Molecular Testing in Colorectal Cancer

Diagnosis of Lynch Syndrome and Personalized Cancer Medicine

Chanjuan Shi, MD, PhD, and Kay Washington, MD, PhD

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CME/SAM

Upon completion of this activity you will be able to:
- define 3 major molecular pathways of colorectal carcinogenesis.
- apply molecular testing to identify patients with Lynch syndrome.
- apply molecular testing to identify colorectal cancer sensitive to anti–epidermal growth factor receptor therapies.

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Abstract

Currently, molecular testing in colorectal cancer (CRC) is aimed at detecting Lynch syndrome and predicting response to anti–epidermal growth factor receptor (EGFR) therapies. However, CRC is a complex disease, with at least 3 molecular pathways of carcinogenesis. The importance of the EGFR signaling pathway in colorectal carcinogenesis is underscored by the availability of anti-EGFR monoclonal antibodies for the treatment of some metastatic CRCs. Potentially, mutations in any of the genes in the EGFR signaling pathway may be associated with prognosis and may predict response to anti-EGFR or other targeted therapies. Although not currently the standard of care, molecular testing of CRCs is expanding to include mutational analysis of the genes in the EGFR pathway, in addition to more widely performed tests for identifying cancers with high microsatellite instability. Multiplex molecular prognostic panels for therapeutic decision making in stage II CRCs also represent expanding use of molecular testing for this common cancer.

Historically, all colorectal cancers (CRCs) have been considered a single disease entity that shares the same cause, clinical characteristics, and treatment outcomes. However, through analysis of precursor lesions and hereditary forms of the disease, it has now become clear that CRC is a complex and heterogeneous disorder. At least 3 distinct molecular pathways to CRC have been described: the conventional suppressor pathway characterized by mutations in *APC* and exemplified by familial adenomatous polyposis coli (FAP), the serrated pathway characterized by aberrant cytosine-guanosine (CpG) island methylation, and the hereditary microsatellite instability (MSI) pathway found in Lynch syndrome.[1-4] To add to the complexity, different genes may be mutated or altered in carcinomas arising via the same genetic pathway. Comprehensive exome sequencing has revealed that individual CRCs harbor an average of 76 gene mutations and the mutated genes in the 2 tumors overlap to only a small extent; a few genes such as *APC* are mutated at high frequency, whereas a much larger number of genes are mutated at relatively low frequency.[5]

While MSI testing has been used for more than a decade for identifying patients with Lynch syndrome, with the recent growth in personalized cancer care, other molecular tests to identify the genetic makeup of individual cancers will become increasingly important in making therapeutic decisions. Current indications for standard-of-care molecular testing in colorectal carcinomas include identifying hereditary cancer syndromes, such as Lynch syndrome, and testing for *KRAS* mutational status as a predictor of response to anti–epidermal growth factor receptor (EGFR) agents such as cetuximab. In addition, sometimes MSI status is tested for prognostic and treatment-related purposes in cases with low suspicion of

Lynch syndrome. Multiple additional biomarkers predicting a response to a certain therapy have been recently described in CRCs but are not yet standard of care or widely used, though these tests may become more important as more targeted therapies make their way into the clinic.[6]

Genetic Pathways to CRCs

The Conventional Suppressor Pathway (Chromosomal Instability Pathway)

In 1988, Vogelstein et al[4] studied genetic alterations in different stages of colorectal neoplasia and found that the steps required for the development of CRC often involve the mutational activation of an oncogene coupled with the loss of several genes that normally suppress tumorigenesis, a genetic model of colorectal tumorigenesis termed the adenoma-carcinoma sequence. This conventional pathway is initiated with inactivation of the APC/β-catenin/Wnt signaling pathway, usually by mutation of 1 copy of the *APC* gene, a tumor suppressor gene, followed by a second event leading to inactivation of the other allele as a result of allelic deletion or an additional mutation. The alterations in the *APC* gene lead to the development of dysplasia in aberrant crypt foci and early adenomas. Sequential accumulation of additional genetic events, including mutations in oncogene *KRAS* as well as in tumor suppressor genes *DCC*, *SMAD4*, and *p53*, drives tumor progression.[7] Approximately 60% of CRCs including those arising from FAP follow this conventional suppressor pathway. Gross chromosomal alterations are common in this group of CRCs.

Hereditary MSI Pathway (Lynch Syndrome)

Lynch syndrome, also known as hereditary nonpolyposis colorectal cancer (HNPCC), is the most common hereditary CRC, accounting for approximately 2% to 7% of all CRCs.[1,3] It is an autosomal dominant disorder usually caused by germline mutation in 1 of 4 mismatch repair (MMR) genes, including *MSH2*, *MLH1*, *MSH6*, and *PSM2*. The well-known 2-hit hypothesis of tumorigenesis typically applies to Lynch syndrome, where germline mutation in 1 copy of 1 *MMR* gene represents the "first hit," and somatic inactivation of the wild type allele the "second hit." Up to 60% of *MMR* mutation carriers develop CRC, commonly before age 50 years. Rarely, biallelic mutations of 1 *MMR* gene have been reported. Constitutional MSH2 promoter hypermethylation of both alleles has recently been identified as a novel mechanism causing Lynch syndrome.[8,9]

MMR genes encode proteins that are critical to the proper repair of DNA sequence mismatch. Loss of MMR function leads to DNA replication errors in simple short-tandem DNA repeat sequences of 1 to 6 bases, called microsatellites, which are scattered throughout the genome, resulting in variable expansion or contraction of microsatellites. In MMR-deficient cells, genes that contain microsatellites in the coding region are more prone to frameshift mutations.[10] One example is frameshift mutations in *TGF-βRII* found in colorectal but not endometrial cancer. Two of the MMR genes, *MSH3* and *MSH6*, themselves contain coding microsatellites that can be mutated in MSI-high (MSI-H) cancers and are thus mutational targets. The *BRAF* gene is almost never mutated in Lynch syndrome–associated CRCs; however, *KRAS* and *p53* mutations can be present.

