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SPECIAL ARTICLE

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

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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.

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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, cochairs, 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 *http://dx.doi.org/10.1016/j.jmoldx.2016.11.001* 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, 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 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]

Table 1 Levels of Evidence*

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)

*Data derived from National Health and Medical Research Council.[10]

Table 2 Grades for Strength of Evidence*

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

*Adapted from Guyatt et al,[11] by permission of BMJ Publishing Group Limited.

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

Table 3 Grades for Strength of Recommendation*

Designation	Recommendation	Rationale
Strong recommendation	Recommend for or against a particular molecular testing practice for colorectal cancer (can include *must* or *should*)	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 *should* or *may*)	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 *should* or *may*)	Serious limitations in strength of evidence (inadequate of 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

*Data derived from Guyatt et al.[11]

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 *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*).	Recommendation
2a. *BRAF* p.V600 [*BRAF* c.1799 (p.V600)] mutational analysis should be performed in colorectal cancer tissue in patients with colorectal carcinoma for prognostic stratification.	Recommendation
2b. *BRAF* p.V600 mutational analysis should be performed in deficient MMR tumors with loss of MLH1 to evaluate for Lynch syndrome risk. Presence of a *BRAF* mutation strongly favors a sporadic pathogenesis. The absence of a *BRAF* 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 *BRAF* 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 *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.	No recommendation
6. There is insufficient evidence to recommend PTEN 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

(*table continues*)

Table 4 (*continued*)

Guideline Statement	Strength of Recommendation
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
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.

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

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 *KRAS* 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; $P < .05$, in favor of Mut−	Median PFS, HR, 0.85; 95% CI, 0.74-0.98; $P < .05$, in favor of Mut−	*KRAS* Mut+: 48.3% vs *KRAS* Mut−: 54.8% (OR, 1.42; 95% CI, 1.05-1.92; $P < .05$)
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, $P < .05$, in favor of G13D	PFS, HR, 0.54; 95% CI, 0.36-0.81, $P < .05$, in favor of G13D	*KRAS* pG13D: 22% *KRAS* 12: 16% *KRAS* Mut−: 44% (pG13D vs 12: RR, 1.64; 95% CI, 1.13-2.38; $P < .05$) pG13D vs Mut−: RR, 0.54; CI, 0.38-0.77; $P < .05$)
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; $P < .05$)	GCN associated with improved PFS (HR, 0.65; 95% CI, 0.47-0.89; $P < .05$)	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, $P < .05$, in favor of cetuximab over BSC in Mut− Panitumumab + BSC compared with BSC alone in Mut−, P = ns	Median PFS, HR, 0.40; 95% CI, 0.30-0.54, $P < .05$, third-line cetuximab + BSC compared with BSC alone in Mut− Median PFS, HR, 0.45; 95% CI, 0.34-0.59, $P < .05$, panitumumab + BSC compared with BSC alone in Mut−	*KRAS* Mut−: 12.8% *KRAS* Mut+: 1.2%, $P < .05$, cetuximab + BSC compared with BSC alone in Mut− *KRAS* Mut−: 10% *KRAS* mut: 0, $P < .05$, 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, $P < .05$) Codon 13 Mut+ vs Mut−: RR, 0.61 (95% CI, 0.45-0.83, $P < .05$)
Zhou et al,[16] 2012	SR: 4 RCTs including 1,270 first-line patients with mCRC (all Mut−)	Oxaliplatin CT ± anti-EGFR mAbs Anti-EGFR + CT vs CT		Mut− only	HR, 1.00; 95% CI, 0.88-1.13, P = ns	HR, 0.86; 95% CI, 0.71-1.04, P = ns	RR, 1.08; 95% CI, 0.86-1.36, P = 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, P = ns Cetuximab + CT vs	Cetuximab + CT vs CT alone, Mut−: HR, 0.64; 95% CI, 0.50-0.84, $P < .05$, favors +cetuximab	Cetuximab + CT vs CT alone: RR, 1.93; 95% CI, 1.14-3.26, $P < .05$, favors +cetuximab

(*table continues*)

Table 5 (*continued*)

