Select Biomarkers for Tumors of the Gastrointestinal Tract

Present and Future

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● Context.—Advances in molecular biomarkers of the gastrointestinal tract have contributed to a decline in the incidence of and mortality from diseases of the gastrointestinal tract. The discovery and clinical validation of new biomarkers are important to personalized cancer therapy, and numerous clinical trials are currently ongoing to help identify individualized therapy affecting these biomarkers and molecular mechanisms they represent. Distinct molecular pathways leading to cancers of the colorectum, esophagus, stomach, small bowel, and pancreas have been identified. Using biomarkers in these pathways to direct patient care, including selection of proper molecular testing for identification of actionable mutations and reporting the results of these biomarkers to guide clinicians and genetic counselors, is paramount.

Objective.—To examine and review select clinically actionable biomarkers of the colon, esophagus, stomach, small bowel, and pancreas, including present and future biomarkers with relevant clinical trials.

Data Sources.—Extensive literature review and practical and consultation experience of the authors.

Conclusions.—Although numerous biomarkers have been identified and are currently guiding patient therapy, few have shown evidence of clinical utility in the management of patients with gastrointestinal cancers. Inconsistent results and discordant proposed algorithms for testing were identified throughout the literature; however, the potential for biomarkers to improve outcomes for patients with gastrointestinal cancer remains high. Continued advances through high-quality studies are needed.


BIOMARKERS IN COLORECTAL TUMORS

Colorectal carcinoma (CRC) is the third most common cancer diagnosed in men and women in the United States, with 96 830 new cases of colon cancer and 40 000 new cases of rectal cancer estimated for 2014. It is the second leading cause of cancer-related deaths in men and women combined (projected total of 50 310 deaths). It is also the third most common cancer worldwide.2 However, widespread screening, major progress in treatment modalities, and advances in molecular biomarkers that individualize therapy have contributed to a decline in the incidence of and mortality from the disease. Most patients with CRC present with localized disease and have the potential for cure by a combination of surgery, chemotherapy, or radiation therapy.

Molecular Pathways

Several distinct molecular pathways leading to colorectal cancer have been identified.3 Alterations in the Wnt signaling pathway are nearly ubiquitous, including germline mutations in the APC gene in familial adenomatous polyposis. By contrast, high levels of microsatellite instability (MSI-H) due to inactivation of the DNA nucleotide mismatch repair (MMR) system are manifested in Lynch syndrome when the initial alteration is in the germline, or are associated with serrated lesions as frequent precursors and with somatic cytosine-guanosine (CpG) island hypermethylation of the MLH1 MMR gene promoter and acquired hypermethylation or mutation of the other allele. Mismatch repair corrects base-base and insertion/deletion mismatches that form as a result of replication errors, which lead to widespread instability in the length of nucleotide repeat sequences termed microsatellites.

Microsatellite Instability and Lynch Syndrome

MSI-H mismatch repair is found in approximately 15% of all colorectal cancers, including in the 3% with Lynch syndrome, and approximately 90% of colorectal carcinomas occurring as the result of Lynch syndrome exhibit MSI-H.4 Lynch syndrome–associated MMR gene mutations include point mutations and large deletions or rearrangements. In addition to germline mutations in MLH1 and MSH2, the most commonly mutated mismatch repair genes in Lynch syndrome, mutations in MSH6 and PMS2, and deletion of the epithelial cell adhesion molecule gene (EpCAM) located...
upstream of MSH2, have been described in a subset of families. These deletions affecting the 3' region of the EPCAM gene mediate epigenetic silencing of the MSH2 allele in a mosaic pattern, leading to loss of MSH2 gene product.5

Women with Lynch syndrome have increased risk for endometrial and ovarian cancer. Other associated cancers include gastric, pancreatic, small bowel, urinary tract, and sebaceous gland tumors.6 The identification of patients with the syndrome is key for proper treatment, as well as the identification of family members with the same germline mutation who require screening and surveillance. In 2009, the Evaluation of Genomic Applications in Practice and Prevention Working Group7 first recommended the evaluation of all colorectal carcinomas by using immunohistochemistry (IHC) or polymerase chain reaction (PCR)–based microsatellite instability testing to identify Lynch syndrome because screening may reduce morbidity and mortality in affected relatives of patients who have colorectal cancer.

