite instability; MSI-H, microsatellite instability high; MSS, microsatellite stable; ns, nonsignificant; OR, odds ratio; OS, overall survival; PCR, polymerase chain reaction; PFS, progression-free survival; RFS, relapse-free survival; SR, systematic review.

with dMMR/MSI colorectal tumors do not have underlying *MLH1* promoter hypermethylation but rather have a germline mutation affecting any one of the four DNA MMR genes noted above. Individuals with germline mutations in the MMR genes are said to have Lynch syndrome, an autosomal dominant disorder that confers dramatically increased risks for colorectal and endometrial cancers and moderately increases risks for a variety of other tumors.⁶³ Diagnosis of Lynch syndrome is important as active management of cancer risks has been demonstrated to benefit gene mutation carriers,^{5,64,65} and establishing a diagnosis creates opportunities for prevention among all at-risk relatives. Testing for dMMR can be performed by immunohistochemistry for the four MMR proteins (MLH1, MSH2, PMS2, and MSH6) or by MSI DNA-based testing, as discussed in detail in a report by Funkhouser et al⁶⁶ (recommendation 11).

A systematic review of 31 studies⁷ reporting survival on 12,782 patients whose tumors were characterized for MSI showed a favorable prognosis, as determined by both OS and DFS (Table 10), but this is dependent on stage. In addition, the presence of MSI in CRC was reported to be predictive for non-response to 5-fluorouracil-based adjuvant chemotherapy of early stage disease,⁶ although this has not been corroborated (Table 10).⁶⁷ Emerging data indicate that MMR status may have predictive value in some settings, specifically in patients with advanced disease being considered for anti-programmed cell death protein-1 (PD-1)/ programmed cell death ligand protein-1 (PD-L1) immune checkpoint inhibitor therapy.⁶⁸⁻⁷⁰

This recommendation is supported by two systematic reviews that included 38 studies and 16,472 patients.^{6,7} Both of these systematic reviews included a well-described and reproducible methods section, and both reported on

potential conflicts of interest. Only one, the systematic review reported by Guastadisegni et al,⁷ reported the source of funding, which was nonindustry. Due to deficits in the reporting, one of these systematic reviews was deemed to have a moderate risk of bias,⁶ and the other was deemed to have a low to moderate risk of bias⁷; however, neither of these were found to have any major methodologic flaws that would cause us to question their findings.

4. No Recommendation: There is insufficient evidence to recommend *BRAF* c.1799 (p.V600) mutational status as a predictive molecular biomarker for response to anti-EGFR inhibitors.

As noted in recommendation 2a, mutations in position p.V600 in *BRAF* are associated with poor prognosis, especially in patients with metastatic disease. Response rates to chemotherapy regimens, including regimens with cetuximab and panitumumab, are lower in patients harboring *BRAF* p.V600 mutations^{51,53,71} (Table 8). Similarly, the PFS and OS after treatment with EGFR monoclonal antibodies in combination with chemotherapy are lower in patients with *BRAF* p.V600 mutations.^{47,48} Many of these analyses used nonrandomized cohorts, thereby making evaluation of the potential predictive value of the *BRAF* p.V600 mutation impossible to discern (Table 8). In addition, the poor prognosis and low mutation prevalence make evaluation of the relative benefit of EGFR inhibitors difficult to evaluate in individual randomized clinical trials.

Meta-analyses of randomized studies of EGFR monoclonal antibodies have been completed to address the question of the predictive role of *BRAF* p.V600 mutations. A meta-analysis of 463 patients with *KRAS* wild-type and

BRAF p.V600 mutated tumors did not provide sufficient evidence to exclude a magnitude of benefits seen in *KRAS/BRAF* wild-type tumors. Nor was there sufficient evidence to identify a statistically significant benefit to this treatment.⁵⁵ A second meta-analysis showed that EGFR monoclonal antibody treatment in patients whose tumors contain a *BRAF* p.V600 mutation was not associated with significant OS ($P = .43$), although there was a trend for better PFS ($P = .07$).⁷² This suggests insufficient evidence to recommend the use of *BRAF* p.V600 as a predictive marker for benefit of anti-EGFR monoclonal antibodies. More data are required to definitively determine the predictive value of *BRAF* mutations relative to anti-EGFR therapy.

This recommendation was supported by five systematic reviews^{47,48,51,53,71} (Table 8). None of these systematic reviews reported forming a multidisciplinary panel, and none reported including patient representatives in developing their research questions or interpreting their outcomes. All of the systematic reviews examined important patient subtypes, and all used well-described and reproducible methods. Only the systematic review by Yuan et al⁴⁸ reported on any potential conflicts of interest, the article by Mao et al⁵¹ stated conflicts were not examined, and the other three did not report anything regarding conflicts.^{47,53,71} Only two, the systematic reviews reported by Yang et al⁷¹ and Yuan et al,⁴⁸ rated the quality of the included evidence, although none of the studies reported on the strength of the evidence. None of the studies discussed any plans for future updating. Four reported nonindustry funding for their systematic reviews,^{48,51,53,71} and one did not report the source of funding, if any.⁴⁷ Two of the systematic reviews were deemed to have a low risk of bias,^{48,71} one was deemed to have a low to moderate risk of bias,⁴⁷ and two were deemed to have a moderate risk of bias.^{51,53} Overall, none of the systematic reviews were found to have methodologic flaws that would raise concerns about their findings.

5. No Recommendation: There is insufficient evidence to recommend *PIK3CA* mutational analysis of colorectal carcinoma tissue for therapy selection outside of a clinical trial.

Note: Retrospective studies have suggested improved survival with postoperative aspirin use in patients whose colorectal carcinoma harbors a *PIK3CA* mutation.

Despite comprehensive *RAS* testing (recommendation 1), many patients still fail to respond to EGFR monoclonal antibody therapy. Additional biomarkers to guide patient selection for such therapy are desired.

PIK3CA mutations are observed in 10% to 18% of patients with CRC, primarily in exons 9 and 20, and lead to a constitutive activation of p100a enzymatic activity, leading to an increased PI3K activity and high oncogenic transformation ability. However, mutations of *KRAS* or *NRAS* and *PIK3CA* mutations can be detected alternatively and, in some

cases, concurrently in a single CRC.^{3,8} *PIK3CA* mutations are positively correlated with *KRAS* exon 12 and 13 mutations.³ Several meta-analyses and one individual patient data large pooled analysis have examined the prognostic role of *PIK3CA* in patients with stage IV CRC, both overall and in the *KRAS* nonmutated/wild-type population. These studies have generally indicated poorer response rate and PFS in patients with the *PIK3CA* mutation, a finding that appears to be driven primarily by patients with exon 20 mutation^{3,33,50,71}

Table 11. These meta-analyses have included many of the same studies, as well as observed and acknowledged between-study heterogeneity, and all have concluded further prospective data are necessary. Contradictory recent studies have also been recently reported.⁷⁴ None of the studies considered the independent role of *PIK3CA* in the context of comprehensive *RAS* testing. De Roock et al³ estimated that comprehensive *PIK3CA* testing would increase response rate in the first-line setting by only 1%. The prognostic impact of *PIK3CA* in stage I to III disease has been inconsistent.⁷⁵⁻⁷⁷

Multiple prospective observational studies have demonstrated an association between aspirin use and decreased CRC mortality.⁷⁸⁻⁸⁰ Data on aspirin as a treatment for CRC (postdiagnosis usage) are more limited and drawn only from observational studies. Domingo et al⁸¹ and Liao et al⁸² found a survival advantage for posttreatment aspirin users only in patients whose tumors exhibit *PIK3CA* mutations; however, a recent cohort study did not validate these observations.⁸³ Multiple prospective studies are under way to address the potential benefit of adding aspirin or other nonsteroidal anti-inflammatory drugs to adjuvant therapy.

This recommendation is supported by two systematic reviews^{33,40} obtained from our systematic review. None reported the composition of a multidisciplinary panel, reported patient representation or study quality, rated strength of the evidence reviewed, or disclosed a plan for updating. However, both systematic reviews did include relevant patient subgroups and included methods that were well described and reproducible. In both systematic reviews, information about the potential conflicts of the panelists was reported, and funding was provided by nonindustry sources. Both were found to have a moderate risk of bias, but neither of the studies providing the evidence base for recommendation 5 were found to have methodologic flaws that would raise concerns about their findings.

At the present time, the retrospective data for the use of *PIK3CA* mutation to deny anti-EGFR antibody therapy in patients with stage IV CRC or as a selection factor for use of aspirin in stage I to III tumors are insufficient for clinical use outside of a clinical trial.

6. No Recommendation: There is insufficient evidence to recommend PTEN analysis (expression by

Table 11

PIK3CA Clinical Practice Guidelines, Systematic Reviews, Meta-Analyses, Prospective Cohort Studies, and Retrospective Cohort Studies

Author, Year	Study Type and Evidence	Comparison	Tests Used	Codons Studied	OS	PFS	ORR
CPGs, systematic reviews, and meta-analyses on <i>PIK3CA</i> Mut+ vs Mut- (n = 5)							
Wu et al, ⁷³ 2013	SR: 8 839 patients with mCRC who all received anti-EGFR mAbs	Mut+ vs Mut-	Sanger, allelic discrimination, direct sequencing, pyrosequencing	Exons 9, 20	HR, 1.28; 95% CI, 1.05-1.56, <i>P</i> < .05, patients with <i>PIK3CA</i> Mut+ had shorter PFS	PFS, HR, 1.53; 95% CI, 1.28-1.84, <i>P</i> < .05, patients with <i>PIK3CA</i> Mut+ had shorter PFS	NR
Mao et al, ³³ 2012	SR: 13 studies including patients all <i>KRAS</i> Mut- treated with anti-EGFR mAbs	E20 Mut+ vs E20 Mut-	NR	Exon 20	HR, 3.29; 95% CI, 1.60-6.74; <i>P</i> < .05	PFS, HR, 2.52; 95% CI, 1.33-4.78, <i>P</i> < .05, <i>PIK3CA</i> exon 20 mutations associated with shorter PFS	ORR%: Exon 20 Mut+: 0; Exon 20 Mut-: 37% RR, 0.25; 95% CI, 0.05-1.19, <i>P</i> = ns (subset: 377 patients)
Lin et al, ⁵⁰ 2011	SR: 4 studies 1,030 patients with mCRC, all <i>KRAS</i> Mut- subgroup analysis, exons 9 and 20	Mut+ vs Mut-	NR	Exons 9, 20	<i>P</i> = ns, no difference between Mut+ and Mut- patients Exon 20 Mut+ predicts poorer survival	<i>P</i> = ns, no difference between Mut+ and Mut- patients Exon 20 Mut+ predicts poorer survival	NR
Baas et al, ²⁰ 2011	SR: 3 studies including 195 patients with mCRC	Mut+ vs Mut-	Sequencing, pyrosequencing	Exons 9, 20	NR	NR	NR
Yang et al, ⁷¹ 2013	SR: 10 studies (patient number = NR)	Mut+ vs Mut-	DS, PCR amplification, AS-PCR, genotyping, RT-PCR, Sanger, DNA sequencing, pyrosequencing	Exons 7, 8, 9, 18, 19, 20	(6 studies) HR, 1.43 (95% CI, 1.02-2.0; <i>P</i> < .05) in favor of Mut-	(6 studies) HR, 1.91 (95% CI, 0.78-4.68; <i>P</i> = ns) <i>P</i> < .05 in favor of exon 9 compared with exon 20 mutations	(6 studies) RD: -23% (-35%, -10%; <i>P</i> < .05) in favor of exon 9 compared exon 20 mutations
Retrospective cohort studies (n = 1)							
Bando et al, ⁴³ 2013	82 samples from 376 patients	All Mut- vs <i>BRAF</i> Mut+ and <i>PIK3CA</i> Mut+	Luminex xMAP vs DS (concordance rate 100%)	Exon 9	All Mut-: 13.8 months (95% CI, 9.2-18.4) vs <i>BRAF/PIK3CA</i> Mut+: 6.3 months (95% CI, 1.3-11.3; <i>P</i> < .05)	All Mut-: 6.1 months (95% CI, 3.1-9.2) vs <i>BRAF/PIK3CA</i> Mut+: 1.6 months (95% CI, 1.5-1.7; <i>P</i> < .05)	All Mut-: 38.8% vs <i>BRAF/PIK3CA</i> Mut+: 0, <i>P</i> < .05

AS-PCR, allele-specific polymerase chain reaction; CI, confidence interval; CPG, clinical practice guideline; DS, direct sequencing; EGFR, epidermal growth factor receptor; HR, hazard ratio; mAbs, monoclonal antibodies; mCRC, metastatic colorectal cancer; Mut-, mutation negative or wild type; Mut+, mutation positive; NR, not reported; ns, non-significant; ORR, objective response rate; OS, overall survival; PCR, polymerase chain reaction; PFS, progression-free survival; *PIK3CA*, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; RD, risk difference; RR, response rate; RT-PCR, reverse transcription polymerase chain reaction; SR, systematic review; xMAP, multiplex assay.

immunohistochemistry [IHC] or deletion by fluorescence in situ hybridization [FISH]) in colorectal carcinoma tissue for patients who are being considered for therapy selection outside of a clinical trial.

PTEN functions as a tumor suppressor gene, and loss of PTEN results in upregulation of the PI3K/AKT pathway. *PTEN* mutations occur in approximately 5% to 14% of

colorectal cancers,^{4,84} and loss of *PTEN* expression can be observed in tumors with *KRAS*, *BRAF*, and *PIK3CA* mutations.