Author, Year	No. of Studies (No. of Patients)	Comparison	Tests Used	Codons Studied	OS	PFS	ORR
					CT alone, mutation: HR, 1.03; 95% CI, 0.74-1.44, P = ns	Cetuximab + CT vs CT alone, mutation: HR, 1.37; 95% CI, 0.81-2.31, P = ns	Cetuximab+CT vs CT alone, Mut—: RR, 1.44; 95% CI, 1.20-1.73, $P < .05$, 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, $P < .05$	First/second line, PFS, HR, 0.83; 95% CI, 0.76-0.90, $P < .05$ Third line, PFS, HR, 0.43; 95% CI, 0.35-0.52, $P < .05$, 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—	—*	12, 13, 61	HR, 1.61; 95% CI, 1.19-2.18, $P < .05$, 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	P = ns	PFS, HR, 0.68, $P < .05$, in favor of adding cetuximab and/or panitumumab to CT in Mut— patients	RR, 1.67, $P < .05$, in favor of adding cetuximab and/or panitumumab to CT in Mut— patients
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	P = 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; $P < .05$, in favor of adding anti-EGFR MAbs to CT in Mut— patients (irinotecan favoring CT, $P < .05$)	RR, 1.17; 95% CI, 1.04-1.33; $P < .05$, in favor of *KRAS* 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, P = ns	No pooling performed in this comparison AIO trial, P = ns	NR

(*table continues*)

Table 5 (*continued*)

Author, Year	No. of Studies (No. of Patients)	Comparison	Tests Used	Codons Studied	OS	PFS	ORR
					CECOG trial, $P <$ .05 in favor of cetuximab + FOLFOX in Mut− patients	CECOG trial, $P =$ ns in favor of cetuximab + FOLFOX in Mut− patients	
Petrelli et al,[15] 2011	SR: 7 trials including 5,212 patients with advanced CRC, *KRAS* Mut− only	Cetuximab or panitumumab + CT vs BSC	NR	NR	HR, 0.84; 95% CI, 0.73-0.98, $P <$ .05, in favor of anti-EGFR mAbs vs no mAbs in Mut −patients	PFS, HR, 0.65; 95% CI 0.51-0.83, $P <$ .05, in favor of anti-EGFR mAbs vs no mAbs in Mut− patients	RR, 1.69; 95% CI, 1.20-2.38; $P <$ .05, in favor of anti-EGFR
Mao et al,[33] 2012	SR: 13 studies including 576 patients with mCRC, all *KRAS* Mut −treated with anti-EGFR MAbs	Mut+ vs Mut−	Direct sequencing, survey analysis, alleic discrimination, Sanger	*PIK3CA* exon 9, 20	HR, 3.29; 95% CI, 1.60-6.74; $P <$ .05	PFS, HR, 2.52; 95% CI, 1.33-4.78, $P <$ .05, *PIK3CA* exon 20 mutations associated with significantly shorter PFS duration	RR, 0.25; 95% CI, 0.05-1.19; $P <$ .05, *PIK3CA* 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	$P =$ ns	PFS, HR, 0.66; 95% CI, 0.53-0.82, $P <$ .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− *KRAS*	Panitumumab-based treatment vs control	NR	NR	$P =$ ns	PFS, HR, 0.58; 95% CI, 0.36-0.93; $P <$ .05, in favor of adding panitumumab to CT in Mut− patients	OR, 1.08; 95% CI, 0.75-1.58; $P =$ ns
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, $P =$ ns in Mut− patients	PFS, HR, 2.22; 95% CI, 1.74-2.84, $P <$ .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) *KRAS* 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 *KRAS* Mut+/Mut− between primary and metastases	Sequencing, pyrosequencing, PCR-RFLP, SSCP, AS-PCR, ASO	*KRAS*, *PIK3CA*, *BRAF*, 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, $P <$ .05, in favor of anti-EGFR mAbs in Mut− patients	RD, 15%; 95% CI, 8%-22%, $P <$ .05, in favor of *KRAS* Mut− + anti-EGFR treatment

(*table continues*)

Table 5 (*continued*)