**Assays for MSI-H and Lynch Syndrome.**—Screening methods used to assess tumors for MMR deficiency include multiplex PCR-based MSI testing and IHC for nuclear MMR gene product expression that can be performed on formalin-fixed, paraffin-embedded tissue. Carcinomas are preferable for testing because a far lower percentage of adenomas in Lynch syndrome have abnormalities. For MSI testing, DNA extracted from a section of tumor with adequate cellularity and from matched nonneoplastic control tissue is amplified with PCR, generally for 5 to 7 microsatellite markers, with subsequent sizing of the PCR products. The patterns obtained from tumor and control tissue are compared, and the tumor is classified as MSI-H if 2 or more markers, representing at least 30% of evaluable microsatellites, are altered in length; MSI-L (low) if only 1 marker, or less than 30%, is altered, or MSS (stable) if no markers are altered. A combination of mononucleotide and dinucleotide markers may be used (eg, 2 mononucleotide markers [BAT-25 and BAT-26] with 3 dinucleotide markers [D2S123, D5S346, and D17S250]); alternatively, a commercially available panel of 5 mononucleotide markers (BAT-25, BAT-26, MONO-27, NR-21, and NR24; Promega, Madison, Wisconsin) is available. For rectal cancers that have been treated with preoperative neoadjuvant chemoradiation with insufficient neoplastic cellularity, the pretreatment biopsy specimen should be used.

Immunohistochemistry testing is performed by using commercially available antibodies to examine the expression of MLH1, MSH2, MSH6, and PMS2 proteins in tumor tissue sections. This methodology has the advantages over PCR-based testing of being more widely available, simpler, cheaper, and having faster turnaround time. Any expression in tumor nuclei usually indicates intact protein product of the corresponding MMR gene, but expression can be heterogeneous or patchy. Interpretation of expression loss in tumor cells should be made only if expression is present in adjoining or intraepithelial nonneoplastic cells that serve as an internal positive control, such as the nuclei of stromal, inflammatory, or nonneoplastic epithelial cells. Clinical history is important, however, as Lynch syndrome cancers with intact IHC expression do occur, owing to retained expression of mutated nonfunctional MMR protein, which could potentially lead to misclassification of a patient’s tumor.6,8 Colorectal adenomas of patients with Lynch syndrome often retain protein expression. As well, substantial reduction of MSH6 expression has been reported in a small percentage of colorectal carcinomas with somatic mutations of the coding region microsatellites of the MSH6 gene in MLH1/PMS2-deficient carcinomas.9 Nucleolar expression or complete loss of MSH6 expression has been described in sporadic colorectal cancer cases with prior radiation or chemotherapy.10,11

**BRAF Mutation Testing.**—Loss of expression of MLH1 may be due to germline mutation in Lynch syndrome, or somatic hypermethylation of the MLH1 promoter region in sporadic MSI colorectal carcinoma. A specific BRAF gene mutation (V600E) is present in many sporadic cases, but extremely rare in familial cancers. BRAF encodes a cytoplasmic serine/threonine kinase that is part of the RAS-RAF-MEK-ERK cascade and acts as an oncogene. The cascade leads to the activation of cell growth and differentiation. The V600E mutation promotes tumor cell viability, proliferation, and growth,12 and occurs in approximately 10% of colorectal cancers13 and up to 70% of MSI-H tumors with loss of MLH1.14

**Germline Testing for MMR Mutation.**—Once the MMR-deficient gene is identified in the tumor by IHC, the specific mutation can often be identified in DNA from peripheral blood mononuclear cells via targeted gene sequencing combined with multiplex ligation-dependent probe amplification. Detection of the EphCAM mutation can be performed by sequencing or IHC.15 Genetic testing is ultimately required to identify patients with Lynch syndrome, but the absence of a detectable germline mutation does not exclude the syndrome because of technical difficulties in identifying some mutations.