Serrated Pathway

The most common carcinomas arising via the serrated pathway begin with serrated polyps, especially sessile serrated adenomas (SSAs) bearing an activating mutation in the *BRAF* gene.[2] SSAs are prone to hypermethylation of a number of genes rich in CpG islands in the promoter region. This aberrant CpG island hypermethylation is associated with transcriptional inactivation and loss of gene function. The CpG island methylator phenotype (CIMP+) makes up about 35% of total CRCs. Depending on which gene(s) are silenced by hypermethylation, the arising carcinoma can be either microsatellite stable (MSS) cancer, which accounts for about 60% of CIMP+ CRCs, or MSI-H, which represents approximately 40% of CIMP+ CRCs. Most sporadic MSI-H CRCs, which account for about 12% to 15% of total CRCs, result from epigenetic silencing of the *hMLH1* gene because of hypermethylation of the 3' end of the promoter. It has been proposed that the loss of hMLH1 protein in SSAs leads to the rapid accumulation of mutations of other genes that drive tumor development, including transforming growth factor-β (*TGFβ*) and *BAX*.[2] Morphologically, SSAs with *hMLH1* hypermethylation are characterized by cytologic dysplasia, which is followed by rapid development of frank malignant transformation **Image 1**. Alternatively, CpG island methylation may occur in tumor suppressor genes rather than *hMLH1*, an event leading to inactivation of tumor suppressor genes and eventually resulting in CIMP+ MSS carcinomas.

Molecular Testing in CRC

Identification of MSI-H CRC

MSI-H CRCs may be sporadic (~12%) or Lynch syndrome–associated (~3%). MSI testing is conducted with a polymerase chain reaction (PCR)–based assay and/or immunohistochemical staining for MMR protein expression for screening of MSI-H CRCs. Further studies are always necessary to differentiate sporadic from hereditary MSI-H CRCs. In addition, to diagnose Lynch syndrome, detection of MSI-H cancers has prognostic and, more controversially, predictive significance, which may be used in clinical decision making.

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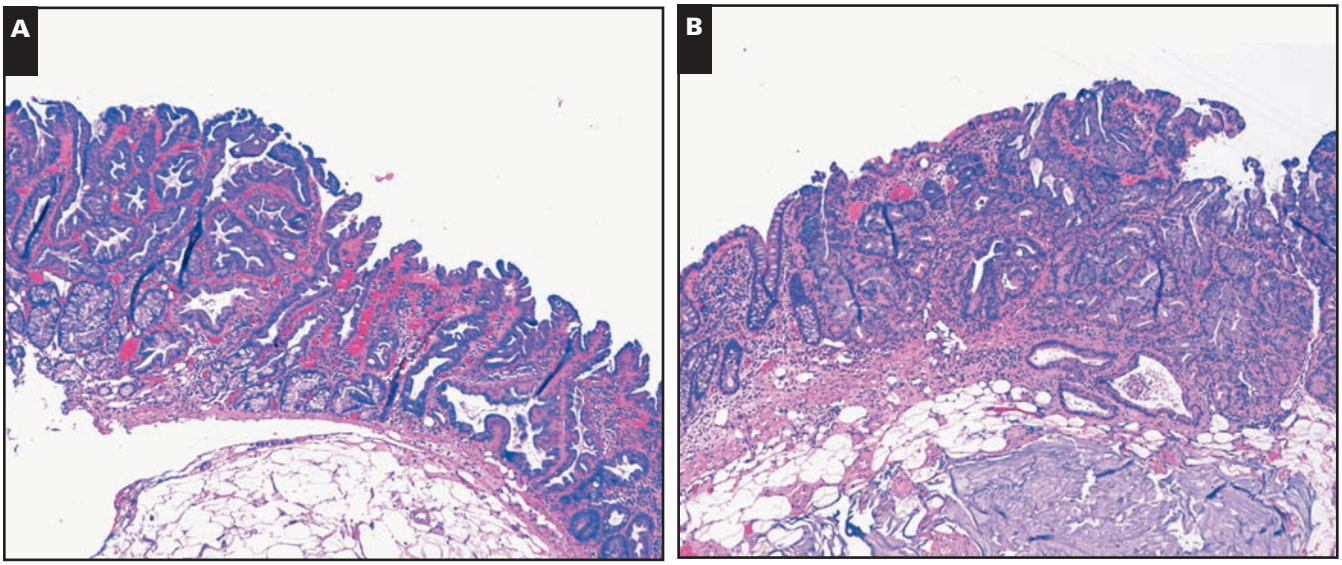

Image 1 Progression of a sessile serrated adenoma (SSA) to invasive carcinoma (H&E, ×40). **A**, An example of SSA with low-grade cytologic dysplasia. **B**, The same lesion with high-grade cytologic dysplasia and invasive carcinoma in the submucosa.

MSI Testing

The original Bethesda guidelines for identifying individuals with HNPCC proposed a panel of 5 markers, including 2 mononucleotide markers (BAT-25 and BAT-26) and 3 dinucleotide markers (D2S123, D5S346, and D17S250), for PCR-based detection of MSI.[11] If 2 or more microsatellite markers are mutated, the tumor is considered MSI-H; if only 1 is mutated, then the tumor is classified MSI-low (MSI-L) and additional testing with other microsatellite sequences is recommended for definitive classification. Tumors showing no microsatellite mutations are considered MSS. Because mononucleotide markers are more sensitive than di- or trinucleotide microsatellites, the revised Bethesda guidelines following a 2002 National Cancer Institute workshop recommended that a secondary panel of mononucleotide markers such as BAT-40 be used to exclude MSI-L cases in which only the dinucleotide repeats are mutated. The revised guidelines were effective in identifying MLH1/MSH2 mutation carriers with a sensitivity of approximately 82% and specificity of approximately 98%. However, a pentaplex of mononucleotide repeats (BAT-25, BAT-26, NR-21, NR-22, and NR-24) has a better performance, with a sensitivity of more than 95% and specificity of more than 98% for detecting MMR-deficient CRCs.[12]