Although there is evidence suggesting that PTEN is a critical factor in cancer development, the association between PTEN expression and predictive/prognostic value remains controversial, with several studies suggesting an

association with poorer prognosis and others finding no association at all. Four systematic reviews were obtained that reported on loss of PTEN expression compared with normal PTEN expression and 31 primary studies, including a total of 2,545 patients^{20,50,85,86} (Supplemental Table 14). Tests used included IHC and FISH. Of the four studies that reported overall survival rates,^{20,50,85,86} three studies reported on pooled outcomes.^{50,85,86} One study reported a significant difference in favor of normal PTEN expression,⁸⁶ and the others reported no significant differences.^{20,50,85} For PFS, three studies pooled outcomes,^{50,85,86} two detected a significant difference in favor of normal PTEN expression,^{85,86} and one showed no significant difference.⁵⁰ For ORR, two studies pooled outcomes, and both found loss of PTEN expression associated with a poorer response.^{85,86}

Several studies have shown an association between PTEN loss and local recurrence, advanced TNM stage, lymph node metastasis, and a lower 5-year survival rate.⁸⁷⁻⁹⁰ However, several other studies have found no correlation between PTEN status and patient survival, tumor grade, TNM stage, lymphatic invasion, and liver metastasis.⁹¹⁻⁹³ Regarding response to EGFR-targeted therapies, several studies have shown an association with PTEN loss and lack of response to cetuximab and panitumumab.⁹⁴⁻⁹⁷ However, other published studies failed to demonstrate a clear correlation between loss of PTEN expression and response to anti-EGFR therapy.⁹⁸⁻¹⁰⁰ Given the significant discordance in results, the role of PTEN as a prognostic or predictive biomarker in CRC is still largely unknown, and research into the prognostic and predictive significance of PTEN is ongoing.

This recommendation is supported by 20 studies,^{4,20,50,84-100} four^{20,50,85,86} of which met the inclusion criteria for inclusion in our systematic review. All four of these were systematic reviews and included 42 studies and 3,412 patients. None of these systematic reviews reported using a multidisciplinary panel or reported including the patient perspective or a plan for future updating. Three^{50,85,86} reported on important patient subgroups. All four had well-described and reproducible methods sections. Three^{20,50,86} reported that potential conflicts of interest were examined. Only two^{50,86} rated the quality of the included evidence, and these same two were also the only two that rated the strength of the evidence. Only three^{20,50,86} reported on the source of any funding, but all three reported nonindustry funding. One was deemed to have a low risk of bias,⁵⁰ one was deemed to have a low to moderate risk of bias,⁸⁶ and two were deemed to have a moderate risk of bias.^{20,85} None of the studies were found to have any methodologic flaws that would bring doubt to their findings.

Table 12
Concordance Rates Between Primary and Metastatic Lesions^a

Genes Tested (n)	Concordance Rate, %
<i>KRAS</i> (117) ¹⁰¹	91.0
<i>KRAS</i> , <i>NRAS</i> , <i>BRAF</i> (84) ¹⁰²	98.8
<i>PIK3CA</i> (117) ¹⁰¹	94.0
<i>PIK3CA</i> (84) ¹⁰²	92.8
<i>PTEN</i> IHC (117) ¹⁰¹	66.0

^aSummary of two randomized clinical trials where comparison of mutation in *KRAS*, *NRAS*, *BRAF*, and *PIK3CA* was performed for paired primary tumor and metastatic lesions. Immunohistochemistry for PTEN was done in Cejas et al.¹⁰¹ In the study by Cejas et al,¹⁰¹ metastases were synchronous or metachronous. DNA was extracted from formalin-fixed, paraffin-embedded tissue, and mutational analysis was performed with a polymerase chain reaction–direct sequencing assay. *KRAS* mutations were detected in 42% of metastatic lesions and 39% of primary tumors. In the study by Vakiani et al,¹⁰² DNA was extracted from frozen tissue, and the iPLEX (Agena Bioscience, San Diego, CA) assay was used to examine the following mutations: *KRAS* 12, 13, 22, 61, 117, and 146; *NRAS* 12, 13, and 61; *BRAF* 600; and *PIK3CA* 345, 420, 542, 545, 546, 1043, and 1047.

7. Expert Consensus Opinion: Metastatic or recurrent colorectal carcinoma tissues are the preferred specimens for treatment predictive biomarker testing and should be used if such specimens are available and adequate. In their absence, primary tumor tissue is an acceptable alternative and should be used.

In clinical practice, one or more specimens of CRC from an individual patient may become available for molecular testing during the course of the disease. These specimens may include initial diagnostic biopsy or surgical resection specimens of the primary tumor and resection, biopsy, or cytologic specimens from metastatic and recurrent tumor. Discordance between primary and metastatic lesions may be attributed to a number of mechanisms, including tumor heterogeneity already present in the primary tumor, tumor evolution, where novel mutations are acquired, and, in some cases, the presence of separate primaries. The systematic literature review for the CRC guideline was done to identify studies that compared the mutational status of primary vs metastatic CRC.

An earlier systematic literature search that was conducted to include studies testing concordance of *KRAS*, *BRAF*, *PIK3CA*, and loss of PTEN expression in CRC²⁰ reported the results of 21 studies, with an overall concordance rate of 93% (range, 76%-100%) for *KRAS*, 93% for *BRAF* status, a range of 89% to 94% for *PIK3CA*, and 68% for loss of PTEN. **Table 12** shows the summary of two subsequent studies where *KRAS*, *NRAS*, *BRAF*, and *PIK3CA* mutation and *PTEN* expression were compared in paired primary vs metastatic tumor lesions.^{101,102} Overall concordance rates between primary and metastatic lesions were high with more than 90% concordance (Table 12).^{101,102} In the study by Lee et al, analysis of

KRAS mutation in primary and recurrent tumors after radical resection showed 20.3% discordance.¹⁰³

This recommendation was supported by two retrospective cohort studies^{101,102} that were obtained in the systematic review. Both of these studies compared results within a single cohort. The study reported by Cejas et al¹⁰¹ reported at least partial industry funding, and the study reported by Vakiani et al¹⁰² did not report the source of funding, if any. The study by Cejas et al¹⁰¹ was deemed to have a low to moderate risk of bias, and the study by Vakiani et al¹⁰² was deemed to be low. Overall, neither of these studies had any methodologic flaws that would raise concerns about the reported findings.

In summary, given that discordance of mutational status between primary and metastatic or recurrent CRC lesions may occur in a number of cases, metastatic or recurrent CRC tissues are the preferred specimens for treatment predictive biomarker testing. However, if these specimens are not available, primary tumor tissue is an acceptable alternative, given the overall high rates of concordance for the mutation status of *EGFR* pathway genes.

8. Expert Consensus Opinion: Formalin-fixed, paraffin-embedded (FFPE) tissue is an acceptable specimen for molecular biomarker mutational testing in colorectal carcinoma. Use of other specimens (eg, cytology specimens) will require additional adequate validation, as would any changes in tissue-processing protocols.

The systematic review identified a number of studies, summarized in **Table 13**, where CRC *KRAS* mutational testing was performed using FFPE specimens as well as fresh or frozen specimens. Recommendation 17 highlights the importance of review of stained sections of tumor selected for testing by a pathologist to verify the tumor cell content population of the sample and demarcate regions for potential macrodissection or microdissection to enrich for cancer cells. Biopsy and resection specimens are similarly acceptable, as long as sufficient tumor cells are present (Table 13). Cytology specimens may be adequate for testing but will require proper validation. The use of FFPE cell blocks allows for the evaluation of tumor cell content and viability.¹⁰⁴ Laboratories will need to establish the minimum tumor cell content for specimens based on the performance characteristics of their validated assay.^{105,126}

Liquid biopsy tests use serum or plasma and may be used for monitoring tumor recurrence and emergence of treatment resistance. The noninvasive nature of this approach (monitoring through blood testing) offers great potential for clinical use.¹⁰⁶ However, at the present time, the clinical application of liquid biopsy assays awaits robust validation and further studies to determine their clinical utility.

9. Strong Recommendation: Laboratories must use validated colorectal carcinoma molecular biomarker testing methods with sufficient performance characteristics for the intended clinical use. Colorectal carcinoma molecular biomarker testing validation should follow accepted standards for clinical molecular diagnostics tests.

Clinical validation assesses the molecular biomarker testing method in light of clinical characteristics of the disease or marker being tested, to ensure the test is “fit for purpose.” Elements of clinical validation include analytical sensitivity, analytical specificity, clinical sensitivity, and clinical specificity. Data for clinical validation can be obtained from studies performed by the laboratory, studies reported in peer-reviewed literature, or other reliable sources. CLIA requires clinical laboratories to have a qualified laboratory director who is responsible for ensuring that the laboratory provides quality laboratory services for all aspects of test performance.¹⁰⁷ Rigorous validation should be performed to ensure all molecular marker testing methods, such as those used for colorectal carcinoma, are ready for implementation in the clinical laboratory. To reach that goal, each step of the testing process must be carefully evaluated and documented. Excellent and comprehensive documents have been published on this topic, and a detailed review is provided under recommendation 10. Our systematic review of the available literature provided information regarding the performance characteristics of molecular marker testing methods of colorectal carcinoma in clinical use for *RAS* mutational testing (Table 13). Most studies reported the performing characteristic of assays that detected *KRAS* exon 2 mutations, as detailed in Table 13. Direct sequencing of genomic DNA, even after polymerase chain reaction (PCR) amplification of the fragment of interest, has low analytical sensitivity requiring a mutant allele frequency of about 20% for mutation detection. A number of more sensitive assays have been developed for *RAS* testing, including those listed in Table 13.

Sanger sequencing was used as the most common baseline assay for comparison against other molecular detection methods for *KRAS* mutations. Testing methods vary widely, including direct Sanger sequencing, amplification refractory mutation system, real-time PCR–high-resolution melting (HRM) assays, allele-specific PCR, Luminex (Austin, TX) bead microarray, PCR restriction fragment length polymorphism strip assays, pyrosequencing, and, more recently, NGS. Population or clinical sensitivity of the testing methods for *KRAS* mutations as shown in Table 13 ranged between 36% and 59%. Assay sensitivity ranged from 84.4% to 100%, with Sanger sequencing on the lower end of the range. Analytical sensitivity, defined as the lowest

Table 13
Comparison of Test Performing Characteristic of Assays for KRAS Mutation Detection

Author, Year	No.	Comparison	Testing Method	Codons	Tissue Site	Procedure
Ma et al, 2009 ¹³⁰	100	Sequencing	HRM	12, 13	Primary	NR
Pinto et al, 2011 ¹³¹	372	Consensus ^c	Sequencing	12, 13	NR	NR
	184		DxS			
	182		HRM			
	372		Snapshot			
Tol et al, 2010 ¹³²	511	Sequencing	DxS	12, 13	Primary	Resection
Buxhofer-Ausch et al, 2013 ¹³³	60	Sequencing	SA	12, 13	Primary	NR
Chang et al, 2010 ¹³⁶	60	Sequencing	MPCR PE	12, 13, 61	Primary	NR
Chen et al, 2009 ¹³⁷	90	Sequencing	SSCP	12, 13	Primary	NR
Chow et al, 2012 ¹³⁸	204	Sequencing	ASP	12, 13	NR	NR
Sundstrom et al, 2010 ¹⁴²	100	DxS	Pyro	12, 13, 61	Primary or met	Biopsy
Franklin et al, 2010 ¹²⁸	59	Sequencing	HRM	12, 13	Primary	Resection
	59	Sequencing	ARMS	12, 13		NR
Laosinchai-Wolf et al, 2011 ¹²⁹	86	Sequencing	BMA	12, 13	Primary	NR
Carotenuto et al, 2010 ¹³⁴	540	Sequencing	DxS	12, 13	Primary	NR
	540	Sequencing	Sanger			
Cavallini et al, 2010 ¹³⁵	112	DxS	SA	12, 13	NR	NR
	112	DxS	PCR-RFLP			
Kristensen et al, 2010 ¹³⁹	61	COLD-PCR	DxS	12, 13	Primary	Resection
	61	PCR	MCA			
Kristensen et al, 2012 ¹⁴⁰	100	CADMA	DxS	12, 13	Primary	Resection
	100	DxS	CADMA			
Lang et al, 2011 ¹⁴¹	125	Sequencing	ASP	12, 13	Primary	Resection

ARMS, amplification refractory mutation system; ASP, allele specific (nonquantitative); BMA, Luminex bead microarray; CADMA, competitive amplification of differentially melting amplicons; COLD-PCR, coamplification at lower denaturation temperature-PCR; DxS, QIAGEN method; FFPE, formalin fixed, paraffin embedded;

HRM, high-resolution melting; M, missing; MCA, melting curve analysis; met, metastatic; MPCR PE, multiplex polymerase chain reaction (*KRAS*, *NRAS*, *HRAS*) and primer extension; NPV, negative predictive value; NR, not reported; PPV, positive predictive value; PCR-RFLP, polymerase chain reaction–restriction fragment length polymorphism; PCS, prospective cohort study; Pyro, pyrosequencing; RCS, retrospective cohort study; SA, *KRAS-BRAF* strip assay; SSCP, single-strand conformation polymorphism.

^aPopulation or clinical sensitivity of testing method (%) of cases positive for *KRAS* mutation tested.

^bFour (9.5%) of 42 samples negative for *KRAS* mutation by direct sequencing were positive for *KRAS* mutations by HRM analysis.

^cTotal of 84.4% of consensus mutation result.

^dDetected one mutation in 23 Mut– alleles.

^eVariable concordance for different tumor percentage in the sample.