**Complexities of Testing.**—Loss of MSH2 expression strongly suggests Lynch syndrome. Loss of MSH6 or PMS2 alone is usually observed with a germline mutation in the respective gene, but with retained expression of MSH2 or MLH1. Tumors of some patients with Lynch syndrome due to germline mutation of the MSH6 gene may be MSS or MSI-L. Polymerase chain reaction–based MSI and IHC testing have comparable analytic sensitivity, but as described above, neither technique alone will identify all patients with MMR deficiency and Lynch syndrome. MSI-H cases may have retention of MMR proteins, especially noted in patients with Lynch syndrome, and tumors with loss of MMR protein, especially MSH6 in Lynch syndrome, may lack MSI-H.16,17 This situation has led many institutions to perform universal testing on all colorectal cancers by using both PCR-based MSI and IHC testing. This approach is not feasible for all laboratories, and the optimal algorithm for testing has been debated. There is also debate as to whether all patients with colorectal cancer should be tested, or if defined age limits, such as younger than 50 or 60 years, is most cost-effective and just as clinically effective. One study18 found that imposing an age cutoff of 50 years reduced the cost of the screening program to 16% of a program with no cutoff, but it missed more than half of the cases. Regardless of the algorithm used for screening, it has been demonstrated that the highest detection rate of Lynch syndrome in patients with colorectal cancer is through an integrated approach with cooperation among pathologists, clinicians, and genetic counselors19 (Table).

Somatic hypermethylation of the MLH1 gene and the presence of the V600E (c.1799T>A) mutation in the BRAF gene are highly predictive of sporadic colorectal cancers with loss of expression of MLH1. When loss of MLH1 expression is identified by IHC, testing for the V600E mutation should be performed to distinguish a Lynch syndrome.
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Abbreviations: EGFR, epidermal growth factor; 5-FU, 5-fluorouracil; IHC, immunohistochemistry; MSI, microsatellite instability; MSI-H, high-level MSI; MSI-L, low-level MSI; MSS, microsatellite stable; PCR, polymerase chain reaction.

dysplasia—associated tumor from a sporadic tumor. Although lack of BRAF mutation is highly sensitive for a syndromic tumor, rare BRAF mutations have been described in patients with Lynch syndrome.\(^{14,20}\) As well, BRAF V600E mutations have been described in Lynch syndrome pedigrees with monoallelic PMS2 mutations.\(^{21}\) Testing for BRAF mutations can be performed by Sanger sequencing and pyrosequencing, allele-specific reverse transcription–PCR, mass spectrometry–based sequencing, high-resolution melting curve analysis, and next-generation sequencing methods using microfluidics technology, among others.

**Immunohistochemistry for BRAF V600E Protein.**—Recently, several studies\(^{22-26}\) have also validated the use of IHC, using a V600E-specific VE1 clone to identify mutated protein expression with excellent sensitivity and specificity (98%–100% and 95%–988%, respectively) with 97% concordance rate in comparison to mutational analysis. Other studies\(^{27,28}\) however, have found less concordance, raising questions about the utility for clinical management decisions for individual patients.

**Methylation of MLH1.**—Analysis for MLH1 promoter hypermethylation is also used to identify sporadic tumors to limit unnecessary germline testing for Lynch syndrome. However, hypermethylation has also been identified in Lynch syndrome–associated tumors.\(^{14,29,30}\) Up to 68% of colorectal cancers with MLH1 promoter hypermethylation have the V600E mutation, so cases without this mutation should receive further workup.\(^{7}\) If a BRAF V600E mutation is not detected, methylation analysis of the MLH1 promoter can be performed by using methylation–specific multiplex ligation–dependent probe amplification or methylation-specific PCR. Of note, cases of constitutional MLH1 epimutations have been described where the promoter of one MLH1 allele is hypermethylated in the germline, resulting in transcriptional silencing of the allele in nonneoplastic tissue. These findings have been described in patients with loss of MLH1 and PMS2 on IHC testing, with detection of MLH1 methylation in neoplastic and adjacent nonneoplastic tissue, no BRAF V600E mutation, and no germline mutation of MLH1.\(^{31}\)

**Multimodality Testing.**—The addition of reflex BRAF mutation analysis to the testing of patients with MSI-H tumors with loss of MLH1 and PMS2 reduces the number of patient contacts by 40%, simplifies genetic testing, and leads
to cost and time savings. The advantages and disadvantages of BRAF and/or MLH1 hypermethylation testing of patients with loss of MLH1 have been studied. The sensitivity of absent BRAF mutation and MLH1 hypermethylation in identifying MLH1 mutation carriers is high; however, BRAF mutation specificity is low (22%–100%), with the specificity of MLH1 hypermethylation testing being slightly higher (45%–100%). Although it has been shown that MLH1 hypermethylation testing is also more cost-effective than BRAF mutation analysis in terms of incremental cost, BRAF mutation analysis and testing by IHC is more readily available and simpler to perform for many laboratories.