Author, Year	No. of Studies (No. of Patients)	Comparison	Tests Used	Codons Studied	OS	PFS	ORR
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, $P < .05$, 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; $P < .05$, longer median PFS shown in Mut −patients who received anti-EGFR mAbs + CT	*KRAS* Mut−: 39% *KRAS* Mut+: 14% RR, 0.24; 95% CI, 0.16-0.38, $P < .05$
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, $P < .05$, longer survival detected in Mut− patients treated with cetuximab +irinotecan	Mean PFS, MD, = −3.32; 95% CI, −4.86 to −1.78, $P < .05$, 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	$P < .05$, in favor of treatment with cetuximab + CT in Mut− patients	PFS, $P < .05$, in favor of treatment with cetuximab + CT in Mut− patients	OR, 2.10; 95% CI, 1.42-3.10, $P < .05$
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) $P < .05$, 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) $P < .05$, 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 *KRAS* 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 *RAS* subgroups, including Mut+ vs Mut−	Bidirectional Sanger sequencing, pyrosequencing, MALDI-TOF analysis, and WAVE-based Surveyor analysis	*KRAS/NRAS* 12, 13, 59, 61, 117, 146	*RAS* Mut− vs *RAS* Mut+: HR, 0.72 (95% CI, 0.56-0.92; $P < .01$) *RAS* Mut− superior *KRAS* exon 2 mutant vs new *RAS* mutant: $P =$ ns *RAS* Mut−, anti-EGFR vs no anti-EGFR: HR, 0.87	*RAS* Mut− vs *RAS* Mut+: HR, 0.60 (95% CI, 0.48-0.76; $P < .001$) *RAS* Mut− superior *KRAS* exon 2 mutant vs new *RAS* mutant: $P =$ ns *RAS* Mut−, anti-EGFR vs no anti-EGFR: HR, 0.62	NR

(*table continues*)

Table 5 (*continued*)

Author, Year	No. of Studies (No. of Patients)	Comparison	Tests Used	Codons Studied	OS	PFS	ORR
					(95% CI, 0.77-0.99; $P < .04$) *KRAS* exon 2 Mut−, anti-EGFR vs no anti-EGFR: HR, 0.90 (95% CI, 0.83-0.98; P = ns) Any *RAS* mutant, anti-EGFR vs no anti-EGFR: HR, 1.08 (95% CI, 0.97-1.21; P = ns) *KRAS* exon 2 mutant, anti-EGFR vs no anti-EGFR: HR, 1.05 (95% CI, 0.95-1.17; P = ns)	(95% CI, 0.50-0.76; $P < .001$) *KRAS* exon 2 Mut−, anti-EGFR vs no anti-EGFR: HR, 0.68 (95% CI, 0.58-0.80; $P < .001$) Any *RAS* mutant, anti-EGFR vs no anti-EGFR: HR, 1.12 (95% CI, 0.94-1.34; P = ns) *KRAS* exon 2 mutant, anti-EGFR vs no anti-EGFR: HR, 1.14 (95% CI, 0.95-1.36; P = ns)	
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-PCR; 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; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; mCRC, metastatic colorectal cancer; MD, mean difference; mAbs, monoclonal antibodies; 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 al[37]: hybridization, PCR, direct sequencing, topographic genotyping, AS-PCR, tissue transglutaminase enzyme, high-performance liquid chromatography, pyrosequencing, capillary sequencing.

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 tumor tested for *KRAS* mutations in a Clinical Laboratory Improvements Amendments '88 (CLIA)−accredited laboratory.[2]

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 *http://dx.doi.org/10.1016/j.jmoldx.2016.11.001*). 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 al[44] 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]

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

Table 6 Outcomes of *RAS* Mutations and Anti-EGFR Therapy[12]

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, 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.

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 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 nonindustry 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

Table 7 Prevalence of New *RAS* Mutations Across Studies*

Study	New *RAS* Total,† %	*KRAS* Exon 3,† %	*KRAS* Exon 4,† %	*NRAS* Exon 2,† %	*NRAS* Exon 3,† %	*NRAS* Exon 4,† %
		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‡	3.7§	6.3¶	NR‡	NE
20020408	17.6	4.8‡	5.0	4.2	3.0‡	1.1
20050181	20.5	4.6	7.9	2.3	5.8	0.0
PRIME	17.4	3.7‡	5.6	3.4	4.1‡	0.0
FIRE-3	16.0	4.3‡	4.9§	3.8	2.0‡	0.0
PEAK	20.1	4.1	7.7	5.4	5.9	0.0
COIN	8.4	2.1c	NE	0.9‖	3.0‡	NE
CRYSTAL	14.7	3.3	5.6	3.5	2.8	0.9
Summary (95% CI)**	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, 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.