^fThe sensitivity was increased by 5- to 100-fold for melting temperature decreasing mutations when using COLD-PCR compared with standard PCR. Mutations, undetectable by the TheraScreen (QIAGEN, Valencia, CA) kit in clinical samples, were detected by COLD-PCR followed by HRM and verified by sequencing. Sequencing (PCR of fragment of interest followed by sequence analysis) described as direct sequencing.

detectable mutant allele fraction, was between 0.5% and 20% across all testing methods, with most methods performing between 1% and 5% mutant allele fraction. Specificity was between 98% and 100% for most assays, with two studies demonstrating lower specificity. Positive predictive value percentages varied between 66% and 100%, with most studies reporting between 99% and 100%. Negative predictive value percentages were between 97% and 100%. Minimal tumor percentages reported varied widely between studies. Concordance between assays was between 93% and 100%, with some variability noted in two retrospective cohort studies. The available evidence from assays to detect *KRAS* mutations supports the use of a number of alternative assays, as long as their performing characteristics, adjusted for sample type and percent tumor purity, meet the clinical sensitivity with acceptable specificity. Recently, NGS has been used in a number of studies and in laboratory practice

for solid tumor mutational analysis.¹⁰⁸ NGS has shown to meet the sensitivity of detection used in CRC clinical trials (detecting at least 5% mutant alleles), permitting simultaneous testing of hundreds of mutations, and is becoming widely used. Testing for mutations in multiple genes or gene loci with multiplex assays such as NGS and other methods should be done on patients at the time of metastases to obtain comprehensive genomic information and identify mutations beyond *RAS/BRAF* status that might be able to be targeted if conventional therapies become ineffective.

10. Strong Recommendation: Performance of molecular biomarker testing for colorectal carcinoma must be validated in accordance with best laboratory practices.

Proper validation of CRC biomarker testing is important to ensure appropriate patient care. If validation

Sample Type	Population Sensitivity of Testing Method, % ^a	Sensitivity of Assay	Analytical Sensitivity, % (Mutant Allele Fraction)	Specificity, %	PPV, %	NPV, %	Minimal Tumor, %	Concordance Between Assays, %	Study
FFPE	59	Increased ^b (>100)	5-10	98	NR	NR	30	95	PCS
FFPE	36.4	84.4 ^c	15-20	NR	NR	NR	>50	NR	PCS
	43.1	96	1	NR	NR	NR		NR	
	42.7	98	3-10	NR	NR	NR		NR	
	43.3	99	5	NR	NR	NR		NR	
Frozen	39.4	96.5	1	99.7	99.5	97.2	3-90	95.30	PCS
Biopsy	47.0	100	1	100	NR	NR	At least 50	100	PCS
Frozen	34.0	100	NR ^d	100	100	100	NR	100	PCS
Fresh	36.0	100	NR	100	100	100	NR	100	PCS
FFPE	40.7	100	1.25-2.5	100	100	100	NR	NR	PCS
	39.0	91	1.25-2.5; 1.25	NR	NR	NR	NR	NR	PCS
FFPE	54.0	100	1	87	81	100	1-90	NR	RCS
	43	100	5	71	66	100	1-90	93	RCS
FFPE	45.0	100	1	100	100	100	NR	NR or M	RCS
FFPE	38.6	95.8	1	100	100	97.3	<30 vs > 70	Variable ^e	RCS
		98.6	NR	100	100	99.1	NR	NR	RCS
FFPE		92.5-100	NR	100	NR	NR	70	NR	RCS
	92.5-100	NR	100	NR	NR	NR	NR	RCS	
FFPE	NR	93	0.1-5	100	NR	NR	NR	^f	RCS
		97	5-10	100	NR	NR	NR		RCS
FFPE	44.4	98	0.50	98	NR	NR	NR	95.9	RCS
		99	NR	100	NR	NR	NR	NR	RCS
FFPE	36.8	95.7 ^e	1	NR	NR	NR	>50	NR	RCS

is inadequate, this can lead to erroneous results and improper diagnosis, prognosis, and/or therapeutic intervention. For example, with regard to *RAS* testing, a false-positive result would lead to an improper withholding of therapy, whereas a false-negative result would lead to distribution of an ineffective therapy, resulting in increased costs and unnecessary side effects. As molecular oncology testing grows more complex with NGS, thorough and proper validation of preanalytical (specimen type and processing), analytical (assay performance), and postanalytical (bioinformatics, annotation, and reporting) steps is imperative.^{109,110}

The design of a validation study somewhat depends on the analyte (gene), mutations, or molecular alterations assessed and chosen platform and technology. However, assay validation should be done using best laboratory practices in accordance with CLIA (42 CFR 493.1253(b)(2)), also known as Title 42 Chapter IV Subchapter G Part 493 Subpart K§493.1253)¹¹¹ as applicable to the assay type. Laboratories should comply with CLIA and their individual accrediting agency (eg, CAP, New York State) to fulfill requirements for validation.^{111,112} Additional resources for establishing clinical molecular testing are available to assist laboratories.¹¹³ For the US Food and Drug Administration

(FDA)–cleared/approved assays (without any modification), verification of test specifications, including accuracy, precision, reportable range, and reference range, only needs to be done.¹¹⁴ For nonwaived, non-FDA-approved assays (laboratory-developed procedures or LDPs), validation must be performed. Validation design must include the required elements of analytical accuracy (specificity and sensitivity), precision, and analytical sensitivity (limit of detection) and interfering substances and reportable range as applicable. Clinical sensitivity and specificity, as well as positive and negative predictive value, should be considered additions.

Additional considerations should include specimen processing (including microdissection or macrodissection, histologic processing, and fixation times) and reagent stability and storage. Proper controls should be introduced and used to assess as many of the potential mutations detected by the assay and to verify the limit of detection identified in the validation. With high-throughput (NGS) sequencing, assessing all possible mutations through control material and specimens is impossible, and continuing validation may need to occur. If NGS is used, bioinformatics pipelines should be properly validated using multiple types of mutations (single-nucleotide variants and insertions/deletions).

Finally, reporting should be carefully considered during the validation process. Resources to assist laboratories with solid tumor molecular testing have also been made available through the CLSI.¹¹⁵

Preanalytical Variables

Histologic or preanalytical processing should be considered and representative processes should be included in the validation set. Specific specimen types should also be properly validated. Most tissue used in CRC biomarker testing is derived from FFPE tissue. Formalin fixation results in fragmentation of DNA as a result of histone protein fixation to the DNA. Therefore, most assays for FFPE tissue are designed to amplify products less than 200 base pairs. Length of formalin fixation and age of blocks may also be factors to consider in validation of FFPE tissues. Other tissue sources should also be separately validated if offered as clinical tests, especially cytology-based specimens. Various cytology fixative preparations should be validated as used by the laboratory. If cell-free assays are considered, these should be validated as a separate source. Finally, testing should be limited to invasive carcinoma with exclusion of adenomatous tissue and benign background tissue cellular components (eg, normal mucosa, muscularis, inflammation) as much as possible.

Analytical Variables

Careful specimen selection should be undertaken to cover as many of the potential detected mutations and expected specimen types as possible to ensure analytical accuracy. A gold-standard method (dideoxy sequencing or other validated test method) and/or interlaboratory comparison should be used to verify accuracy of the assay. For example, the CAP Laboratory Accreditation Program COM.40350 indicates that at least 20 specimens (including positive, low-positive, and negative specimens) should be included for qualitative and quantitative assays.¹¹² More specimens may be required. If it is a single-gene assay, the design should include as many of the mutations covered by the assay as possible. If it is a real-time–based allele-specific assay, all mutations for which a primer probe reaction is built should be analyzed as reasonably as possible. If it is a pyrosequencing-based assay, similarly, all of the possible common mutations for which targeted therapies are indicated should be tested. Multigene assays based on NGS or other technology (such as SNaPshot [ThermoFisher Scientific, Waltham, MA]) require an increased number of specimens to test as many of the hotspot regions as possible in all genes included in the assay. With such assays, not all possible mutations can be validated. It is recommended that an ongoing validation occur after initial validation, with verification of novel mutations by either dideoxy sequencing or

real-time PCR, depending on the laboratory capability and limit of detection. Depending on the technology employed, important parameters (eg, variant allele frequency, cyclic threshold values, allele coverage) should be monitored for interrun and intrarun precision.

CRC specimens can vary from large primary resection blocks with plenty of tumor cells to small primary tumor or metastatic CRC liver biopsy specimens to rectal specimens, after neoadjuvant therapy with minimal tumor percentage. Many of these tests are ordered for metastatic disease, for which only a small needle core biopsy specimen or cytologic sampling is available. Presently, tissue volume and accessibility are decreasing while ancillary testing (IHC and molecular studies) is increasing. The ability of an assay to be highly analytically sensitive is important if a laboratory is to test specimens with low tumor burden. It is recommended that an assay be able to identify a mutation in a specimen that has at minimum 20% tumor cells (mutant allele frequency of 10% assuming heterozygosity). With NGS and highly sensitive PCR technologies, mutations should be identifiable in specimens with as little as 10% tumor (mutant allele frequency of 5% assuming heterozygosity and diploidy). Lower analytically sensitive assays, such as dideoxy sequencing, can be used, but it is recommended that PCR enrichment strategies (eg, coamplification at lower denaturation temperature-PCR) be used to increase the analytical sensitivity of the test and require less tumor percentage. A proper validation study should use cell line DNA (preferably FFPE treated) or reference control material manufactured by good manufacturing processes to assess limit of detection for as many mutations as possible. Importantly, the limit of detection may differ for mutations of varying types (small indels vs point mutations).

Postanalytical Variables

Postanalysis is as important to consider in validation as preanalytical and analytical variables. For single-gene assays, the software used in analysis should be validated, with verification of updates. If NGS is used, the bioinformatics pipeline should be thoroughly and rigorously validated, include potential problematic mutations (eg, large indels), and be verified or revalidated for new upgrades as applicable to the change. Any analysis should be performed on validation specimens as it would be for clinical specimens.

Reporting format should also be considered and decided during validation. Interpretation comments for inclusion in the patient report to ensure that the reports are correctly understood should be developed during the validation process.¹¹² Human Genome Organisation (HUGO)–based nomenclature should be used for reports and a designated National Center for Biotechnology Information

(NCBI) transcript number (NM_##) should be used within the validation and report.¹¹⁶ For multigene panels based on NGS, reporting protocols and any used software should be included in the validation procedure. Databases and annotation guidelines should be discussed and included in the validation as one prepares to report variants based on NGS data. In addition, decisions should be made during the validation process as to whether normal tissue will be tested to assist in variant interpretation with NGS.

In conclusion, validation of assays used in CRC molecular testing is extremely important for accuracy of reporting and proper patient care. There are several documents (eg, CLIA, CAP, and CLSI)^{111-113,115} available to assist in proper validation, which should be consulted to validate according to best laboratory practices.

11. Strong Recommendation: Laboratories must validate the performance of IHC testing for colorectal carcinoma molecular biomarkers (currently IHC testing for MLH1, MSH2, MSH6, and PMS2) in accordance with best laboratory practices).

Four proteins (MLH1, MSH2, MSH6, and PMS2) are currently considered important in the normal biochemistry of DNA MMR.¹¹⁷⁻¹¹⁹ As detailed in recommendation 2b, altered DNA mismatch repair proteins due to mutation or epigenetic silencing result in interference with normal MMR protein heterodimerization and loss of normal repair of mispaired bases and short insertions/deletions, resulting in MSI,^{119,120} overall categorized as dMMR. Loss of MMR function usually correlates with loss of protein expression, such that immunohistochemical testing for MMR proteins is optimized to detect loss of MMR protein expression in tumor cell nuclei. Each of these four proteins can be detected in paraffin sections using commercially available primary and secondary antibodies, standardized antigen retrieval, and 3,3'-diaminobenzidine chromogen detection. Development of anti-MMR protein antibody staining protocols follows a standard approach that involves (1) demonstration of absent background noise with secondary antibody alone and (2) empirical optimization of the signal-to-noise ratio by testing different antibody concentrations, antigen retrieval buffers, and reaction conditions, taking advantage of internal control cells, including lymphocytes, stromal cells, and other nonneoplastic nuclei.

Validation of the final staining protocol is required prior to implementation for clinical use. Peer-reviewed literature-based guidelines for validation and revalidation of immunohistochemical tests have been defined as 14 recommendations and expert consensus opinions.¹²¹ Concordance with internal or external known comparator tests is required to exceed 90%. Proficiency testing is a good approach to confirm interlaboratory test reproducibility. Test result

concordance across laboratories implies accuracy of participant laboratory diagnosis.

Once the protocol is defined and validated for a given primary antibody clone and antigen retrieval conditions, a known positive external control (eg, tonsil) is routinely run in parallel with each unknown. This demonstrates that the MMR protein was detectable on that staining run and allows trust in a loss of expression result in the unknown specimen. Each of the four MMR proteins is expressed in nonneoplastic tissue, in most lymphocytes, and overexpressed in germinal centers, such that most colon block sections will also have positive internal control staining.

Overall, validated immunohistochemical detection of MMR proteins is a trustworthy method for identification of loss of expression of individual MMR proteins in paraffin sections of CRC. In most CRCs with high-level microsatellite instability (MSI-H), the loss of DNA MMR protein expression in tumor cell nuclei by immunohistochemical detection is uniform throughout the tumor.^{122,123} Rare cases of MSI tumors have been reported to show heterogeneous staining.¹²⁴ Loss of MMR protein expression usually correlates with MSI, particularly for MSI-H tumors, and is indicative of dMMR. If MSH2 or MLH1 shows loss of expression due to loss of function, then their heterodimer partners (MSH6 and PMS2, respectively) will also not be expressed. In contrast, inactivation of MSH6 or PMS2 results in loss of expression of the individual MMR protein MSH6 or PMS2, respectively.