A fluorescent multiplex PCR-based method is used for detecting MSI. A tumor tissue specimen (with tumor cellularity of >20%) and normal tissue specimen are amplified using PCR for 5 to 7 microsatellite markers. Fluorescently labeled products are sized by capillary electrophoresis. Patterns of normal and tumor genotypes are compared for each marker and scored as MSI-H, MSI-L, or MSS **Figure 1**. Surgical resections are usually the best source for this material. However, for rectal cancers treated with neoadjuvant therapy with

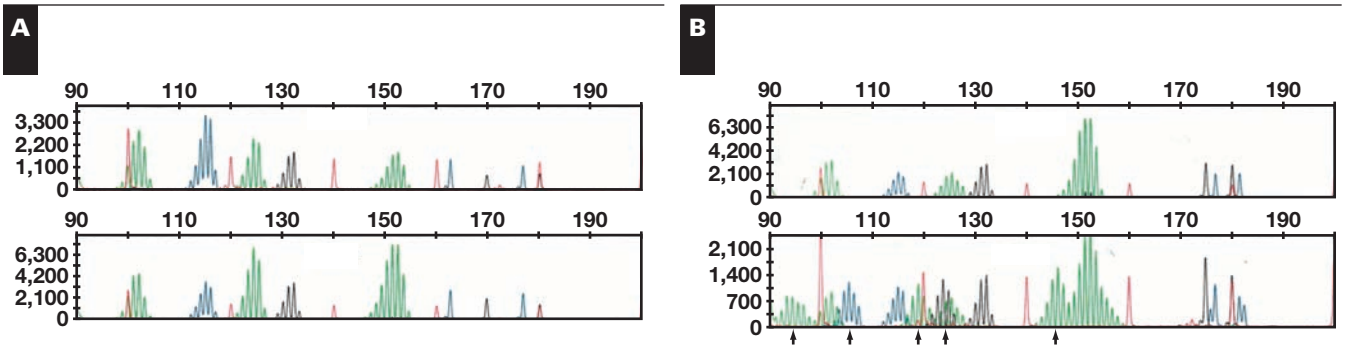

Figure 1 Fluorescent multiplex polymerase chain reaction–based microsatellite instability (MSI) testing. **A**, An example of microsatellite-stable tumor sample with chromatography findings identical to those seen in matched normal tissue sample. **B**, An example of MSI-high tumor with deletions (arrows) in 5 of 5 mononucleotide microsatellite loci compared with matched normal tissue sample.

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minimal residual tumor, the pretreatment biopsy is usually a better source for tumor tissue than the surgical resection. Currently many molecular diagnostic laboratories use a commercially available kit from Promega (Madison, WI), with 5 mononucleotide markers (BAT-25, BAT-26, MON0-27, NR-21 and NR-24) for the detection of MSI and 2 pentanucleotide markers (Penta C and Penta D) used as specimen identification markers to ensure that the tumor and normal DNA samples are derived from the same patient (Figure 1). Although normal matching DNA samples for the tumors being tested is not required in most instances, comparison of allelic profiles of the microsatellite markers generated by amplification from matching pairs of test samples might facilitate the identification of MSI, especially in tumors with relatively low tumor cellularity.

Diagnosis of Lynch Syndrome

Detection of defects in the MMR system in CRCs is important for detecting Lynch syndrome, and it is recommended that the tissue sample be examined for defective DNA MMR using MSI testing or immunohistochemical staining of MMR proteins if any of the criteria in the revised Bethesda guidelines are met.[13] These guidelines recommend testing for MSI in the following situations: (1) CRC in a patient younger than 50 years of age; (2) synchronous or metachronous colorectal or other HNPCC-related tumors, such as endometrial, small bowel, gastric, ovarian, pancreatic, biliary, ureteral, or renal pelvis carcinomas, brain tumors, sebaceous gland adenomas, and keratoacanthomas, in a patient of any age; (3) CRC with histologic features associated with MSI-H status (medullary, mucinous, or signet ring cell differentiation, presence of numerous tumor infiltrating lymphocytes, or presence of Crohn disease–like peritumoral lymphocytic reaction) in a patient younger than 60 years of age[14,15]; (4) CRC in 1 or more first-degree relatives with an HNPCC-associated tumor, with one of the patients being diagnosed before age 50 years; and (5) CRC in 2 or more first- or second-degree relatives with HNPCC-related tumors, regardless of age.

Presymptomatic detection of carriers leading to increased surveillance can potentially reduce morbidity and mortality from CRCs and other cancers in these patients. The specificity of MSI testing can be increased by using primarily at-risk populations such as patients with CRC younger than 50 years of age or patients with a strong family history of HNPCC-associated tumors (eg, colorectal, endometrial, gastric, or upper urinary tract urothelial carcinoma).[13]

Unfortunately, application of the revised Bethesda criteria does not lead to identification of all patients with Lynch syndrome. One study has demonstrated that 14.3% of patients with Lynch syndrome do not fulfill the criteria,[16] and other studies have also suggested that the criteria are relatively inaccurate, especially in cases with germline mutations in MSH6 and PMS2.[17,18] A large population-based cohort with a low prevalence of Lynch syndrome has shown that routine molecular screening for patients with CRC for Lynch syndrome has better sensitivity for detecting mutation carriers than the Bethesda guidelines,[16] and recent cost-effectiveness studies support the recommendation to offer testing for Lynch syndrome to all newly diagnosed patients with CRC.[19] Therefore, in many institutions, testing for MSI or immunohistochemical staining for MMR proteins is routinely done for all resected CRCs.