*Modified from Sorich et al[12] by permission of Oxford University Press on behalf of the European Society for Medical Oncology.

†New *RAS* mutations are reported as a proportion of the *KRAS* exon 2 nonmutated/wild-type group.

‡*KRAS* and *NRAS* codon 59 mutation was not evaluated.

§*KRAS* codon 117 mutation was not evaluated.

¶Exon 3 codon 61 mutations in addition to the exon 2 mutations.

‖Only *NRAS* mutation G12C evaluated.

**Random-effects meta-analysis summary estimates.

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 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 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 al[20] 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

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
CPGs, systematic reviews, and meta-analyses (n = 8)			
Parsons et al,[52] 2012	SR: 36 studies including 4,562 CRC tumors (*BRAF*), 43 studies including 2,975 CRC tumors (*MLH1*)	Correlation study	NR
Mao et al,[51] 2011	SR: 11 studies including 1,046 patients with mCRC	Mut+ vs Mut−	NR
Lin et al,[50] 2011	SR: 1 study of 649 patients with mCRC, all *KRAS* Mut−; 6.5% were *BRAF* Mut+	Mut+ vs Mut−	NR
Baas et al,[20] 2011	SR: 7 studies including 538 patients with mCRC	Mut+ vs Mut−	Sequencing, pyrosequencing
Cui et al,[53] 2014	SR: 4 studies including 1,245 patients	Mut+ vs Mut− CT ± anti-EGFR mAbs	PCR
Yang et al,[71] 2013	SR: 17 studies (patients, n = NR)	Mut+ vs Mut−	—*
Yuan et al,[48] 2013	SR: 21 studies including 5,229 patients	Mut+ vs Mut−	NR
Xu et al,[47] 2013	SR: 19 studies including 2,875 patients	Mut+ vs Mut−	NR
Prospective cohort studies (n = 1)			
Etienne-Grimaldi et al,[45] 2014	251 patients	Mut+ vs Mut−	NR
Retrospective cohort studies (n = 1)			
Bando et al,[43] 2013	82 samples from 376 patients	All Mut- vs *BRAF* Mut+ and *PIK3CA* Mut−	Luminex xMAP vs DS (concordance rate 100%)

(*table continues*)

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.

*Yang et al[71]: 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.

Table 8 (*continued*)

Codons Studied	OS	PFS	ORR
BRAF p.V600E, *MLH1*	NR	NR	NR
V600E	NR	NR	*BRAF* Mut+: 0 *BRAF* Mut−: 36.3%; $P < .05$; RR, 0.14; 95% CI, 0.04-0.53
V600E	Shorter duration in *BRAF* Mut+ patients, difference 28 weeks, $P < .05$	PFS, shorter duration in *BRAF* Mut+ patients, difference 18 weeks, $P < .05$	NR
V600E	NR	NR	NR
V600E	NR	NR	Mut+ vs Mut− (all *KRAS* Mut−): RR, 0.43 (95% CI, 0.16-0.75; $P < .05$) in favor of Mut− Mut ± vs CT ± anti-EGFR mAbs (all *KRAS* Mut−): RR, 0.38 (95% CI, 0.20-0.73; $P < .05$) in favor of Mut− Mut+ and CT ± anti-EGFR mAbs; $P =$ ns Mut− and *KRAS* Mut− and CT ± anti-EGFR mAbs: RR, 1.48 (95% CI, 1.28-1.71; $P < .05$) in favor of *BRAF* Mut− with CT + anti-EGFR mAbs
V600E, 599, 466, 469	(7 studies) *BRAF* Mut ±: HR, 2.74 (95% CI, 1.79-4.19; $P < .05$) in favor of *BRAF* Mut−	(8 studies) *BRAF* Mut ±: HR, 2.59 (1.67, 4.03; $P < .05$) in favor of *BRAF* Mut−	*BRAF* Mut−: 46.4% *BRAF* Mut: 18.5% $P < .05$ in favor of *BRAF* Mut−
V600E	HR, 0.35 (95% CI, 0.29-0.42; $P < .05$) in favor of *BRAF* Mut−	HR, 0.38 (95% CI, 0.29-0.51; $P < .05$) in favor of *BRAF* Mut−	RR, 0.31 (95% CI, 0.18-0.53; $P < .05$) in favor of *BRAF* and *KRAS* Mut−
V600E, K601E (1 study), D549C (1 study)	HR, 2.85 (95% CI, 2.31-3.52; $P < .05$) in favor of *BRAF* Mut−	HR, 2.98 (95% CI, 2.07-4.27; $P < .05$) in favor of *BRAF* Mut−	ORR, 0.58 (95% CI, 0.35-0.94; $P < .05$) in favor of *BRAF* Mut−
BRAF p.V600E	NR	Shorter RFS in *KRAS* Mut− and *BRAF* Mut− patients with stage III tumors ($P < .05$)	
600	All Mut−: 13.8 months (95% CI, 9.2-18.4) vs *BRAF/PIK3CA* Mut: 6.3 months (95% CI, 1.3-11.3; $P < .05$)	All Mut−: 6.1 months (95% CI, 3.1-9.2) vs *BRAF/PIK3CA* Mut+: 1.6 months (95% CI, 1.5-1.7; $P < .05$)	All Mut−: 38.8% vs *BRAF/PIK3CA* Mut+: 0%, $P < .05$