Although loss of MMR protein immunoreactivity is generally detected in dMMR CRC, normal immunoreactivity can be seen in up to 10% of dMMR cases¹²⁵; therefore, MSI DNA testing may be performed either stepwise or as a concurrent test.

12. Expert Consensus Opinion: Laboratories must provide clinically appropriate turnaround times and optimal utilization of tissue specimens by using appropriate techniques (eg, multiplexed assays) for clinically relevant molecular and immunohistochemical biomarkers of CRC.

Expediency in reporting of biomarker results for colorectal tumors is dictated primarily by two factors: need for patient management decisions and, more generally, patient anxiety. Consequently, results of such evaluations should be available within a timeframe for the involved clinician to relay this information to the patient. This need is compounded by the patient's need to receive a complete understanding of his or her diagnosis and treatment plans going forward. A reasonable benchmark is that nonacute biomarker results be available to the treating physician within 10 working days of receipt in the molecular diagnostics laboratory. This turnaround time has been recommended in other guidelines for molecular tumor testing.^{105,126,127}

Ideally, the transitional time between test ordering, tissue block selection, block retrieval, and shipment to the performing laboratory should be included in the 10-day timeframe. Consequently, laboratories should make every effort to minimize delays in securing appropriate tissue blocks for testing. Testing laboratories should make every effort to minimize processing time and return of results.

The availability of tumor tissue for biomarker evaluation is generally not limiting in most cases of resected CRC. Occasionally, following neoadjuvant therapy, the amount of residual tumor in resection specimens can be very small and focal. Similarly, the amount of tumor tissue obtained by biopsy or fine-needle aspiration procedures from primary or metastatic foci can be very small and challenging to test for the desired biomarkers. In such circumstances, available tissue blocks should be sectioned judiciously, reserving sufficient sections for testing by molecular methods or immunohistochemical techniques, as deemed appropriate to secure as accurate and informative an evaluation as possible.

Test turnaround times for *RAS* testing in instances of advanced stage tumors are dictated by the need to select and initiate appropriate chemotherapy options. Ideally, such information should be available either at the time of postoperative oncology evaluation, where decisions regarding therapeutic options are entertained, or at the tumor boards where patient treatment options are discussed. In some institutions, these discussions may occur in the week following surgery or biopsy and probably no later than in the second week following tissue diagnosis and staging. Here, too, a timeframe of no more than 10 days would seem an appropriate benchmark for biomarker result availability.

In exceptional circumstances, even shorter test turnaround times may be called for. Occasional patients have histories sufficiently suggestive of Lynch syndrome that prompt consideration and discussion regarding extent of surgery (ie, complete colectomy or prophylactic hysterectomy in select affected patients). Efforts should be made in such circumstances to obtain appropriate test results as rapidly as possible to allow for informed decision making. MMR immunohistochemistry can be performed and reported with a turnaround time of 48 hours or less, and in the appropriate clinical context, a result of preserved expression of MMR proteins would argue against Lynch syndrome. Conversely, any loss of MMR protein expression will need to be integrated with additional clinical information, family history, and further testing such as *BRAF* mutation, MLH1 methylation testing, and potential germline genetic testing. Furthermore, DNA MMR status, performed by MMR immunohistochemistry or by MSI DNA tests, as a good prognostic biomarker for CRC overall, should be

available within the recommended 10 working day turnaround time for test results.

13. Expert Consensus Opinion: Molecular and IHC biomarker testing in colorectal carcinoma should be initiated in a timely fashion based on the clinical scenario and in accordance with institutionally accepted practices.

Note: Test ordering can occur on a case-by-case basis or by policies established by the medical staff.

Molecular and IHC biomarker testing is increasingly being used in patient management. Prognostic biomarkers are being used for early stage disease to guide decisions on the use of adjuvant chemotherapy. Such discussions require the availability of tests in a timely manner, and delays in initiation of therapy have been associated with worse outcomes.¹²⁷ Predictive biomarkers, such as those for EGFR monoclonal antibody therapy, should be initiated in a timely fashion to guide chemotherapy options and long-term treatment planning. Institutional policies and practices that encourage the rapid initiation of appropriate molecular and IHC marker testing should be encouraged. Such policies may include reflexive ordering of molecular and IHC markers as guided by the clinical scenario and incorporation of testing initiation by multiple members of the multidisciplinary team, as noted in recommendation 15.

14. Expert Consensus Opinion: Laboratories should establish policies to ensure efficient allocation and utilization of tissue for molecular testing, particularly in small specimens.

The number of molecular and immunohistochemical tests becoming available that have a direct benefit to patient care will continue to increase. Most of these tests are performed on FFPE specimens, the most common preservation technique, including pretreatment and posttreatment biopsies and resections (Table 13). Tissues from patients with cancer should be processed according to established laboratory protocols, which include quality controls of preservation materials, tissue dissection, time to fixation, fixation time, and processing.

Laboratory protocols need to include procedures for handling small samples such as endoscopic or core biopsy specimens and fine-needle aspirate samples of metastatic lesions (eg, from liver or lung). Limiting the number of tissue fragments per individual cassette is encouraged. Established protocols may allow upfront ordering of required tissue sections (eg, extra unstained slides), which limit tissue wasting and improve turnaround time of final results. Immunohistochemistry studies, if needed to diagnose metastatic CRC, should be limited in scope and standardized to preserve tissues.

It is imperative to identify suspected metastatic CRC specimens at specimen accessioning to limit unneeded ancillary tests, such as liver biopsy special stains. Recognition of previous CRC diagnoses from the patient clinical history should limit the need for immunohistochemistry profiles in many cases. Established laboratory procedures to identify patients undergoing cancer biopsy or fine-needle aspiration specifically for predictive molecular biomarker assessments need to be in place.

Laboratories must maintain appropriate cataloguing and storage of tissue specimens and diagnostic slides to allow for retrospective timely testing of cancer samples.

This recommendation is supported by 15 studies,¹²⁸⁻¹⁴² comprising eight prospective cohort studies^{130-133,136-138,142} and seven retrospective cohort studies.^{128,129,134,135,139-141}

For the eight prospective cohort studies,^{130-133,136-138,142} all reported balance between the treatment and assessment groups, as all but one¹³² used a single cohort design allowing for within-group comparisons. Only this single study, reported by Tol et al,¹³² would have required making adjustments for imbalances between the treatment and assessment groups, but none were needed. Five studies^{130,133,136-138} reported nonindustry funding, one¹³² reported at least partial industry funding, one¹⁴² reported industry funding, and one¹³¹ did not disclose the source of funding, if any. Seven^{130,131,133,136-138,142} were deemed to have a low risk of bias, and one¹³² was deemed to have a low to moderate risk of bias.

For the seven retrospective cohort studies,^{128,129,134,135,139-141} all used a single cohort design allowing for within-group comparisons. Four reported nonindustry funding,^{134,135,139,140} one reported industry funding,¹²⁹ and two did not disclose the source of funding, if any.^{128,141} Six were deemed to have a low risk of bias,^{128,134,135,139-141} and one was deemed to have a moderate risk of bias.¹²⁹

All of the evidence that supported this recommendation was assessed, and none had methodologic flaws that would raise concerns about their findings.

15. Expert Consensus Opinion: Members of the patient's medical team, including pathologists, may initiate colorectal carcinoma molecular biomarker test orders in accordance with institutionally accepted practices.

For patients with CRC, timely diagnosis or therapeutic initiation is critical, and molecular testing that is to be considered should be ordered as efficiently as possible in accordance with institutional practices and guidelines. MSI testing is often ordered at the time of diagnosis to identify patients with Lynch syndrome, direct adjuvant chemotherapy, or determine prognosis. Many institutions employ

algorithms to ensure that all colorectal cancers are evaluated for MMR deficiency, and these are often initiated by pathologists when the diagnosis occurs after joint general process approval by pathologists, oncologists, and other members of the patient medical team. Molecular testing that is performed to direct targeted therapy (eg, *RAS*) may be ordered at a later date than the primary diagnosis, at metastatic presentation, for example, and so institutions may differ as to whether one should order such testing upfront on the primary diagnostic biopsy or resection specimen or wait until metastatic disease arises requiring targeted therapy. Often oncologists order predictive molecular assays since they are used to direct therapy, but this should not necessarily be limited to oncologists, as pathologists serve as important stewards of the tissue and make the tumor diagnosis. There are also issues to consider, including logistical issues, cost-effectiveness, patient access to molecular testing in rural or underserved areas, and even heterogeneity considerations between primary and metastatic tumor. Since each institution differs in patient population, facilities, departmental organization, regulatory and reimbursement climates, and practitioner preference, whether to submit testing at initial diagnosis of a primary lesion or when a metastatic lesion arises should be discussed collaboratively between oncologists, pathologists, and medical executive or hospital committees as applicable.

“Reflex” testing, a testing policy that does not require a separate clinician order for each case, is appropriate if agreed upon by the CRC care team as an institutionally approved standing order and may help to ensure expedited and consistent routing of specimens for molecular testing. However, some patients may not be candidates for targeted therapy for clinical reasons, and good communication between the clinical care team and the testing laboratory is needed to ensure testing is performed for patients whose management will be affected by the test result. Specifically, testing is not necessary for patients with stage IV disease who are being considered for palliative or hospice care only. Similarly, in settings in which reflex testing is the practice, a mechanism should be provided for the clinical care team to communicate to the pathologist examining a small biopsy or cytology sample when a more suitable diagnostic specimen (eg, a resection) is expected to be obtained, and the molecular testing should be deferred to the subsequent, more generous sample. All reflex testing should be approved institutionally by the hospital or institution's medical executive committee as local policies dictate.

16. Expert Consensus Opinion: Laboratories that require send out of tests for treatment predictive

biomarkers should process and send colorectal carcinoma specimens to reference molecular laboratories in a timely manner.

Note: It is suggested that a benchmark of 90% of specimens should be sent out within 3 working days.

It is critical to provide the results of molecular tests in a timely fashion to start the most appropriate cancer treatment option for each patient. Delays in initiation of therapy have been associated with worse outcomes.¹²⁷ To date, laboratories have had limited guidance on the recommended timing or turnaround time of molecular test results, and studies addressing the impact of specific turnaround times have not been conducted. Therefore, the panel reached an expert consensus opinion, based on each panel member's practical experience in the laboratory and clinical setting.

For laboratories that do not perform molecular testing and/or biomarker immunohistochemistry for CRC therapy selection, the consensus opinion was that send out of specimens should occur within 3 working days, starting from the day the test order was received in the laboratory, provided the specimens (eg, biopsy or resection specimens) are received at the same time of the test order or specimens are already in the laboratory (eg, archived paraffin blocks). The underlying rationale stems from the usual workflow for tissue processing. In practice, the longest process would be the processing of large surgical specimens, such as colectomies. A possible approach is to obtain a designated molecular tissue block at the time of specimen grossing, and molecular protocols for obtaining tissue sections may be used to have the necessary sections for test send-out in a timely fashion by the third working day for most cases. Another scenario may be the retrieval of archived tissue paraffin blocks that may be stored outside of the laboratory location. In this case, a protocol for block retrieval for molecular testing may be operationalized to streamline the process and reach the desired turnaround time for send-out. This turnaround time of 3 working days was also recommended for *RAS* testing of colorectal carcinoma in the guidance document from the Association of Clinical Pathologists Molecular Pathology and Diagnostics Group in the United Kingdom.¹²⁶

Laboratories should develop written policies as part of their quality assurance program to monitor turnaround times for all cancer therapeutic and prognostic biomarkers.

17. Expert Consensus Opinion: Pathologists must evaluate candidate specimens for biomarker testing to ensure specimen adequacy, taking into account tissue quality, quantity, and malignant tumor cell fraction. Specimen adequacy findings should be documented in the patient report.

It is critical that pathologists selecting blocks for biomarker testing understand the specimen requirements of the method being employed in terms of total tissue amount (a reflection of the total amount of DNA required for the assays) and the fraction of malignant tumor cells in the specimen focus to be evaluated. The total amount of tissue selected for evaluation is significant in two respects. First, the amount of tissue sampled should be of sufficient quantity to produce a result that is reliably representative of the entire tumor. While recent evidence indicates that some genes continue to evolve during tumor progression, leading to substantial tumor genetic heterogeneity, those driver mutations of importance to CRC are usually, but not always, homogeneous throughout the tumor. The amount of tumor necessary, however, for a particular analytical method can vary and demands knowledge and due attention to the indicated tissue requirements for the specific assay employed. The minimal required proportion of tumor DNA in a sample from cancer is dictated by the analytical sensitivity of the particular validated assay. As shown in Table 13, the amount of tumor used in the analyses of *KRAS* mutations in several studies comparing the test-performing characteristics of various assays varied widely, ranging from 1% to 90%.

The proportion of malignant tumor cells (as opposed to tumor-associated nonmalignant cells, eg, stromal fibroblasts, endothelial cells, infiltrating inflammatory cells) should be evaluated as accurately as possible and documented. This evaluation is most readily performed by estimating the proportion of malignant cell nuclei to nonmalignant cell nuclei within the focus selected for evaluation.¹⁴³ Understanding that the number of mutated alleles for a particular gene may represent as few as half of the alleles in diploid tumor cells, a tumor cell focus with a nominal proportion of 50% tumor cells would have a mutant allele fraction of 25%, a value approaching the analytical sensitivity of some molecular assays. So, while variety of molecular methods can be used to evaluate tissue specimens, it is critical that these be carefully matched to their specific tissue and tumor cell proportion requirements. When adhered to, all these of these methods can produce accurate and reliable results.