Examination of expression of MLH1, MSH2, MSH6, and PMS2 using commercially available antibodies is the most common immunohistochemical testing method used for suspected MSI-H cases. A positive reaction in the nuclei of tumor cells is considered intact expression (normal), and it is common for intact staining to be somewhat patchy. Intact expression of all 4 proteins indicates that mismatch repair enzymes tested are intact but does not entirely exclude Lynch syndrome. This is because 5% to 8% of families may have a missense mutation (especially in *MLH1*) that can lead to a nonfunctional protein with retained antigenicity. Rarely, defects in lesser-known mismatch repair enzymes may also lead to a similar result. Loss of expression of MLH1 may be the result of Lynch syndrome or methylation of the promoter region (as occurs in sporadic MSI CRC). Loss of MSH2 expression essentially always implies Lynch syndrome. Because PMS2 is a binding partner for MLH1, loss of PMS2 is often tied to loss of MLH1 and is only independently meaningful if MLH1 is intact.[20] Expression of MSH6 is similarly related to that of its binding partner MSH2.

BRAF *Gene Mutation Testing in MSI-H CRCs*

Analysis for somatic mutations in the V600E hot spot in the *BRAF* gene may be indicated for tumors that show MSI-H or loss of MLH1 expression, because this mutation has been found in sporadic MSI-H tumors but not in HNPCC-associated cancers.[21] Use of *BRAF* mutational analysis as a step before germline genetic testing in patients with MSI-H tumors may be a cost-effective means of identifying patients with sporadic tumors for whom further testing is not indicated.[22] If *BRAF* is not mutated, methylation analysis of the *hMLH1* promoter can be performed using methylation-specific multiplex ligation-dependant probe amplification or methylation-specific PCR. This is because sporadic MSI-H CRCs result from *hMLH1* promoter hypermethylation, whereas *hMLH1* promoter is rarely methylated in Lynch syndrome–associated CRCs.

Germline mutation analysis is required for MSI-H CRCs that are *BRAF* wild type and lack *hMLH1* promoter methylation because of the high probability of Lynch syndrome in such cases. Pathologic mutations of the *MMR* genes include (1) nonsense or frameshift mutations, causing a truncated protein, (2) missense mutations, resulting in a dysfunction protein,

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and (3) large deletion (accounting for 20% of the mutations). Sequence analysis of exons and intron-exon boundaries of the implicated gene is performed, usually using a peripheral blood sample, to detect small insertion/deletion and missense mutation. Multiplex ligation-dependent probe amplification is used for large deletions. About 40% of pedigrees that meet the Amsterdam criteria and have a MSI-H CRC(s) do not have deleterious mutation in one of the MMRs.[23] Sequencing the entire genes may increase the sensitivity. However, alterations in other genes than the *MMR* genes may be responsible for Lynch syndrome in some cases. For example, germline deletions of the last exons of *TACSTD1*, a gene directly upstream of *MSH2* encoding Ep-CAM, can cause MSH2 promoter hypermethylation. Deletions of *TACSTD1* has recently been discovered in Dutch and Chinese families with *MSH2*-deficient tumors.[9] The sequential study has demonstrated that the constitutional 3' end deletions of *TACSTD1* are associated with a high risk of CRCs and endometrial cancer.[24]

Other Significances of Identification of MSI-H tumors

Accumulated evidence indicates that patients with an MSI-H CRC have favorable stage-adjusted prognosis,[25-27] possibly related to a more robust immunologic response to the tumor. MSI-H CRCs commonly have greater numbers of activated, cytotoxic tumor-infiltrating lymphocytes, a reaction independently associated with better survival.[28,29] In a large series involving 2,141 patients with stage II and III CRC, Sinicrope et al[25] demonstrated that patients with MMR-deficient colon cancers had reduced rates of tumor recurrence, delayed time to recurrence, and improved survival rates, compared with patients with MMR-proficient CRCs. In addition, several studies have described a lower incidence of MSI-H CRCs in stage III or stage IV disease, suggesting that they have a lower propensity to metastasize.[30-32]

The association between MSI status and response to chemotherapy continues to be an active area of investigation. In vitro studies have shown that CRC cell lines with MMR deficiency have a reduced response to alkylating agents and platinum compounds[33-36] and are resistant to 5-fluorouracil (5-FU),[37-40] an observation supported by several randomized clinical trials.[41,42] Patients with an MSI-H CRC who received 5-FU chemotherapy had no survival benefit compared with those who did not receive 5-FU chemotherapy, whereas those with an MSS tumor showed a survival benefit from 5-FU treatment. Further, some studies even suggest reduced survival in patients with an MSI-H CRC receiving 5-FU chemotherapy compared with those who did not.[42,43] However, other trials such as the Quick and Simple and Reliable (QUASAR) trial suggest that MMR status may not predict benefit from chemotherapy.[30]

A number of chemotherapeutic agents other than standard 5-FU regimens are being investigated for use in MSI-H CRCs. MSI-H cell lines are more sensitive to irinotecan, a topoisomerase inhibitor, than MSS CRC cell lines. Complete response to preoperative chemoradiotherapy with irinotecan has been reported in 60% of patients with MSI-H compared with 20% of those with MSS tumors.[44] Patients with stage III MSI-H tumors showed improved disease-free survival when treated with irinotecan, 5-FU, and leucovorin compared with those with MSS tumors; the same association between improved survival and microsatellite instability status was not observed for patients receiving 5-FU and leucovorin without irinotecan.[45]

Biomarkers for Targeted Therapies

The EGFR signaling pathway plays an essential role in carcinogenesis and progression of CRCs, making it an attractive therapeutic target. EGFR activation leads to autophosphorylation of its c-terminal tyrosine residues, which serve as docking sites that bind to several intracellular proteins, consequently activating a number of downstream signaling pathways, including the RAS-RAF-MAR kinase signaling pathway and the PI3K-AKT-mammalian target of rapamycin (mTOR) signaling pathway. The PI3K-AKT-mTOR signaling pathway can also be activated by KRAS. Both signaling pathways are involved in tumor cell proliferation, invasion, migration, and inhibition of apoptosis **Figure 2**.