Table 9 Summary of Frequencies of Tumor V600E Mutation Status*

Sample Group	No. of Studies	Positive p.V600E, No.	Negative *BRAF* p.V600E, No.	*BRAF* p.V600E, % (95% CI)
Known negative MMR mutation status				
MSI-H known mutation status	11	115	216	36.10 (20.95-52.84)
MLH1 methylation or *MLH1* 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.

*Adapted from Parsons et al[52] by permission from BMJ Publishing Group Limited.

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 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 al[66] (recommendation 11).

A systematic review of 31 studies[7] reporting survival on 12,782 patients whose tumors were characterized for MSI showed a favorable prognosis, as determined by both OS

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 = ns; no significant benefit of chemotherapy in MSI-H patients	MSI-H: RFS, HR, 0.96; 95% CI, 0.62-1.49, P = 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.

and DFS (Table 10), but this is dependent on stage. In addition, the presence of MSI in CRC was reported to be predictive for nonresponse 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 bias[7]; 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 al[48] reported on any potential conflicts of interest, the article by Mao et al[51] 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 al[71] 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

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
CPGs, systematic reviews, and meta-analyses on *PIK3CA* 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
Mao et al,[33] 2012	SR: 13 studies including patients all *KRAS* Mut−treated with anti-EGFR mAbs	E20 Mut+ vs E20 Mut−	NR
Lin et al,[50] 2011	SR: 4 studies 1,030 patients with mCRC, all *KRAS* Mut− subgroup analysis, exons 9 and 20	Mut+ vs Mut−	NR
Baas et al,[20] 2011	SR: 3 studies including 195 patients with mCRC	Mut+ vs Mut−	Sequencing, pyrosequencing
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
Retrospective cohort studies (n = 1)			
Bando et al,[43] 2013	82 samples from 376 patients	All Mut−vs *BRAF* Mut+ and *PIK3CA* Mut+	Luminex xMAP vs DS (concordance rate 100%)

(*table continues*)

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, 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; RD, risk difference; RR, response rate; RT-PCR, reverse transcription polymerase chain reaction; SR, systematic review; xMAP, multiplex assay.

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 al[3] 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 al[81] and Liao et al[82] 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 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

Table 11 (*continued*)

Codons Studied	OS	PFS	ORR
Exons 9, 20	HR, 1.28; 95% CI, 1.05-1.56, $P < .05$, patients with *PIK3CA* Mut+ had shorter PFS	PFS, HR, 1.53; 95% CI, 1.28-1.84, $P < .05$, patients with *PIK3CA* Mut+ had shorter PFS	NR
Exon 20	HR, 3.29; 95% CI, 1.60-6.74; $P < .05$	PFS, HR, 2.52; 95% CI, 1.33-4.78, $P < .05$, *PIK3CA* 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, P = ns (subset: 377 patients)
Exons 9, 20	P = ns, no difference between Mut+ and Mut− patients Exon 20 Mut+ predicts poorer survival	P = ns, no difference between Mut+ and Mut- patients Exon 20 Mut+ predicts poorer survival	NR
Exons 9, 20	NR	NR	NR
Exons 7, 8, 9, 18, 19, 20	(6 studies) HR, 1.43 (95% CI, 1.02-2.0; $P < .05$) in favor of Mut−	(6 studies) HR, 1.91 (95% CI, 0.78-4.68; P = ns) $P < .05$ in favor of exon 9 compared with exon 20 mutations	(6 studies) RD: −23% (-35%, -10%; $P < .05$) in favor of exon 9 compared exon 20 mutations
Exon 9	All Mut−: 13.8 months (95% CI, 9.2-18.4) vs *BRAF/PIK3CA* Mut+: 6.3 months (95% CI, 1.3-11.3; $P < .05$)	All Mut−: 6.1 months (95% CI, 3.1-9.2) vs *BRAF/PIK3CA* Mut+: 1.6 months (95% CI, 1.5-1.7; $P < .05$)	All Mut-: 38.8% vs *BRAF/PIK3CA* Mut+: 0, $P < .05$