Pathologists evaluating tissue section for biomarker evaluation should also be aware that necrosis and tissue degeneration can lead to erroneous results, and foci demonstrating significant necrosis should be avoided for molecular testing. Any amount of necrosis in the sample selected for biomarker testing should be estimated and documented.

18. Expert Consensus Opinion: Laboratories should use colorectal carcinoma molecular biomarker testing methods that are able to detect mutations in specimens with at

least 5% mutant allele frequency, taking into account the analytical sensitivity of the assay (limit of detection or LOD) and tumor enrichment (eg, microdissection).

Note: It is recommended that the operational minimal neoplastic carcinoma cell content tested should be set at least two times the assay's LOD.

Since the accuracy and results of testing for molecular markers are dependent on both tumor cell content and the assay-specific sensitivity in the identification of a mutant allele against a background of wild-type/nonmutated alleles, it is suggested that laboratories should establish minimum acceptable tumor cell content as a component of their specimen requirements. It is recommended that a pathologist reviews all cases for tumor cell content and quality. Due to the stochastic nature of mutant allele identification at the lower LOD, it is recommended that the minimal tumor cell content be at least two times the lower LOD of a validated molecular method or assay. This LOD was also recommended for *RAS* testing of colorectal carcinoma in the guidance document from the United Kingdom.¹²⁶ Hence, if a particular assay has a lower limit of mutant allele detection of 5%, then the minimum tumor cell content in samples analyzed by this assay should be at least 10% to reliably detect heterozygous mutations in those neoplasms. Due to intratumoral heterogeneity, subclones, and the nature of tissue sampling, clinical trials have used 5% as the lower LOD, and for clinical purposes, it is recommended that the lower LOD for a mutant allele be at least 5%.¹² Therefore, the utilization of methods such as PCR, HRM, single-strand conformation polymorphism, pyrosequencing, or commercially available kits that achieve this level of sensitivity is recommended^{130,137,138,142} (Table 13).

This recommendation is supported by four prospective cohort studies^{130,137,138,142} and two retrospective cohort studies.^{102,144} The four prospective cohort studies all studied a single cohort, allowing for within-group comparisons. For this reason, all were balanced between comparison groups, and no adjustments were needed to account for baseline differences. All four reported nonindustry funding, and all were deemed to have a low risk of bias.

The two retrospective cohort studies^{102,144} also used single cohorts, allowing for within-group comparisons only. One¹⁰² did not report the source of funding, while the other¹⁴⁴ reported nonindustry funding. Both were deemed to have a low risk of bias.

None of the studies had methodologic flaws that would raise concerns about their findings.

19. Expert Consensus Opinion: Colorectal carcinoma molecular biomarker results should be made available as promptly as feasible to inform therapeutic decision making, both prognostic and predictive.

Note: It is suggested that a benchmark of 90% of reports be available within 10 working days from date of receipt in the molecular diagnostics laboratory.

Combined chemotherapy, including anti-EGFR therapy, in patients with CRC in the absence of mutations in the EGFR signaling pathway is associated with significant survival advantage. No significant therapeutic benefit is derived from anti-EGFR therapy in the presence of mutations in *KRAS* and *NRAS*.⁴⁴ The presence of deficient MMR in stage II CRC indicates a good prognosis and identifies patients for whom adjuvant 5-fluorouracil monobased therapies have no significant benefit.^{145,146} The presence of deficient MMR or *BRAF* p.V600E mutation in proficient MMR CRCs has important prognostic significance.⁵⁴

In the absence of published data establishing an evidence-based recommendation, it is our expert consensus opinion that the above results, regardless of testing methods, be available from test ordering in the initial diagnostic pathology laboratory to the clinical team within 2 weeks (10 working days). The 10 working days does not include the time before the tissue specimen is available for testing (ie, from diagnostic procedure to receipt in laboratory) or time to retrieve tissue samples from an outside laboratory. Laboratories unable to maintain this standard, either through in-house testing or use of a reference laboratory, need to implement measures to improve test result turnaround time. A turnaround time of 7 working days was recommended for *RAS* testing of colorectal carcinoma in the guidance document from the Association of Clinical Pathologists Molecular Pathology and Diagnostics Group in the United Kingdom.¹²⁶

This recommendation is supported by evidence from one randomized controlled trial, reported by Douillard et al.⁴⁴ This report used prospective patient data collected within the PRIME trial. While it did not report details on the randomization, blinding, statistical power calculation, sample size, or length of follow-up, it did report on baseline characteristics and was otherwise well reported. Funding was reported to be partially from industry sources. Overall, this trial was found to have a low to moderate risk of bias and did not have methodologic flaws that would raise concerns about its findings.

Each laboratory should develop a quality assurance program to monitor turnaround times for all cancer therapeutic and prognostic biomarkers.

20. Expert Consensus Opinion: Colorectal carcinoma molecular biomarker testing reports should include a results and interpretation section readily understandable by oncologists and pathologists. Appropriate Human Genome Variation Society (HGVS) and HUGO nomenclature must

be used in conjunction with any historical genetic designations.

Reporting of molecular results is becoming more complex as new information and clinical utility are discovered for somatic variants. Single-gene assays are still being widely used, but multiplexing has allowed for multiple possible results. With the introduction of NGS into the clinical setting, multiple somatic mutations with clinical significance may be identified. However, panel assays by NGS can also reveal variants with unknown clinical significance. As pathogenic genes and somatic mutations have been discovered over the past 30 years, there has been divergent nomenclature employed, making clinical reporting and clinical analysis difficult. Presently and in the future, as national databases are constructed annotating clinical somatic variants, it is imperative that standardized nomenclature be employed to identify the clinical significance of rare variants.

Clinicians want a report that is easily readable and understandable but that gives pertinent clinical information concisely, accurately, and thoroughly. Reported variants should be identified using both DNA and protein nomenclature. Citing codon positivity only is not encouraged (eg, positive for a *KRAS* codon 12 mutation); the specific mutation should be explained using standardized nomenclature, preferably HUGO gene nomenclature.^{112,147} Historical designations (eg, historical HER-2/neu, for HUGO *ERBB2*) should also be included as appropriate in the report to avoid confusion among oncologists. Importantly, the messenger RNA transcript number (NM_#) from the NCBI, used to designate the specific codon numbering, should be named in the report since numbering can differ between the different/alternative transcript designations for the same gene. If using NGS, variants should at least be classified as pathogenic, likely pathogenic, variant of unknown significance, likely benign, or benign, but classification of somatic mutations is still awaiting specifically approved guidelines.¹⁴⁸ However, a numerical classification scheme for somatic variants has been proposed, taking into consideration actionability of the variant in the patient's tumor type vs other tumor types, predicted pathogenicity (using programs such as SIFT and PolyPhen 2) in the patient's tumor type vs other tumor types, variant recurrence in a certain cancer type, or unknown significance.¹⁴⁹ Such a classification scheme may be better suited to somatic variants considering the indications for which most of these assays are being ordered.

Reports should contain the analytical result, the method used, and information about the genes and loci tested or included in the assay; the assay limit of detection; and any disclaimers (eg, ASR) that are required to meet regulations. When reasonable and applicable, an interpretive comment

should be given to ensure that results are correctly understood.¹¹² Such an interpretive comment may include information regarding therapeutic implications, prognostic implications, and/or pathogenic significance of the mutation and, when appropriate or desired, potential applicable clinical trials.

In summary, molecular reports should be easily understandable by clinical oncologists and use standardized nomenclature outlined by HGVS/HUGO. All reports should contain the elements of result, interpretation, variant classification, and information as applicable; limit of detection of the assay and methods to assist the oncologist in understanding the test result; and limitations as they consider the result in a clinical context.

21. Strong Recommendation: Laboratories must incorporate colorectal carcinoma molecular biomarker testing methods into their overall laboratory quality improvement program, establishing appropriate quality improvement monitors as needed to ensure consistent performance in all steps of the testing and reporting process. In particular, laboratories performing colorectal carcinoma molecular biomarker testing must participate in formal proficiency testing programs, if available, or an alternative proficiency assurance activity.

Proficiency testing (PT) is an important component of quality assurance for laboratory tests in general and applies to the molecular tests discussed in the current CRC molecular testing guidelines. These include mutational as well as immunohistochemical testing. Participation in PT allows the assessment and comparison of test performance among different clinical laboratories and technologies and allows verification of accuracy and reliability of laboratory tests.¹⁵⁰

From a regulatory standpoint, PT in the United States is a requirement for accreditation by the Centers for Medicare & Medicaid Services. Participation in PT may be done through CAP PT programs or through other providers accepted by CLIA.¹⁵¹ Other countries—namely, the United Kingdom—follow similar guidelines, recommending that laboratories providing *RAS* testing of CRC should demonstrate successful participation in a relevant external quality assurance scheme and be appropriately accredited.¹²⁶

Formal external proficiency testing programs for analytes other than *KRAS*, MSI, MMR, and *BRAF* may not be available at the time of this publication. Alternative proficiency testing activities should be used. Appropriate alternative performance assessment procedures may include split sample analysis with other laboratories or, if that is not available, assessment of split samples with an established in-house method and previously assayed material, which are

run and interpreted by laboratory personnel who do not have access to the prior results.¹⁵¹ If exchanging specimens with other laboratories is the laboratory proficiency approach, this should be done with one or more other laboratories at least twice per year.¹⁰⁵ Methods-based proficiency testing (MBPT) refers to a testing approach that is based on method, rather than based on each individual analyte tested. MBPT is well established for several pathology subspecialty areas, and the concept of MBPT complies with federal laboratory regulations.¹⁵¹

Discussion on Emerging Biomarkers

Numerous studies have reported potential molecular biomarkers for CRC prognosis, while fewer studies evaluated markers that could be predictive of response to specific treatments. Many published studies are limited due to early exploratory and retrospective analyses, and those biomarkers, while of potential interest, have not made it to clinical practice. Our systematic review identified several CRC molecular biomarkers that showed either prognostic or treatment predictive characteristics in single studies (Supplemental Table 15). Most of the molecular biomarkers reported in the studies listed in the Supplemental Table 15 were tested for expression by immunohistochemistry. Immunohistochemistry is notable for its widespread availability in pathology laboratories but has limited quantitative capabilities due to difficult standardization of quantitative or semiquantitative scoring, and is fraught by significant interobserver variability. A problem of quantitative assays, such as gene expression, microRNA expression, and methylation levels, tested in solid tumors, results from the intrinsic mixed nature of the tissue with significant variability of tumor and nontumor tissue content. Another limitation of molecular biomarker discovery approaches that rely on expression levels is that these biomarkers have not been evaluated in the context of complex molecular regulation of individual cancer subtypes. Their fruitful use in the clinic may require further studies that take into account computational predictions of biological behavior and validation in prospective cohorts.

A great deal of interest has been raised recently for non-invasive prognostic and/or therapy-predictive molecular biomarkers, such as those tested in circulating tumor cells or circulating nucleic acids, either as free nucleic acid in serum or associated with extracellular vesicles or exosomes. This has been referred to as “liquid biopsy.”¹⁵² Liquid biopsies may be particularly useful in the management of patients with CRC to identify recurrence, *RAS* mutation testing for emergence of treatment resistance associated with anti-EGFR therapy, and potential early cancer detection in defined subpopulations, such as those at high risk of CRC. Overall, molecular

biomarkers for colorectal cancer tested in liquid biopsy samples are promising but await further validation.

Emerging data indicate that MMR status may have predictive value in some settings, specifically in patients with advanced disease being considered for anti-PD-1/PD-L1 therapy.^{68,69}

Conclusions

Evidence supports mutational testing of specific genes in the EGFR signaling pathway, since they provide clinically actionable information for targeted therapy of CRC with anti-EGFR monoclonal antibodies. Mutations in some of the biomarkers have clear prognostic value (*BRAF*, MMR), and at least two (*KRAS* and *NRAS*) have relatively strong evidence as negative predictors of benefit to anti-EGFR therapies and should be used to guide the use of these agents. *BRAF* mutations are consistently associated with poor outcomes in patients with metastatic CRC, including those who relapse after adjuvant therapy. Patients with localized colon cancer and dMMR have improved outcomes. Emerging data suggest that MMR status has predictive value in some settings, specifically in patients with advanced disease being considered for anti-PD-1/PD-L1 therapy.

Laboratory approaches to operationalize molecular testing for predictive and prognostic molecular biomarkers involve selection of assays, type of specimens to be tested, timing of ordering of tests, and turnaround time for testing results. A number of alternative technical approaches can effectively be used as long as test specificity and sensitivity meet the clinical needs. While earlier testing approaches were focused on one or a few testing targets (eg, *BRAF* p.V600 mutations), currently, new approaches are using gene panels such as targeted NGS cancer panels, which can range from a few to hundreds of genes and amplicons with known mutational hotspots in cancer.

These guidelines will be subjected to regular updates, such that new advances in the field can be captured and integrated in the guidelines in a timely manner.

Corresponding author: Antonia R. Sepulveda, MD, PhD, Dept of Pathology and Cell Biology, 630 W 168th St VC 14-212, Columbia University, New York, NY 10032; as4400@cumc.columbia.edu.

This guideline was developed through collaboration between the American Society for Clinical Pathology, College of American Pathologists, Association for Molecular Pathology, and American Society of Clinical Oncology and has been jointly published by invitation and consent in the American Journal of Clinical Pathology, Archives of Pathology & Laboratory Medicine, Journal of Molecular Diagnostics, and

Journal of Clinical Oncology. It has been edited in accordance with standards established at the American Journal of Clinical Pathology.