EGFR

EGFR gene mutation is very rare in CRCs, unlike in lung cancers, and small molecular tyrosine kinase inhibitors show minimal activity as a single agent in metastatic CRCs (mCRCs).[46,47] However, some CRCs have increased copy numbers of the *EGFR* gene[48] and overexpression of EGFR can be demonstrated with immunohistochemical staining,[49]

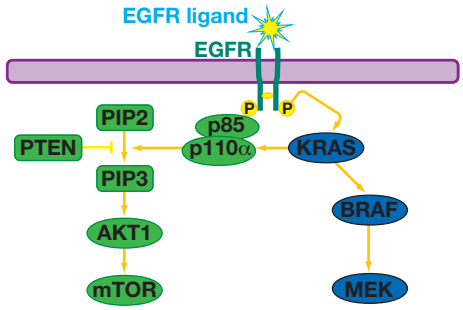

Figure 2 Epidermal growth factor receptor (EGFR) signaling pathway. Activation of EGFR by its ligands leads to activation of the RAS-RAF-MAP kinase signaling pathway and the PI3K-AKT1-mTOR signaling pathway, which are involved in tumor cell proliferation, invasion, migration, and inhibition of apoptosis.

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though such testing does not predict clinical response to anti-EGFR blockade[50-52] and is not useful in clinical practice. Cetuximab and panitumumab, anti-EGFR monoclonal antibodies (MoAbs) that can recognize and inactivate the extracellular domain of EGFR, are proven to be effective in treating some mCRCs. In addition to inhibiting downstream signaling pathways, these agents have a potential therapeutic effect through antibody-dependent cell-mediated cytotoxicity. It is now clear that mutations in downstream genes such as *KRAS* as well as expression levels of EGFR ligands can affect the sensitivity of CRCs to anti-EGFR therapy.[53,54]

KRAS

KRAS, a small G protein that functions as a signal transducer and downstream integrator of EGFR, is a key component in the EGFR signaling cascade. In sporadic CRCs, activating *KRAS* mutations involving codons 12, 13, or 61 have been detected in roughly 40% to 50% of tumors ▮**Table 1**▮,[55,56] with approximately 85% to 90% of mutations occurring in codons 12 and 13. Activating mutations involving codon 146 of exon 4 of the *KRAS* gene have recently been described with frequencies ranging from 1% to 6.6% in CRCs.[57-59] Activating mutations in *NRAS*, a member of the RAS family have been found in approximately 3% of CRCs (Table 1).[60] These mutations disable the intrinsic GTPase activity of RAS, causing accumulation of the active GTP-bound conformation, thereby constitutively activating the downstream signaling pathway. *KRAS* mutations occur in both MSS and sporadic MSI-H tumors as well as in HNPCC.[61,62] However, sporadic MSI-H CRCs, which harbor a higher frequency of *BRAF* mutations, have a lower frequency of *KRAS* mutations, because mutations in the *KRAS* and *BRAF* genes are mutually exclusive.

KRAS mutations have been convincingly associated in randomized clinical trials with poor response to cetuximab and panitumumab. Activating mutations in *KRAS* serve to isolate this signaling pathway from the effects of EGFR and render EGFR inhibition ineffective. Recent advances have shown that only tumors with wild-type *KRAS* show significant response to these agents. Accumulating data from both randomized and nonrandomized studies,[55,63-66] reviewed by Jimeno et al,[67] suggest that patients with CRC whose tumors show *KRAS* mutations should not receive EGFR-targeting MoAb therapy. *KRAS* mutation testing is therefore increasingly recommended to facilitate selection of the most appropriate patients for treatment with anti-EGFR antibodies. Although clinical guidelines for *KRAS* mutational analysis in CRC are evolving, current provisional recommendations from the American Society for Clinical Oncology are that all patients with stage IV CRC who are candidates for anti-EGFR antibody therapy should have their tumors tested for *KRAS* mutations (http://www.asco.org/portal/site/ASCO/). Anti-EGFR antibody therapy is not recommended for patients whose tumors show mutation in *KRAS* codons 12 or 13. However, *KRAS* mutation at c.38G>A (p.G13D) may not influence responsiveness to EGFR-targeted therapy in the

▮Table 1▮
Gene Mutations in the EGFR Signaling Pathway in CRCs

Gene	Mutation Frequency (%)	Location of Mutations	Poor Prognosis Marker	Resistance to Anti-EGFR	Clinical Trial
EGFR	0.3	Exon18	Unknown	Unknown	Ongoing clinical trials on EGFR tyrosine kinase inhibitors in patients with stage I-III CRC
KRAS	40-50	Codon12, 13 (most common) Codon 61 Codon 146	Controversial	Yes* (not for G13D; controversial for codon 146)	Completed clinical trial on Ras peptide cancer vaccine for patients with locally advanced or metastatic CRCs
NRAS	3	Codon 12 Codon 13	Remains to be validated	Suggested by a few studies	No
BRAF	5-22	V600E (vast majority) Others (rare)	Yes† in MSS tumors	Suggested by previous studies but not confirmed in recent studies	Ongoing phase II clinical trial on small molecule BRAF inhibitors combined with chemotherapy, cetuximab, or another new biologic agent targeting a downstream protein (eg, MEK inhibitor)
PIK3CA	10-30	Exon 9 (most common) Exon 20 Exon 1, 2 (rare)	Reported by most but not all studies	Reported in CRCs with mutations in exon 20 by few studies	Ongoing clinical trials on PI3K inhibitors with an MEK inhibitor in patients with advanced cancer; ongoing clinical trials on PI3K inhibitors, dual catalytic site inhibitors in solid tumors including CRCs
PTEN	5 (18 in MSI-H CRCs)	Exon 7, 8	Remains to be validated	Reported by some studies	
AKT1	6	Codon 49	Remains to be validated	Remains to be validated	

CRC, colorectal carcinoma; EGFR, epidermal growth factor receptor; MSI-H, microsatellite instability–high; MSS, microsatellite stable.
* *KRAS* mutation is associated with resistance to anti-EGFR.
† *BRAF* is a poor prognosis marker (in MSS tumors).