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.

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 CRC[20] 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 al[101] reported at least partial industry funding, and the study reported by Vakiani et al[102] did not report the source of funding, if any. The study by Cejas et al[101] was deemed to have a low to moderate risk of bias, and the study by Vakiani et al[102] 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.

Table 12 Concordance Rates Between Primary and Metastatic Lesions*

Genes Tested (n)	Concordance Rate, %
KRAS (117)[101]	91.0
KRAS, *NRAS*, *BRAF* (84)[102]	98.8
PIK3CA (117)[101]	94.0
PIK3CA (84)[102]	92.8
PTEN IHC (117)[101]	66.0

*Summary 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.

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 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 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,

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

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

(*table continues*)

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.

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

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

‡Total of 84.4% of consensus mutation result.

§Detected one mutation in 23 Mut- alleles.

¶Variable concordance for different tumor percentage in the sample.

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

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

Table 13 (*continued*)

Population Sensitivity of Testing Method, %*	Sensitivity of Assay	Analytical Sensitivity, % (Mutant Allele Fraction)	Specificity, %	PPV, %	NPV, %	Minimal Tumor, %	Concordance Between Assays, %	Study
59	Increased[†] (>100)	5-10	98	NR	NR	30	95	PCS
36.4	84.4[‡]	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	
39.4	96.5	1	99.7	99.5	97.2	3-90	95.30	PCS
47.0	100	1	100	NR	NR	At least 50	100	PCS
34.0	100	NR[§]	100	100	100	NR	100	PCS
36.0	100	NR	100	100	100	NR	100	PCS
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
54.0	100	1	87	81	100	1-90	NR	RCS
43	100	5	71	66	100	1-90	93	RCS
45.0	100	1	100	100	100	NR	NR or M	RCS
38.6	95.8	1	100	100	97.3	<30 vs >70	Variable[¶]	RCS
	98.6	NR	100	100	99.1	NR	NR	RCS
	92.5-100	NR	100	NR	NR	70	NR	RCS
	92.5-100	NR	100	NR	NR	NR	NR	RCS
NR	93	0.1-5	100	NR	NR	NR	[‖]	RCS
	97	5-10	100	NR	NR	NR		RCS
44.4	98	0.50	98	NR	NR	NR	95.9	RCS
	99	NR	100	NR	NR	NR	NR	RCS
36.8	95.7[§]	1	NR	NR	NR	>50	NR	RCS

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 cases[125]; 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 one[132] 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, one[132] reported at least partial industry funding, one[142] reported industry funding, and one[131] 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 one[132] 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. One[102] did not report the source of funding, while the other[144] 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 mono-based 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 noninvasive 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.

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Supplemental Data

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Appendix 1 Disclosed Interests and Activities 2010 — June 2016