The authors gratefully acknowledge the following: expert and advisory panel members Kevin Halling, MD, Charles Blanke, MD, Pr. Jean-François Fléjou, Heather Hampel, MS, CGC, J. Randolph Hecht, MD, Kazunori Kanehira, MD, Faye Kastrinos, MD, MPH, Carla B. MacLeod, MD, Peter J. Odwyer, MD, Weijing Sun, MD, Josep Tabernero, MD, PhD, Laura H. Tang, MD, PhD, Mary Kay Washington, MD, PhD, Shuji Ogino, MD, PhD, MS, Loren Joseph, MD, Kim Ryan, and Pamela McAllister, PhD, for their review of the key questions, recommendations, and draft manuscript, as well as Kaitlin Einhaus, Lisa A. Fatheree, SCT(ASCP), Jeff Jacobs, MA, Sandra Larsen, MBA, MT(ASCP), Melvin Limson, PhD, John Olsen, MD, Mrudula Pullambhatla, MS, Shiwen Song, MD, Megan Wick, MT(ASCP), and Mary Steele Williams, MNA, MT(ASCP)SM, CAE, for their support throughout the guideline development process.

References

1. Febbo PG, Ladanyi M, Aldape KD, et al. NCCN task force report: evaluating the clinical utility of tumor markers in oncology. *J Natl Compr Cancer Netw*. 2011;9(suppl 5):S1-S33.
2. Grothey A. EGFR antibodies in colorectal cancer: where do they belong? *J Clin Oncol*. 2010;28:4668-4670.
3. De Roock W, Claes B, Bernasconi D, et al. Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. *Lancet Oncol*. 2010;11:753-762.
4. De Roock W, De Vriendt V, Normanno N, et al. KRAS, BRAF, PIK3CA, and PTEN mutations: implications for targeted therapies in metastatic colorectal cancer. *Lancet Oncol*. 2011;12:594-603.
5. Rubenstein JH, Enns R, Heidelbaugh J, et al. American Gastroenterological Association Institute guideline on the diagnosis and management of Lynch syndrome. *Gastroenterology*. 2015;149:777-782.
6. Des Guetz G, Schischmanoff O, Nicolas P, et al. Does microsatellite instability predict the efficacy of adjuvant chemotherapy in colorectal cancer? A systematic review with meta-analysis. *Eur J Cancer*. 2009;45:1890-1896.
7. Guastadisegni C, Colafranceschi M, Ottini L, et al. Microsatellite instability as a marker of prognosis and response to therapy: a meta-analysis of colorectal cancer survival data. *Eur J Cancer*. 2010;46:2788-2798.
8. Gavin PG, Colangelo LH, Fumagalli D, et al. Mutation profiling and microsatellite instability in stage II and III colon cancer: an assessment of their prognostic and oxaliplatin predictive value. *Clin Cancer Res*. 2012;18:6531-6541.
9. Institute of Medicine. *Clinical Practice Guidelines We Can Trust*. Washington, DC: National Academies Press; 2011.
10. National Health and Medical Research Council. A guide to the development, implementation and evaluation of clinical practice guidelines. 1999. https://www.nhmrc.gov.au/_files_nhmrc/publications/attachments/cp.30.pdf. Accessed March 23, 2016.
11. Guyatt GH, Oxman AD, Vist GE, et al; GRADE Working Group. GRADE: an emerging consensus on rating quality of evidence and strength of recommendations. *BMJ*. 2008;336:924-926.
12. 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;26:13-21.
13. Ibrahim EM, Abouelkhair KM. Clinical outcome of panitumumab for metastatic colorectal cancer with wild-type KRAS status: a meta-analysis of randomized clinical trials. *Med Oncol*. 2011;28(suppl 1):S310-S317.
14. Modest DP, Brodowicz T, Stintzing S, et al. Impact of the specific mutation in KRAS codon 12 mutated tumors on treatment efficacy in patients with metastatic colorectal cancer receiving cetuximab-based first-line therapy: a pooled analysis of three trials. *Oncology*. 2012;83:241-247.
15. Petrelli F, Borgonovo K, Cabiddu M, et al. Cetuximab and panitumumab in KRAS wild-type colorectal cancer: a meta-analysis. *Int J Colorectal Dis*. 2011;26:823-833.
16. Zhou SW, Huang YY, Wei Y, et al. No survival benefit from adding cetuximab or panitumumab to oxaliplatin-based chemotherapy in the first-line treatment of metastatic colorectal cancer in KRAS wild type patients: a meta-analysis. *PLoS One*. 2012;7:e50925.
17. 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;359:1757-1765.
18. Adelstein BA, Dobbins TA, Harris CA, et al. A systematic review and meta-analysis of KRAS status as the determinant of response to anti-EGFR antibodies and the impact of partner chemotherapy in metastatic colorectal cancer. *Eur J Cancer*. 2011;47:1343-1354.
19. 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;27:2091-2096.
20. Baas JM, Krens LL, Guchelaar HJ, et al. Concordance of predictive markers for EGFR inhibitors in primary tumors and metastases in colorectal cancer: a review. *Oncologist*. 2011;16:1239-1249.
21. Chen J, Ye Y, Sun H, et al. Association between KRAS codon 13 mutations and clinical response to anti-EGFR treatment in patients with metastatic colorectal cancer: results from a meta-analysis. *Cancer Chemother Pharmacol*. 2013;71:265-272.
22. Dahabreh IJ, Terasawa T, Castaldi PJ, et al. Systematic review: anti-epidermal growth factor receptor treatment effect modification by KRAS mutations in advanced colorectal cancer. *Ann Intern Med*. 2011;154:37-49.
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;304:1812-1820.
24. Health Quality Ontario. KRAS testing for Anti-EGFR therapy in advanced colorectal cancer: an evidence-based and economic analysis. *Ont Health Technol Assess Ser*. 2010;10:1-49.

25. Hoyle M, Crathorne L, Peters J, et al. The clinical effectiveness and cost-effectiveness of cetuximab (mono- or combination chemotherapy), bevacizumab (combination with non-oxaliplatin chemotherapy) and panitumumab (monotherapy) for the treatment of metastatic colorectal cancer after first-line chemotherapy (review of technology appraisal No.150 and part review of technology appraisal No. 118): a systematic review and economic model. *Health Technol Assess.* 2013;17:1-237.
26. Ibrahim EM, Zekri JM, Bin Sadiq BM. Cetuximab-based therapy for metastatic colorectal cancer: a meta-analysis of the effect of K-ras mutations. *Int J Colorectal Dis.* 2010;25:713-721.
27. Jiang Z, Li C, Li F, et al. EGFR gene copy number as a prognostic marker in colorectal cancer patients treated with cetuximab or panitumumab: a systematic review and meta analysis. *PLoS One.* 2013;8:e56205.
28. Ku GY, Haaland BA, de Lima Lopes G Jr. Cetuximab in the first-line treatment of K-ras wild-type metastatic colorectal cancer: the choice and schedule of fluoropyrimidine matters. *Cancer Chemother Pharmacol.* 2012;70: 231-238.
29. Lin AY, Buckley NS, Lu AT, et al. Effect of KRAS mutational status in advanced colorectal cancer on the outcomes of anti-epidermal growth factor receptor monoclonal antibody therapy: a systematic review and meta-analysis. *Clin Colorectal Cancer.* 2011;10:63-69.
30. Linardou H, Dahabreh IJ, Kanaklopiti D, et al. Assessment of somatic k-RAS mutations as a mechanism associated with resistance to EGFR-targeted agents: a systematic review and meta-analysis of studies in advanced non-small-cell lung cancer and metastatic colorectal cancer. *Lancet Oncol.* 2008;9:962-972.
31. Loupakis F, Cremolini C, Salvatore L, et al. Clinical impact of anti-epidermal growth factor receptor monoclonal antibodies in first-line treatment of metastatic colorectal cancer: meta-analytical estimation and implications for therapeutic strategies. *Cancer.* 2012;118:1523-1532.
32. Mao C, Huang YF, Yang ZY, et al. KRAS p.G13D mutation and codon 12 mutations are not created equal in predicting clinical outcomes of cetuximab in metastatic colorectal cancer: a systematic review and meta-analysis. *Cancer.* 2013;119:714-721.
33. Mao C, Yang ZY, Hu XF, et al. PIK3CA exon 20 mutations as a potential biomarker for resistance to anti-EGFR monoclonal antibodies in KRAS wild-type metastatic colorectal cancer: a systematic review and meta-analysis. *Ann Oncol.* 2012;23:1518-1525.
34. Petrelli F, Barni S. Resectability and outcome with anti-EGFR agents in patients with KRAS wild-type colorectal liver-limited metastases: a meta-analysis. *Int J Colorectal Dis.* 2012;27:997-1004.
35. Petrelli F, Coinu A, Cabiddu M, et al. KRAS as prognostic biomarker in metastatic colorectal cancer patients treated with bevacizumab: a pooled analysis of 12 published trials. *Med Oncol.* 2013;30:650.
36. Qiu LX, Mao C, Zhang J, et al. Predictive and prognostic value of KRAS mutations in metastatic colorectal cancer patients treated with cetuximab: a meta-analysis of 22 studies. *Eur J Cancer.* 2010;46:2781-2787.
37. Ren J, Li G, Ge J, et al. Is K-ras gene mutation a prognostic factor for colorectal cancer: a systematic review and meta-analysis. *Dis Colon Rectum.* 2012;55:913-923.
38. Tsoukalas N, Tzovaras AA, Tolia M, et al. Meta-analysis of the predictive value of KRAS mutations in treatment response using cetuximab in colorectal cancer. *J BUON.* 2012;17:73-78.
39. Vale CL, Tierney JF, Fisher D, et al. Does anti-EGFR therapy improve outcome in advanced colorectal cancer? A systematic review and meta-analysis. *Cancer Treat Rev.* 2012;38:618-625.
40. Yang ZY, Shen WX, Hu XF, et al. EGFR gene copy number as a predictive biomarker for the treatment of metastatic colorectal cancer with anti-EGFR monoclonal antibodies: a meta-analysis. *J Hematol Oncol.* 2012;5:52.
41. Zhang L, Ma L, Zhou Q. Overall and KRAS-specific results of combined cetuximab treatment and chemotherapy for metastatic colorectal cancer: a meta-analysis. *Int J Colorectal Dis.* 2011;26:1025-1033.
42. Ross JS. Clinical implementation of KRAS testing in metastatic colorectal carcinoma: the pathologist's perspective. *Arch Pathol Lab Med.* 2012;136:1298-1307.
43. Bando H, Yoshino T, Shinozaki E, et al. Simultaneous identification of 36 mutations in KRAS codons 61 and 146, BRAF, NRAS, and PIK3CA in a single reaction by multiplex assay kit. *BMC Cancer.* 2013;13:405.
44. 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.
45. Etienne-Grimaldi MC, Mahamat A, Chazal M, et al. Molecular patterns in deficient mismatch repair colorectal tumours: results from a French prospective multicentric biological and genetic study. *Br J Cancer.* 2014;110: 2728-2737.
46. Van Cutsem E, Lenz HJ, Kohne CH, et al. Fluorouracil, leucovorin, and irinotecan plus cetuximab treatment and RAS mutations in colorectal cancer. *J Clin Oncol.* 2015;33:692-700.
47. Xu Q, Xu AT, Zhu MM, et al. Predictive and prognostic roles of BRAF mutation in patients with metastatic colorectal cancer treated with anti-epidermal growth factor receptor monoclonal antibodies: a meta-analysis. *J Dig Dis.* 2013;14:409-416.
48. 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:e65995.
49. Forbes SA, Bhamra G, Bamford S, et al. The catalogue of somatic mutations in cancer (COSMIC). *Curr Protoc Hum Genet.* 2008;chap 10:unit 10.11.
50. Lin JS, Webber EM, Senger CA, et al. Systematic review of pharmacogenetic testing for predicting clinical benefit to anti-EGFR therapy in metastatic colorectal cancer. *Am J Cancer Res.* 2011;1:650-662.
51. Mao C, Liao RY, Qiu LX, et al. BRAF V600E mutation and resistance to anti-EGFR monoclonal antibodies in patients with metastatic colorectal cancer: a meta-analysis. *Mol Biol Rep.* 2011;38:2219-2223.
52. Parsons MT, Buchanan DD, Thompson B, et al. Correlation of tumour BRAF mutations and MLH1 methylation with germline mismatch repair (MMR) gene mutation status: a literature review assessing utility of tumour features for MMR variant classification. *J Med Genet.* 2012;49:151-157.