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same manner as other *KRAS* codon 12 and 13 mutations.[68] Codon 61 mutations predict for lack of response to cetuximab similar to codon 12 and 13 mutations.[57,60] The effect of codon 146 mutations remains controversial.

Although the predictive value of *KRAS* mutations in MoAb-based anti-EGFR therapy is now well established, the prognostic value of *KRAS* mutations, independent of treatment, remains controversial. A large series of more than 3,400 CRC patients found that only the glycine-to-valine mutation on codon 12, found in 8.6% of all cases, had a significant effect on disease-free and overall survival.[69] This mutation appeared to have a more significant negative effect on patients with stage III disease, compared with those with stage II tumors. Richman et al[70] also reported that *KRAS* mutations in advanced CRCs were associated with poor prognosis. However, several retrospective subset analyses from large randomized studies have failed to confirm this finding, including studies in which no difference relative to *KRAS* mutational status was observed in patients treated with the best supportive care.[55,63,71]

BRAF

BRAF, a serine/threonine protein kinase, is an immediate downstream effector of KRAS in the MAP kinase signaling pathway. Five percent to 22% of CRCs have a *BRAF* mutation (V600E) with oncogenic activation (Table 1),[31,60,70,72] more frequently detected in sporadic MSI-H CRCs than in MSS CRCs.[72-75] Because *BRAF* mutations almost never occur in HNPCCs, mutational analysis of the *BRAF* gene can therefore be used to differentiate sporadic MSI-H tumors from HNPCCs.

Some studies have suggested that *BRAF* mutations are associated with a more aggressive phenotype and a shorter overall survival.[54,70,74,76] However, other studies indicate that the prognostic effect of *BRAF* mutations depends on the MSI status; MSI-L or MSS tumors with *BRAF* mutations do poorly compared with those with wild type *BRAF*, whereas *BRAF* status has no or less prognostic effect on MSI-H tumors.[31,74]

In a retrospective study of 113 cases, Di Nicolantonio and colleagues[54] demonstrated that *BRAF* status may also predict response to anti-EGFR therapy, with wild-type *BRAF* required for response to panitumumab or cetuximab in mCRCs. They found that none of the *BRAF*-mutated tumors responded to treatment with panitumumab or cetuximab, whereas none of the responders carried mutant *BRAF*.[54] *BRAF* mutation is thought to be responsible for an additional 12% to 15% of patients with wild-type *KRAS* tumors who fail to respond to anti-EGFR MoAb treatment. The National Comprehensive Cancer Network colon cancer guideline 2010 update states that testing for mutations in *BRAF* should occur when *KRAS* testing indicates *KRAS* wild type. However, data published since this recommendation regarding predictive value of *BRAF* V600E have been inconsistent.[77] Currently *BRAF* mutational analysis is not required for treatment decision making[78]; however, it may be useful as a prognostic factor, especially for CRCs without MMR deficiency.

BRAF is an appealing potential therapeutic target in the treatment of CRCs. A small molecular BRAF inhibitor, in combination with either FOLFOX, FOLFIRI, cetuximab, or another new biologic agent targeting a downstream protein, eg, MEK inhibitor, is currently being tested in a phase II clinical trial.[78] A selective BRAF inhibitor, GSK22118436, has shown a 63% response rate in the cohort of patients with V600E *BRAF* mutant melanoma, which frequently harbors mutant *BRAF*. In preclinical models of *BRAF*-mutant CRCs, a BRAF inhibitor, vemurafenib, in combination with standard-of-care or novel targeted therapies, led to enhanced and sustained clinical antitumor efficacy.[79] However, patients with colon cancer harboring V600E mutation show only a limited response to this drug, probably because of a rapid feedback activation of EGFR.[80]

Genes in the PI3K Signaling Pathway

PI3K.—Three classes of PI3Ks (I, II, and III) have been described, each of which is composed of 2 subunits: regulatory subunit (p85) and catalytic subunit (p110). There are 3 catalytic isoforms: p110α, p110β and p110γ, which are the products of 3 genes, PIK3CA, PIK3CB, and PIK3CD. Class IA PI3Ks, composed of a p85 and a p110α subunit, can be activated by receptor tyrosine kinases (RTKs), including EGFR, and KRAS. Activated PI3K, in turn, phosphorylates the 3′-OH group on phosphatidylinositols in the plasma membrane to produce phosphatidylinositol-3,4,5-triphosphate (PI(3,4,5)P3), consequently leading to recruitment of the protein serine/threonine kinase, AKT, to the cell membrane where AKT becomes activated (Figure 2).

Mutations in the *PIK3CA* gene are the most common genetic alterations in the PI3K signaling pathway in CRCs, and are detected in 10% to 30% of CRCs (Table 1).[81-85] *PIK3CA* mutations are detected in exons 1, 2, 9, and 20 with most occurring in exons 9 (helical domain, 60%-65%) and 20 (kinase domain, 20%-25%). It has been reported that mutations in the *PIK3CA* gene occur more frequently in well-differentiated tumors than in moderately to poorly differentiated tumors, and more frequently in mucinous tumors than in nonmucinous tumors. In addition, predominance of *PIK3CA* mutations was observed in the MSI subgroup by Abubaker et al.[84] Although most studies have suggested that *PIK3CA* mutation is associated with a poor prognosis in CRCs,[83,85-87] some have concluded that the *PIK3CA* mutation does not seem to be related to prognosis.

The clinical effect of *PIK3CA* mutations on resistance to anti-EGFR MoAb therapy is an area of current investigation. Although results from the early studies were

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contradictory,[88,89] recent studies have shown that CRCs with mutations in *PIK3CA* exon 20 are associated with a lack of response to anti-EGFR therapy, and the response is retained in *KRAS* wild-type CRCs with *PIK3CA* exon 9 mutations.[60] The constitutive activation of PIK3CA associated with mutations in exon 20 may explain the resistance of CRCs with such mutations to anti-EGFR therapy. On the other hand, mutations in exon 9 disrupt its interaction with KRAS, consequently preventing the activation of PIK3CA by KRAS; however, the activation of PIK3CA by EGFR-RTK itself is still intact, which explains why CRCs with mutations in exon 9 may still respond to cetuximab or panitumumab. *PIK3CA*-mutant CRCs may also have either *KRAS* or *BRAF* mutations, and therefore *PIK3CA* mutation cannot be used as a single marker for prediction of resistance to cetuximab or panitumumab in patients with mCRC.