**BRAF Mutation as a Prognostic and Predictive Markers.**—Although the clinical utility of BRAF mutation testing has primarily focused on Lynch syndrome screening, the BRAF mutation has also shown prognostic value for overall survival in early-stage and metastatic colorectal cancer in patients with MSS CRC. In addition, the BRAF V600E mutation has been associated with a limited clinical response to epidermal growth factor receptor (EGFR)–targeted therapies (cetuximab or panitumumab). Several studies found that these mutations are linked to shorter progression-free survival and overall survival in patients with KRAS wild-type chemotherapy-refractory metastatic colorectal cancer given panitumumab or cetuximab-based treatment. There are numerous BRAF inhibitors currently being studied in clinical trials. These inhibitors have shown promising results in melanoma but have not been effective in colorectal carcinoma. Of note, concurrent mutations of KRAS and BRAF are extremely rare, and thus testing of BRAF mutations should not be routinely performed in patients with known KRAS-mutated tumors unless needed for a clinical trial (Table).

**Testing for KRAS Mutation**

KRAS is an oncogene located on chromosome 12 and mutated in 35% to 45% of colorectal cancers. The KRAS protein is a membrane-bound guanosine triphosphate/guanosine diphosphate (GTP/GDP)–binding GTPase involved in signal transduction and activation of the EGFR-signaling pathway. Codons 12 and 13 are the 2 mutation hotspots occurring in colorectal cancers, accounting for 95% of mutations, with codons 59, 61, 117, and 146, and 154 representing most of the remaining mutations. Most of the mutations are single-nucleotide point mutations, with the p.G12D and p.G12V being the most frequent mutations in codon 12 and the p.G13D, the most frequent in codon 13. Mutated KRAS results in a constitutively active GTP-bound state and the activation of proliferative signaling pathways. KRAS mutations are strongly associated with lack of response to anti-EGFR antibodies (cetuximab and panitumumab) and decreased efficacy of chemotherapies. These findings are primarily based on several studies in which patients with KRAS-mutated CRC showed no response to anti-EGFR therapies with cetuximab and panitumumab. Of note, however, recent studies have suggested that KRAS codon 13 mutations have similar behavior to KRAS wild-type mutations, and that the glycine to aspartate (G13D) mutation exhibits weaker transforming activity than codon 12 mutations. As well, KRAS p.G13D–mutated tumor cells may respond to EGFR-targeted therapy. Several studies have also found that patients with codon 13 KRAS mutations benefit from anti-EGFR therapies, with increases in overall survival, progression-free survival, and overall response rate. Although therapy for patients with codon 13 mutations is still under investigation, several studies have also found that patients with these mutations in KRAS exhibit worse overall prognosis with short overall survival times under standard chemotherapy.

The National Comprehensive Cancer Network (NCCN) and American Society of Clinical Oncology have added KRAS testing to their 2009 clinical practice guidelines and stated that only patients with wild-type KRAS should receive anti-EGFR treatment in advanced colorectal cancer. Since these guidelines were published, laboratory testing for KRAS mutation has increased, but the optimal procedure has yet to be established. Testing may be performed on the metastatic site or on the original biopsy site at diagnosis. Multiple studies have confirmed that testing the primary tumor predicts well the mutational (about 90%) status of the corresponding metastasis in the liver, and most of the lymph node and pulmonary metastasis.

**Biopsy and Resection Specimens.**—Although a diagnostic biopsy specimen can be very small in comparison to the surgical resection specimen, studies with genome-wide sequencing showed that somatic mutations of early driver genes are present in all colorectal tumor cells. KRAS testing can also be performed on cytology specimens from metastatic sites. In all cases, the pathologist should select samples with sufficient cellularity for the assay being performed, usually greater than 20% to 40% neoplastic cellularity depending on the assay used. KRAS testing is performed on formalin-fixed, paraffin embedded tissue by various methods including Sanger sequencing, pyrosequencing, Scorpion amplified refractory mutation system, real-time PCR, chip array hybridization, high-resolution melting analysis, and next-generation sequencing, among others.

**NRAS Mutation and Extended RAS Panels.**—NRAS in the RAS gene family is located on chromosome 1. Not surprisingly in light of the biochemical similarities to KRAS, recent studies have shown that NRAS mutation has influence on response to anti-EGFR therapy, like KRAS mutation. These findings led to recent proposals for testing a broad KRAS and NRAS panel to include codons 12, 13, 61, and 146, of both RAS genes, termed the extended RAS panel (Table).