53. Cui D, Cao D, Yang Y, et al. Effect of BRAF V600E mutation on tumor response of anti-EGFR monoclonal antibodies for first-line metastatic colorectal cancer treatment: a meta-analysis of randomized studies. *Mol Biol Rep.* 2014;41:1291-1298.
54. Lochhead P, Kuchiba A, Imamura Y, et al. Microsatellite instability and BRAF mutation testing in colorectal cancer prognostication. *J Natl Cancer Inst.* 2013;105:1151-1156.
55. Rowland A, Dias MM, Wiese MD, et al. Meta-analysis of BRAF mutation as a predictive biomarker of benefit from anti-EGFR monoclonal antibody therapy for RAS wild-type metastatic colorectal cancer. *Br J Cancer.* 2015;112:1888-1894.
56. Cremolini C, Loupakis F, Antoniotti C, et al. FOLFOXIRI plus bevacizumab versus FOLFIRI plus bevacizumab as first-line treatment of patients with metastatic colorectal cancer: updated overall survival and molecular subgroup analyses of the open-label, phase 3 TRIBE study. *Lancet Oncol.* 2015;16:1306-1315.
57. Corcoran RB, Atreya CE, Falchook GS, et al. Phase 1-2 trial of the BRAF inhibitor dabrafenib (D) plus MEK inhibitor trametinib (T) in BRAF V600 mutant colorectal cancer (CRC): updated efficacy and biomarker analysis [ASCO meeting abstract 3517]. *J Clin Oncol.* 2014;32:5s. <http://meetinglibrary.asco.org/content/131743-144>. Accessed November 18, 2016.
58. Bendell JC, Atreya CE, André T, et al. Efficacy and tolerability in an open-label phase I/II study of MEK inhibitor trametinib (T) BRAF inhibitor dabrafenib (D), and anti-EGFR antibody panitumumab (P) in combination in patients (pts) with BRAF V600E mutated colorectal cancer (CRC) [ASCO meeting abstract 3515]. *J Clin Oncol.* 2014;32:5s. <http://meetinglibrary.asco.org/content/131642-144>. Accessed November 18, 2016.
59. Temraz S, Mukherji D, Shamseddine A. Dual inhibition of MEK and PI3K pathway in KRAS and BRAF mutated colorectal cancers. *Int J Mol Sci.* 2015;16:22976-22988.
60. Taieb J, Zaanan A, Le Malicot K, et al. Prognostic effect of BRAF and KRAS mutations in patients with stage III colon cancer treated with leucovorin, fluorouracil, and oxaliplatin with or without cetuximab: a post hoc analysis of the PETACC-8 trial [published online January 14, 2016]. *JAMA Oncol.*
61. Barras D. BRAF mutation in colorectal cancer: an update. *Biomark Cancer.* 2015;7:9-12.
62. Geiersbach KB, Samowitz WS. Microsatellite instability and colorectal cancer. *Arch Pathol Lab Med.* 2011;135:1269-1277.
63. Barrow E, Hill J, Evans DG. Cancer risk in lynch syndrome. *Fam Cancer.* 2013;12:229-240.
64. Schmeler KM, Lynch HT, Chen LM, et al. Prophylactic surgery to reduce the risk of gynecologic cancers in the Lynch syndrome. *N Engl J Med.* 2006;354:261-269.
65. Vasen HF, Blanco I, Aktan-Collan K, et al. Revised guidelines for the clinical management of Lynch syndrome (HNPCC): recommendations by a group of European experts. *Gut.* 2013;62:812-823.
66. Funkhouser WK Jr, Lubin IM, Monzon FA, et al. Relevance, pathogenesis, and testing algorithm for mismatch repair-defective colorectal carcinomas: a report of the Association for Molecular Pathology. *J Mol Diagn.* 2012;14:91-103.
67. Kawakami H, Zaanan A, Sinicrope FA. Implications of mismatch repair-deficient status on management of early stage colorectal cancer. *J Gastrointest Oncol.* 2015;6:676-684.
68. Diaz LA Jr, Le DT. PD-1 blockade in tumors with mismatch-repair deficiency. *N Engl J Med.* 2015;373:1979.
69. Le DT, Uram JN, Wang H, et al. PD-1 blockade in tumors with mismatch-repair deficiency. *N Engl J Med.* 2015;372:2509-2520.
70. Le DT, Uram JN, Wang H, et al. Programmed death-1 blockade in mismatch repair deficient colorectal cancer [ASCO meeting abstract 103]. *J Clin Oncol.* 2016;34. <http://meetinglibrary.asco.org/content/167415-176>. Accessed November 18, 2016.
71. Yang ZY, Wu XY, Huang YF, et al. Promising biomarkers for predicting the outcomes of patients with KRAS wild-type metastatic colorectal cancer treated with anti-epidermal growth factor receptor monoclonal antibodies: a systematic review with meta-analysis. *Int J Cancer.* 2013;133:1914-1925.
72. Pietrantonio F, Petrelli F, Coinu A, et al. Predictive role of BRAF mutations in patients with advanced colorectal cancer receiving cetuximab and panitumumab: a meta-analysis. *Eur J Cancer.* 2015;51:587-594.
73. Wu S, Gan Y, Wang X, et al. PIK3CA mutation is associated with poor survival among patients with metastatic colorectal cancer following anti-EGFR monoclonal antibody therapy: a meta-analysis. *J Cancer Res Clin Oncol.* 2013;139:891-900.
74. Karapetis CS, Jonker D, Daneshmand M, et al. PIK3CA, BRAF, and PTEN status and benefit from cetuximab in the treatment of advanced colorectal cancer—results from NCIC CTG/AGITG CO.17. *Clin Cancer Res.* 2014;20:744-753.
75. Liao X, Morikawa T, Lochhead P, et al. Prognostic role of PIK3CA mutation in colorectal cancer: cohort study and literature review. *Clin Cancer Res.* 2012;18:2257-2268.
76. Ogino S, Liao X, Imamura Y, et al. Predictive and prognostic analysis of PIK3CA mutation in stage III colon cancer intergroup trial. *J Natl Cancer Inst.* 2013;105:1789-1798.
77. Ogino S, Noshu K, Kirkner GJ, et al. PIK3CA mutation is associated with poor prognosis among patients with curatively resected colon cancer. *J Clin Oncol.* 2009;27:1477-1484.
78. Flossmann E, Rothwell PM. Effect of aspirin on long-term risk of colorectal cancer: consistent evidence from randomised and observational studies. *Lancet.* 2007;369:1603-1613.
79. Jacobs EJ, Thun MJ, Bain EB, et al. A large cohort study of long-term daily use of adult-strength aspirin and cancer incidence. *J Natl Cancer Inst.* 2007;99:608-615.
80. Chan AT, Giovannucci EL, Meyerhardt JA, et al. Aspirin dose and duration of use and risk of colorectal cancer in men. *Gastroenterology.* 2008;134:21-28.

81. Domingo E, Church DN, Sieber O, et al. Evaluation of PIK3CA mutation as a predictor of benefit from nonsteroidal anti-inflammatory drug therapy in colorectal cancer. *J Clin Oncol*. 2013;31:4297-4305.
82. Liao X, Lochhead P, Nishihara R, et al. Aspirin use, tumor PIK3CA mutation, and colorectal-cancer survival. *N Engl J Med*. 2012;367:1596-1606.
83. Kothari N, Kim R, Jorissen RN, et al. Impact of regular aspirin use on overall and cancer-specific survival in patients with colorectal cancer harboring a PIK3CA mutation. *Acta Oncol*. 2015;54:487-492.
84. Berg M, Danielsen SA, Ahlquist T, et al. DNA sequence profiles of the colorectal cancer critical gene set KRAS-BRAF-PIK3CA-PTEN-TP53 related to age at disease onset. *PLoS One*. 2010;5:e13978.
85. Shen Y, Yang J, Xu Z, et al. Phosphatase and tensin homolog expression related to cetuximab effects in colorectal cancer patients: a meta-analysis. *World J Gastroenterol*. 2012;18:2712-2718.
86. Wang ZH, Gao QY, Fang JY. Loss of PTEN expression as a predictor of resistance to anti-EGFR monoclonal therapy in metastatic colorectal cancer: evidence from retrospective studies. *Cancer Chemother Pharmacol*. 2012;69:1647-1655.
87. Nassif NT, Lobo GP, Wu X, et al. PTEN mutations are common in sporadic microsatellite stable colorectal cancer. *Oncogene*. 2004;23:617-628.
88. Sawai H, Yasuda A, Ochi N, et al. Loss of PTEN expression is associated with colorectal cancer liver metastasis and poor patient survival. *BMC Gastroenterol*. 2008;8:56.
89. Lin MS, Huang JX, Chen WC, et al. Expression of PPARgamma and PTEN in human colorectal cancer: an immunohistochemical study using tissue microarray methodology. *Oncol Lett*. 2011;2:1219-1224.
90. Li XH, Zheng HC, Takahashi H, et al. PTEN expression and mutation in colorectal carcinomas. *Oncol Rep*. 2009;22:757-764.
91. Colakoglu T, Yildirim S, Kayaselcuk F, et al. Clinicopathological significance of PTEN loss and the phosphoinositide 3-kinase/Akt pathway in sporadic colorectal neoplasms: is PTEN loss predictor of local recurrence? *Am J Surg*. 2008;195:719-725.
92. Eklof V, Wikberg ML, Edin S, et al. The prognostic role of KRAS, BRAF, PIK3CA and PTEN in colorectal cancer. *Br J Cancer*. 2013;108:2153-2163.
93. Price TJ, Hardingham JE, Lee CK, et al. Prognostic impact and the relevance of PTEN copy number alterations in patients with advanced colorectal cancer (CRC) receiving bevacizumab. *Cancer Med*. 2013;2:277-285.
94. Frattini M, Saletti P, Romagnani E, et al. PTEN loss of expression predicts cetuximab efficacy in metastatic colorectal cancer patients. *Br J Cancer*. 2007;97:1139-1145.
95. Perrone F, Lampis A, Orsenigo M, et al. PIK3CA/PTEN deregulation contributes to impaired responses to cetuximab in metastatic colorectal cancer patients. *Ann Oncol*. 2009;20:84-90.
96. Sartore-Bianchi A, Martini M, Molinari F, et al. PIK3CA mutations in colorectal cancer are associated with clinical resistance to EGFR-targeted monoclonal antibodies. *Cancer Res*. 2009;69:1851-1857.
97. Negri FV, Bozzetti C, Lagrasta CA, et al. PTEN status in advanced colorectal cancer treated with cetuximab. *Br J Cancer*. 2010;102:162-164.
98. Laurent-Puig P, Cayre A, Manceau G, et al. Analysis of PTEN, BRAF, and EGFR status in determining benefit from cetuximab therapy in wild-type KRAS metastatic colon cancer. *J Clin Oncol*. 2009;27:5924-5930.
99. Tol J, Dijkstra JR, Klomp M, et al. Markers for EGFR pathway activation as predictor of outcome in metastatic colorectal cancer patients treated with or without cetuximab. *Eur J Cancer*. 2010;46:1997-2009.
100. Ulivi P, Capelli L, Valgiusti M, et al. Predictive role of multiple gene alterations in response to cetuximab in metastatic colorectal cancer: a single center study. *J Transl Med*. 2012;10:87.
101. Cejas P, Lopez-Gomez M, Aguayo C, et al. Analysis of the concordance in the EGFR pathway status between primary tumors and related metastases of colorectal cancer patients: implications for cancer therapy. *Curr Cancer Drug Targets*. 2012;12:124-131.
102. Vakiani E, Janakiraman M, Shen R, et al. Comparative genomic analysis of primary versus metastatic colorectal carcinomas. *J Clin Oncol*. 2012;30:2956-2962.
103. Lee KH, Kim JS, Lee CS, et al. KRAS discordance between primary and recurrent tumors after radical resection of colorectal cancers. *J Surg Oncol*. 2015;111:1059-1064.
104. Aisner DL, Deshpande C, Baloch Z, et al. Evaluation of EGFR mutation status in cytology specimens: an institutional experience. *Diagn Cytopathol*. 2013;41:316-323.
105. Lindeman NI, Cagle PT, Beasley MB, et al. Molecular testing guideline for selection of lung cancer patients for EGFR and ALK tyrosine kinase inhibitors: guideline from the College of American Pathologists, International Association for the Study of Lung Cancer, and Association for Molecular Pathology. *Arch Pathol Lab Med*. 2013;137:828-860.
106. Bettgowda C, Sausen M, Leary RJ, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. *Sci Transl Med*. 2014;6:224ra224.
107. Clinical Laboratory Improvement Amendments of 1988. 42 CFR §493.1351-1495 (1992).
108. Sakai K, Tsurutani J, Yamanaka T, et al. Extended RAS and BRAF mutation analysis using next-generation sequencing. *PLoS One*. 2015;10:e0121891.
109. Salto-Tellez M, Gonzalez de Castro D. Next-generation sequencing: a change of paradigm in molecular diagnostic validation. *J Pathol*. 2014;234:5-10.
110. Cottrell CE, Al-Kateb H, Bredemeyer AJ, et al. Validation of a next-generation sequencing assay for clinical molecular oncology. *J Mol Diagn*. 2014;16:89-105.
111. Clinical laboratory improvement amendments of 1988. 42 CFR §493.1253 (2003).
112. College of American Pathologists. CAP laboratory accreditation checklists. <http://www.cap.org/apps/cap.portal>. Accessed November 18, 2016.
113. Clinical and Laboratory Standards Institute. Establishing molecular testing in clinical laboratory environments, approved guideline (MM19-A). Wayne, PA: Clinical Laboratory Standards Institute; 2011.