PTEN.—PTEN, a dual-specific phosphatase, dephosphorylates PI(3,4,5)P3, a PI3K-generated lipid product, thus negatively regulating the PI3K/Akt/mTOR signaling pathway. PTEN is inactivated in 20% to 40% of CRCs. Three main mechanisms are involved in the loss of PTEN function: (1) mutations in the *PTEN* gene (5% in total CRCs vs 18% in MSI-H CRCs, see Table 1)[10,90-92]; (2) allelic loss of chromosome 10q23 (23%); and (3) hypermethylation of the *PTEN* promoter region (19.9% in MSI-H CRCs vs 2.2% in MSS CRCs). CRCs with inactivated PTEN may also harbor *KRAS*, *BRAF*, and *PIK3CA* mutations.[93] In vitro studies have shown that PTEN loss confers resistance to cetuximab-induced apoptosis.[94] Consistent with this result, loss of PTEN expression predicts a lack of response and shorter survival in patients with *KRAS* wild-type mCRC treated with anti-EGFR therapy.[95-99]

AKT.—AKT, a serine/threonine protein kinase, is activated by membrane localization initiated by binding of pleckstrin homology domain (PHD) to PI(3,4,5)P3 or its metabolite PI(3,4)P2 followed by phosphorylation of serine 473 and threonine 308, two regulatory amino acids. Activated mutations in the *AKT1* gene are detected in 6% of CRCs (Table 1).[100] All detected mutations occur in the PHD, with a G>A point mutation at nucleotide 49, resulting in a lysine substitution for glutamic acid at amino acid 17, which facilitates constitutive localization of AKT1 to the membrane. The prognostic and predictive role of this mutation to anti-EGFR therapy has not been studied. However, phosphorylation of AKT-1 may correlate with a better prognosis.[101]

PI3K Pathway as Therapeutic Targets.—A number of potential therapeutics targeting the PI3K/AKT/mTOR signaling pathway, including PI3K inhibitors, dual PI3K-mTOC inhibitors, AKT inhibitors, and mTOR catalytic site inhibitors, are being tested in phase I and II clinical trials of patients with solid tumors, including CRCs. A small phase II trial demonstrated that perifosine, an AKT inhibitor, in combination with capecitabine, showed improvement in overall response rate, time to progression, and overall survival in previously treated mCRC.[102] Combination treatment using everolimus, an mTOR inhibitor, and standard treatment for mCRCs is also in clinical trial.

Analytic Methods to Evaluate Gene Mutations in CRCs

The vast majority of gene mutations occurring in CRCs are single-base substitutions (point mutation). While standard DNA sequencing may be able to detect these mutations, the sensitivity is low, usually requiring more than 25% tumor cells in a given specimen. More sensitive technologies have been developed to detect point mutations, including allele-specific PCR **Figure 3A**, quantitative PCR with melt curve analysis, and pyrosequencing; however, these techniques are not amenable to high throughput and cannot be multiplexed. More recently, assays have been developed to detect multiple mutations simultaneously, including a mass spectrometry-based

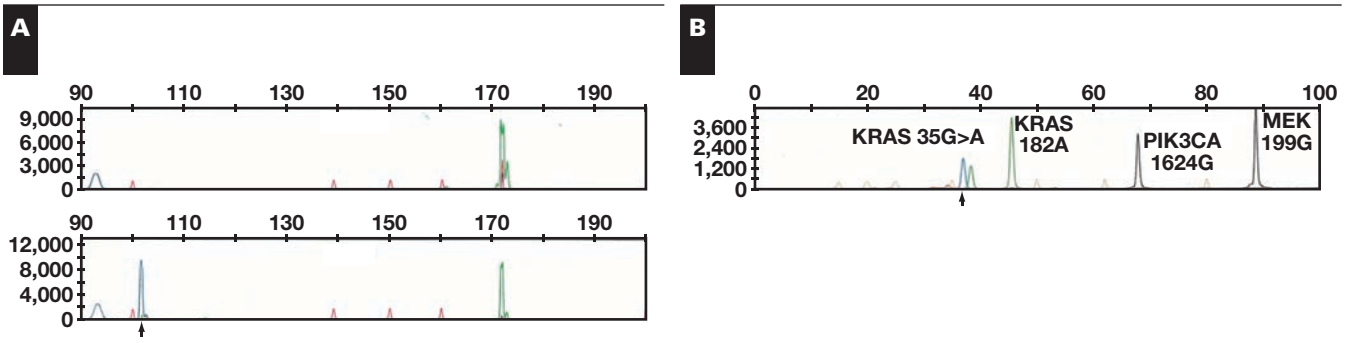

Figure 3 Detection of point mutations. **A**, Allele-specific polymerase chain reaction showing normal tissue sample with wild-type *BRAF* (upper panel with no amplification) and tumor tissue sample with *BRAF* (V600E) mutation (lower panel with amplification, arrow). **B**, Detection of *KRAS* mutation (35G>A, arrow) with the Applied Biosystems SNaPSHOT platform (Applied Biosystems, Foster City, CA).

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assay and Applied Biosystems SNaPSHOT platform (Applied Biosystems, Foster City, CA). Both assays start with multiplex PCR amplification followed by multiplex single-base extension. In the mass spectrometry–based assay, single base differences of the extension products are distinguished according to their mass-charge ratio, whereas in the SNaPSHOT platform, the difference is differentiated by the size and color of fluorescently labeled nucleotides **Figure 3B**.