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

Appendix 2 Disclosed Positions of Interest from 2010 — June 2016

Carmen J. Allegra, MD	NRG Oncology, Deputy Director and GI Committee co-Chair, 2014 — Present
	NSABP Foundation, Senior vice-President; 2014 — Present
	Journal National Cancer Institute, Editor-in-Chief; 2012 — Present
Allison M. Cushman-Vokoun, MD, PhD	CAP Molecular Diagnostic, Genomic and Precision Medicine Resource Guide, CAP PHC Committee
	Molecular Pathology Representative, ASCP Curriculum Committee
	Genetics Representative, Association for Molecular Pathology (AMP) Training and Education Committee
	CAP Personalized Health Care (PHC) Committee
William K. Funkhouser, MD, PhD	Molecular Oncology Committee, College of American Pathologists, 2012-2016
	Association of Directors of Anatomic and Surgical Pathology (ADASP)
Wayne Grody, MD, PhD	President, American College of Medical Genetics and Genomics, 2011-2013
Stanley R. Hamilton, MD, PhD	NCI Colon Task Force, member 2015-present
	Journal of Pathology, Archives of Pathology and Laboratory Medicine 2015-present
	NCI Program for Assessment of Clinical Cancer Tests (PACCT) Working Group 2015-present
	FDA Immunology Devices Panel, Member 2015-present
	Actionable Genome Consortium, Member 2015-present
	MolDX Advisory Panel member, Palmetto Medicare Administrative Contractor (MAC) 2015-present
	College of American Pathologists Co-Chair of Colorectal Molecular Markers Expert Panel, Physician Quality Reporting Committee and Economic Affairs Committee, member 2015-present
	College of American Pathologists Cancer Biomarker Reporting Committee, member 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
	Medical Evidence Development Consortium (MED-C), member 2015-present
	Center for Medicare and Medicaid Services (CMS) Medicare Evidence Development and Coverage Advisory Committee (MEDCAC), member 2015-present
	CMS Advisory Panel on Clinical Diagnostic Laboratory Tests (CDLT), member 2015-present
Scott Kopetz, MD, PhD	NCI Colon Task Force, Translational Sciences Representative, 2012-2016
	NCI Colon Task Force, Genomics Subcommittee, 2012-2016
	Southwest Oncology Group, 2012-2016
	Colon Cancer, National Surgical Adjuvant Breast and Bowel Project (NSABP), the Radiation Therapy Oncology Group (RTOG), and the Gynecologic Oncology Group (GOG) (NRG) Cooperative Group, 2013-2016
	Gastrointestinal Steering Committee, National Cancer Institute, 2014-2016
	Southwest Oncology Group (SWOG), Translational Sciences GI Sub-Committee, 2014-2016
	GI Program, NIH Cancer Center Support Grant (CCSG), 2015-2016

(appendix continues)

Appendix 2 (*continued*)

Christopher Lieu, MD	Southwest Oncology Group (SWOG), Translational Sciences GI Sub-Committee, 2015-2016
Noralane M. Lindor, MD	Genetic Subcommittee of the Cancer Prevention Committee, American Society of Clinical Oncology, 2012-2015
Federico A. Monzon, MD	Association for Molecular Pathology, President, 2017 Association for Molecular Pathology, President-Elect, 2016 Association for Molecular Pathology, Chair, Council of MGP Directors, 2014 CAP-ASCP-AMP Guideline Development for Colorectal Cancer Molecular Testing, AMP Co-chair, 2011-2013 Association for Molecular Pathology, Chair, Solid Tumor Subdivision and Council Member, 2012 College of American Pathologists, Technology Assessment Committee (TAC) member, 2008-2012 Association for Molecular Pathology, Clinical Practice Committee. 2009-2010
Bruce D. Minsky, MD	Chair of the Board, American Society for Radiation Oncology GI Steering Committee, National Cancer Institute
Jan A. Nowak, MD, PhD	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 (LDT) Working Group 2008 — 2010 CAP Molecular Pathology (Molecular Oncology) Resource Committee 2005-2011 Measures and Performance Assessment Working Group, College of American Pathologists Economic Affairs Committee, 2012 — 2013 Council on Governmental and Professional Affairs - PHC Working Group, 2012 — 2016, College of American Pathologists (CAP) Personalized Healthcare Committee (PHC) Archives of Pathology and Laboratory Medicine, Associate Editor for Clinical Pathology, 2012 — current CAP Guideline Metrics Expert Panel, member, 2014 — current AMA CPT Editorial Panel Member (American Hospital Association) 2015 — current AMA CPT Molecular Pathology Advisory Group (MPAG) 2015 — current AMP Professional Relations Committee 2006- 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 AMP Past President 2010 AMP Nominating Committee (chair) 2010 Pathology Coding Caucus — AMP Representative 2005-2008; 2013 — 2015 CMS Medicare Evidence Development and Advisory Committee (MEDCAC) appointee, 2012
Daniel J. Sargent, PhD	Alliance for Clinical Trials in Oncology, 2011-present

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