114. Halling KC, Schrijver I, Persons DL. Test verification and validation for molecular diagnostic assays. *Arch Pathol Lab Med.* 2012;136:11-13.
115. Clinical and Laboratory Standards Institute. Molecular Diagnostic Methods for Solid Tumors (Nonhematological Neoplasms) (MM23-ED 1). Wayne, PA: Clinical Laboratory Standards Institute; 2015.
116. Ogino S, Gulley ML, den Dunnen JT, et al. Standard mutation nomenclature in molecular diagnostics: practical and educational challenges. *J Mol Diagn.* 2007;9:1-6.
117. Fishel R, Lescoe MK, Rao MR, et al. The human mutator gene homolog MSH2 and its association with hereditary nonpolyposis colon cancer. *Cell.* 1993;75:1027-1038.
118. Bronner CE, Baker SM, Morrison PT, et al. Mutation in the DNA mismatch repair gene homologue hMLH1 is associated with hereditary non-polyposis colon cancer. *Nature.* 1994;368:258-261.
119. Liu B, Nicolaides NC, Markowitz S, et al. Mismatch repair gene defects in sporadic colorectal cancers with microsatellite instability. *Nat Genet.* 1995;9:48-55.
120. Gologan A, Sepulveda AR. Microsatellite instability and DNA mismatch repair deficiency testing in hereditary and sporadic gastrointestinal cancers. *Clin Lab Med.* 2005;25:179-196.
121. Fitzgibbons PL, Bradley LA, Fatheree LA, et al. Principles of analytic validation of immunohistochemical assays: guideline from the College of American Pathologists Pathology and Laboratory Quality Center. *Arch Pathol Lab Med.* 2014;138:1432-1443.
122. Sepulveda AR. The importance of microsatellite instability in colonic neoplasms. Medscape. 2008:571610. www.medscape.org/viewarticle/571610. Accessed November 18, 2016.
123. Hatch SB, Lightfoot HM Jr, Garwacki CP, et al. Microsatellite instability testing in colorectal carcinoma: choice of markers affects sensitivity of detection of mismatch repair-deficient tumors. *Clin Cancer Res.* 2005;11:2180-2187.
124. Watson N, Griew F, Morris M, et al. Heterogeneous staining for mismatch repair proteins during population-based pre-screening for hereditary nonpolyposis colorectal cancer. *J Mol Diagn.* 2007;9:472-478.
125. Cicek MS, Lindor NM, Gallinger S, et al. Quality assessment and correlation of microsatellite instability and immunohistochemical markers among population- and clinic-based colorectal tumors results from the Colon Cancer Family Registry. *J Mol Diagn.* 2011;13:271-281.
126. 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;67:751-757.
127. Biagi JJ, Raphael MJ, Mackillop WJ, et al. Association between time to initiation of adjuvant chemotherapy and survival in colorectal cancer: a systematic review and meta-analysis. *JAMA.* 2011;305:2335-2342.
128. Franklin WA, Haney J, Sugita M, et al. KRAS mutation: comparison of testing methods and tissue sampling techniques in colon cancer. *J Mol Diagn.* 2010;12:43-50.
129. Laosinchai-Wolf W, Ye F, Tran V, et al. Sensitive multiplex detection of KRAS codons 12 and 13 mutations in paraffin-embedded tissue specimens. *J Clin Pathol.* 2011;64:30-36.
130. Ma ES, Wong CL, Law FB, et al. Detection of KRAS mutations in colorectal cancer by high-resolution melting analysis. *J Clin Pathol.* 2009;62:886-891.
131. Pinto P, Rocha P, Veiga I, et al. Comparison of methodologies for KRAS mutation detection in metastatic colorectal cancer. *Cancer Genet.* 2011;204:439-446.
132. Tol J, Dijkstra JR, Vink-Borger ME, et al. High sensitivity of both sequencing and real-time PCR analysis of KRAS mutations in colorectal cancer tissue. *J Cell Mol Med.* 2010;14:2122-2131.
133. Buxhofer-Ausch V, Ausch C, Zeillinger R, et al. Duplex reverse-hybridization assay for the simultaneous detection of KRAS/BRAF mutations in FFPE-extracted genomic DNA from colorectal cancer specimens. *Dis Markers.* 2013;34:171-177.
134. Carotenuto P, Roma C, Rachiglio AM, et al. Detection of KRAS mutations in colorectal carcinoma patients with an integrated PCR/sequencing and real-time PCR approach. *Pharmacogenomics.* 2010;11:1169-1179.
135. Cavallini A, Valentini AM, Lippolis C, et al. KRAS genotyping as biomarker in colorectal cancer: a comparison of three commercial kits on histologic material. *Anticancer Res.* 2010;30:5251-5256.
136. Chang YS, Yeh KT, Hsu NC, et al. Detection of N-, H-, and KRAS codons 12, 13, and 61 mutations with universal RAS primer multiplex PCR and N-, H-, and KRAS-specific primer extension. *Clin Biochem.* 2010;43:296-301.
137. Chen YL, Chang YS, Chang JG, et al. Genotyping of K-ras codons 12 and 13 mutations in colorectal cancer by capillary electrophoresis. *J Chromatogr A.* 2009;1216:5147-5154.
138. Chow L, Lin PC, Chang JS, et al. Differences in the frequencies of K-ras c12-13 genotypes by gender and pathologic phenotypes in colorectal tumors measured using the allele discrimination method. *Environ Mol Mutagen.* 2012;53:22-31.
139. Kristensen LS, Daugaard IL, Christensen M, et al. Increased sensitivity of KRAS mutation detection by high-resolution melting analysis of COLD-PCR products. *Hum Mutat.* 2010;31:1366-1373.
140. Kristensen LS, Kjeldsen TE, Hager H, et al. Competitive amplification of differentially melting amplicons (CADMA) improves KRAS hotspot mutation testing in colorectal cancer. *BMC Cancer.* 2012;12:548.
141. Lang AH, Drexel H, Geller-Rhomberg S, et al. Optimized allele-specific real-time PCR assays for the detection of common mutations in KRAS and BRAF. *J Mol Diagn.* 2011;13:23-28.
142. Sundstrom M, Edlund K, Lindell M, et al. KRAS analysis in colorectal carcinoma: analytical aspects of pyrosequencing and allele-specific PCR in clinical practice. *BMC Cancer.* 2010;10:660.

143. Viray H, Li K, Long TA, et al. A prospective, multi-institutional diagnostic trial to determine pathologist accuracy in estimation of percentage of malignant cells. *Arch Pathol Lab Med*. 2013;137:1545-1549.
144. Nardon E, Glavac D, Benhattar J, et al. A multicenter study to validate the reproducibility of MSI testing with a panel of 5 quasimonomorphic mononucleotide repeats. *Diagn Mol Pathol*. 2010;19:236-242.
145. Ribic CM, Sargent DJ, Moore MJ, et al. Tumor microsatellite-instability status as a predictor of benefit from fluorouracil-based adjuvant chemotherapy for colon cancer. *N Engl J Med*. 2003;349:247-257.
146. Jover R, Zapater P, Castells A, et al. The efficacy of adjuvant chemotherapy with 5-fluorouracil in colorectal cancer depends on the mismatch repair status. *Eur J Cancer*. 2009;45:365-373.
147. Human Genome Variation Society. Human Genome Variation Society website. www.hgvs.org. Updated September 25, 2016. Accessed November 18, 2016.
148. 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;17:405-424.
149. Sukhai MA, Craddock KJ, Thomas M, et al. A classification system for clinical relevance of somatic variants identified in molecular profiling of cancer. *Genet Med*. 2016;18:128-136.
150. Kalman LV, Lubin IM, Barker S, et al. Current landscape and new paradigms of proficiency testing and external quality assessment for molecular genetics. *Arch Pathol Lab Med*. 2013;137:983-988.
151. Schrijver I, Aziz N, Jennings LJ, et al. Methods-based proficiency testing in molecular genetic pathology. *J Mol Diagn*. 2014;16:283-287.
152. Diaz LA, Jr, Bardelli A. Liquid biopsies: genotyping circulating tumor DNA. *J Clin Oncol*. 2014;32:579-586.

■ Appendix 1 ■ Disclosed Interests and Activities, 2010–June 2016

Name	Interest/Activity Type	Entity
Federico A. Monzon, MD	Employment, stock options/bonds	InVita, 2011-2015 Castle Biosciences, 2015-present
Antonia R. Sepulveda, MD, PhD	Consultant	American Gastroenterological Society on endoscopy and tissue sampling, 2015
Veena M. Singh, MD	Employment, stock options/bonds Consultant	Biocept bioTheranostics Lab PMM

■ Appendix 2 ■ Disclosed Positions of Interest, 2010–June 2016^a

Name	Interest
Carmen J. Allegra, MD	Deputy Director and GI Committee Co-Chair, NRG Oncology, 2014-Present Senior Vice-President, NSABP Foundation, 2014-Present Editor-in-Chief, <i>Journal of the National Cancer Institute</i> , 2012-Present
Allison M. Cushman-Vokoun, MD, PhD	CAP Molecular Diagnostic, Genomic and Precision Medicine Resource Guide, CAP Personalized Health Care (PHC) Committee Molecular Pathology Representative, ASCP Curriculum Committee Genetics Representative, AMP Training and Education Committee CAP PHC Committee
William K. Funkhouser, MD, PhD	CAP Molecular Oncology Committee, 2012-2016 Association of Directors of Anatomic and Surgical Pathology
Wayne Grody, MD, PhD Stanley R. Hamilton, MD	President, American College of Medical Genetics and Genomics, 2011-2013 Member, National Cancer Institute (NCI) Colon Task Force, 2015-present <i>Journal of Pathology</i> , <i>Archives of Pathology and Laboratory Medicine</i> , 2015-present NCI Program for Assessment of Clinical Cancer Tests Working Group, 2015-present Member, FDA Immunology Devices Panel, 2015-present Member, Actionable Genome Consortium, 2015-present MoDX Advisory Panel Member, Palmetto Medicare Administrative Contractor, 2015-present CAP Co-Chair of Colorectal Molecular Markers Expert Panel, Physician Quality Reporting Committee and Economic Affairs Committee, member, 2015-present Member, CAP Cancer Biomarker Reporting Committee, 2015-present Institute of Medicine Committee on Policy Issues in the Clinical Development and Use of Biomarkers for Molecularly Targeted Therapies, sponsor and testimony on January 29, 2015, 2015-present

(continued)

Appendix 2 (cont)

Name	Interest
Stanley R. Hamilton, MD	Member, Medical Evidence Development Consortium, 2015-present Member, Center for Medicare and Medicaid Services (CMS) Medicare Evidence Development and Coverage Advisory Committee, 2015-present
Scott E. Kopetz, MD, PhD	Member, CMS Advisory Panel on Clinical Diagnostic Laboratory Tests, 2015-present Translational Sciences Representative, NCI Colon Task Force, 2012-2016 NCI Colon Task Force, Genomics Subcommittee, 2012-2016 Southwest Oncology Group (SWOG), 2012-2016 Colon Cancer, National Surgical Adjuvant Breast and Bowel Project, the Radiation Therapy Oncology Group, and the Gynecologic Oncology Group (NRG) Cooperative Group, 2013-2016 Gastrointestinal Steering Committee, NCI, 2014-2016 SWOG, Translational Sciences GI Subcommittee, 2014-2016 GI Program, National Institutes of Health Cancer Center Support Grant, 2015-2016 SWOG, Translational Sciences GI Subcommittee, 2015-2016
Christopher Lieu, MD Noralane M. Lindor, MD Federico A. Monzon, MD	Genetic Subcommittee of the Cancer Prevention Committee, ASCO, 2012-2015 President, AMP, 2017 President-Elect, AMP, 2016 Chair, Council of MGP Directors, AMP, 2014 AMP Co-Chair, CAP-ASCP-AMP Guideline Development for Colorectal Cancer Molecular Testing, 2011-2013 Chair, Solid Tumor Subdivision and Council Member, AMP, 2012 Technology Assessment Committee member, CAP, 2008-2012 AMP, Clinical Practice Committee, 2009-2010 Chair of the Board, American Society for Radiation Oncology GI Steering Committee, NCI
Bruce D. Minsky, MD	CAP Center Committee, Pathology and Laboratory Quality Center, 2009-2015 CAP Public Health Policy Committee (formerly Patient Safety and Performance Measures Committee), 2007-2011 CAP CGPA Molecular Pathology Working Group, 2008-2012 Ad Hoc Committee on Laboratory Quality and Improvement for the 21st Century LQI-21 Laboratory Developed Test Working Group, 2008-2010 CAP Molecular Pathology (Molecular Oncology) Resource Committee, 2005-2011 Measures and Performance Assessment Working Group, CAP Economic Affairs Committee, 2012-2013 Council on Governmental and Professional Affairs, PHC Working Group, 2012-2016, CAP PHC Associate Editor for Clinical Pathology, <i>Archives of Pathology and Laboratory Medicine</i> , 2012-current Member, CAP Guideline Metrics Expert Panel, 2014-current AMA CPT Editorial Panel Member (American Hospital Association), 2015-current AMA CPT Molecular Pathology Advisory Group, 2015-current AMP Professional Relations Committee, 200-2012 AMP Economic Affairs Committee, 2009-current (co-chair, 2013-2014) AMA CPT Editorial Panel Molecular Pathology Coding Working Tier 1 and Tier 2 Working Groups, 2009-2012 Past-President, AMP, 2010 AMP Nominating Committee (chair), 2010 Pathology Coding Caucus—AMP Representative, 2005-2008, 2013-2015 CMS Medicare Evidence Development and Advisory Committee appointee, 2012
Jan A. Nowak, MD, PhD	Alliance for Clinical Trials in Oncology, 2011-present
Daniel J. Sargent, PhD	

AMA, American Medical Association; CGPA, Council on Government and Professional Affairs; CPT, Current Procedural Terminology; FDA, Food and Drug Administration; GI, gastrointestinal; MGP, molecular genetic pathology; NSABP, National Surgical Adjuvant Breast and Bowel Project.

^aExpert panel members and staff Joseph Willis, MD, Jennifer Clark, SCT, MB(ASCP)^{CM}, Carol Colasacco, MLIS, R. Bryan Rumble, MSc, Robyn Temple-Smolkin, PhD, and Christina Ventura, MT(ASCP), have no reported conflicts of interest to disclose. The information above reflects disclosures that were collected and reviewed by the College of American Pathologists (CAP), the American Society for Clinical Pathology (ASCP), the American Society of Clinical Oncology (ASCO), and the Association for Molecular Pathology (AMP). The disclosures that appear in the individual journals of the societies may vary based on journal-specific policies and procedures.