Refinements in DNA extraction techniques from formalin-fixed paraffin-embedded tissue blocks have increased the sensitivity of DNA testing and eliminated the need for fresh or frozen tissue samples. Currently most assays can be performed on small quantities of formalin-fixed paraffin-embedded–derived tumor DNA. The pathologist must carefully select the tumor block to minimize dilution of tumor DNA by contaminating normal cells such as fibroblasts, endothelial cells, and inflammatory cells; a target of at least 10% tumor cells is recommended for most assays. Sometimes regions harboring a high density of tumor cells may need to be macrodissected to reach an optimal tumor cellularity.

Which Tissue Sample to Test?

Several studies suggest high concordance in terms of mutational status of the key genes in the EGFR signaling pathway between primary CRCs and their corresponding liver metastases.[96,103,104] Overall rates of concordance between primary and metastatic CRCs in *KRAS*, *BRAF*, and *PIK3CA* status have been reported to be 93%, 98%, and 92%, respectively. Primary tumor has therefore routinely been tested to select patients for anti-EGFR therapy, though testing of metastases has been acceptable if the primary tumor is not available. However, a recent study found that substantial genetic differences may exist between the primary tumor and its metastasis,[105] suggesting that targeted therapies be chosen based on the genetic properties of the metastatic rather than the primary tumor. In addition, genetic heterogeneity in a single tumor has been demonstrated in CRCs.[106] One specific clone may be more prone to metastases, which could be a minor component in the primary tumor; mutation(s) specific for this small population of tumor cells may not be detected if the primary tumor sample is used. Therefore, when sufficient metastatic tumor sample is available for testing, the metastatic sample may be preferred over the primary tumor sample for molecular testing, especially in patients in whom the metastatic tumor is the only site of the disease.

Biomarkers in Stage II CRCs

Many clinical trials have demonstrated the benefit of adjuvant therapy in patients with stage III CRCs. However, only a small, if any, survival benefit for adjuvant chemotherapy was demonstrated in patients with stage II CRCs,[107,108] perhaps because of the heterogeneity in this stage grouping. National Comprehensive Cancer Network clinical practice guidelines recommend adjuvant chemotherapy only for patients with stage II CRCs with high-risk features, defined as lymphovascular invasion, poorly differentiated histologic characteristics, T4 classification, obstruction/perforation, or inadequate lymph node sampling.[109] However, adjuvant chemotherapy may not substantially improve overall survival in stage II colon cancer with these poor prognostic features.[110] This suggests that clinicopathologic features alone are not a sufficient basis for treatment selection. Therefore new prognostic tools that can specifically identify stage II patients at particularly high risk who might benefit from adjuvant chemotherapy are urgently needed.

The role of MSI and *KRAS* and *BRAF* status in predicting recurrence risk and chemotherapy benefit was specifically investigated in patients with stage II CRCs **Table 2** in multiple studies. MSI-H is associated with a good prognosis and a low recurrence risk in this group.[30,43,111] Although many studies have shown that patients with MMR deficiency colon cancer do not seem to benefit from adjuvant single-agent fluoropyrimidine therapy,[41-43,112-114] the controversy persists. Tumor MSI status may, therefore, help in making decisions about the use of adjuvant fluoropyrimidine therapy for patients with stage II CRCs. The prognostic role of *KRAS* and *BRAF* in stage II CRCs remains more controversial,[30,31,115] and it appears that neither *KRAS* nor *BRAF* mutations are useful in predicting benefit from chemotherapy in stage II CRC.[30]

Table 2
Potential Prognostic Biomarker Tests for Stage II CRCs

Biomarker Name/Test	Type of Specimen Required	Type of Test	Intended Use
MSI	FFPE	MSI testing or IHC for MMR proteins	Prognostic: validated by multiple studies
Coloprint	Fresh tissue	Oligonucleotide microarray gene expression profile	Prognostic marker for recurrence risk
Oncotype DX colon test	FFPE	RT-PCR gene expression profile	Prognostic marker for recurrence risk

CRC, colorectal carcinoma; FFPE, formalin-fixed paraffin-embedded; IHC, immunohistochemical staining; MSI, microsatellite instability; RT-PCR, reverse transcriptase–polymerase chain reaction.

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New multiplex platforms for gene express profiling stratification for stage II CRCs are currently being evaluated (Table 2)[116-118] with the goal of identifying patients with stage II CRC who are more likely to develop recurrent disease and who would be candidates for adjuvant chemotherapy. Although these tests are currently available on the market, they are not considered standard of care. One such example is ColoPrint (Agendia, Irvine, CA), composed of an optimal set of 18 genes developed using gene expression data from whole genome Agilent (Santa Clara, CA) 44K oligonucleotide arrays.[116,119] A quantitative multigene reverse transcriptase (RT)-PCR-based gene expression assay (Oncotype DX [Genomic Health, Redwood City, CA] colon cancer test) was recently developed to assess recurrence risk and treatment benefit in patients with stage II CRCs, where a recurrence score and a treatment score were calculated from gene expression levels of 7 recurrence genes and 6 treatment benefit genes, respectively. While treatment score was not predictive of chemotherapy benefit, recurrence score was validated in a large prospective study as a predictor of recurrence risk in patients treated with surgery alone.[117]

Conclusion

In summary, current standard-of-care molecular testing of CRC is aimed at detecting Lynch syndrome and *KRAS* mutations. However, with recent rapid development of biological agents targeted against components of the EGFR signaling cascade in the treatment of CRCs, mutational analysis of the genes in the EGFR signaling pathway may become a standard of care for patients with CRC in the near future. Ideally, identifying molecular prognostic and predictive factors may allow us to identify high-risk patients with stage II CRC who will benefit from chemotherapy after surgery. In addition, this may allow us to determine patients' eligibility for targeted biological therapies.

From the Department of Pathology, Microbiology and Immunology, Vanderbilt University Medical Center, Nashville, TN.

Address reprint requests to Dr Shi: Dept of Pathology, Microbiology and Immunology, C-3321 Medical Center North, Vanderbilt University Medical Center, Nashville, TN 37232-2561; chanjuan.shi@vanderbilt.edu.

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