**FUTURE BIOMARKERS IN COLORECTAL ADENOCARCINOMA**

**PIK3CA Mutation**

The PIK3CA gene encodes the catalytic subunit of phosphatidylinositol 3-kinase (PI3K), which is a downstream effector of EGFR-mediated signaling. Its mutated form induces phosphorylation of Akt, which promotes cell growth and inhibits apoptosis. Activating mutations of PIK3CA occur in 10% to 30% of colorectal cancers. More than 80% of PIK3CA mutations in colorectal cancers occur in exon 9 (~60%, helical domain) or exon 20 (~20%, kinase domain).

Studies have provided mixed results regarding PIK3CA mutations and response to EGFR inhibitors. PIK3CA mutations have been found to be associated with lack of response to EGFR monoclonal antibodies, while others found no correlation between PIK3CA mutation status and response to cetuximab. Others suggest that exon 20 mutations of PIK3CA are associated with lack of cetuximab activity in KRAS wild-type tumors, and exon 9 mutations are
associated with KRAS mutations without independent effect on cetuximab efficacy. The reasons for the discrepancy are not clear, and more studies are needed to establish the prognostic and predictive roles of PIK3CA exon 9 and exon 20 mutations. PIK3CA mutation has further been associated with poor survival in resectable stage I to III colon cancer, with the adverse effect of PIK3CA mutation potentially limited to patients with KRAS wild-type tumors.20

The most remarkable association with PIK3CA mutation is with improved survival in patients who took aspirin after surgical resection. Several studies have shown that PIK3CA is a potential biomarker for the benefit of adjuvant aspirin in stage II and III colorectal cancer. One study found that the regular use of aspirin after diagnosis was associated with longer survival among patients with mutated PIK3CA colorectal cancer in terms of colorectal cancer–specific survival and overall survival, but not in patients with wild-type PIK3CA cancer. Another study found that regular use of aspirin after colorectal cancer diagnosis was associated with a reduced rate of colorectal recurrence in patients with PIK3CA-mutant cancers, but not in patients lacking the mutation. Clinical trials to investigate postoperative adjuvant aspirin are underway and in the planning phase (Table).

PTEN Expression

Phosphatase and tensin homologue (PTEN) inhibits PIK3CA-initiated signaling, and its loss on immunohistochemistry is reported in 19% to 42% of colorectal cancers. The role of PTEN loss in CRC prognosis and therapy is currently unclear. It has been suggested that loss is associated with lack of benefit from cetuximab in metastatic colorectal cancer. Some studies have shown that loss of PTEN expression is negatively associated with progression-free survival and poor overall survival. Other investigators, however, could not detect any correlation between loss of PTEN expression or copy number and patient prognosis. Loss of PTEN has been found to co-occur with KRAS, BRAF, and PIK3CA mutations. Up to 70% of patients whose metastatic colorectal carcinoma shows loss of PTEN and mutations of KRAS, BRAF, or PIK3CA are unlikely to respond to EGFR inhibitors. Those lacking alterations in KRAS, BRAF, PTEN, and PIK3CA can be defined as “quadruple negative” and may have the highest probability of response to anti-EGFR therapies.

Several phase I and II clinical trials are currently underway to examine an Akt inhibitor (MK2206) in previously treated colorectal cancer with PTEN loss or PIK3CA mutation, and the combination of neratinib and cetuximab in patients with metastatic colorectal cancer defined as the quadruple wild type. Of note, data on the loss of PTEN expression are frequently discordant in primary and metastatic tumor tissue. In addition, there is currently no standardized method for PTEN expression analysis by IHC.

MEK Gene

MEK inhibitors are being studied as a treatment option for patients with KRAS-mutated colorectal cancers who are not candidates for EGFR-inhibitor therapy. These inhibitors as a single-agent have shown limited efficacy; however, in combination with other agents they may be promising. Results from a recent study demonstrated synergistic antiproliferative effects in response to a combination of an insulin-like growth factor receptor tyrosine kinase inhibitor with a MEK1/2 inhibitor in colorectal cell lines in vitro and in vivo. Several phase I and II clinical trials are underway exploring MEK inhibitors in combination with BRAF inhibitors, anti-EGFR antibodies, as well as fluorouracil and radiation therapy in colorectal cancer.

**ERBB2 (HER2/neu) TESTING IN ESOPHAGO-GASTRIC ADENOCARCINOMA**

The number of new esophageal and gastric cancer cases in the United States in 2014 is estimated at 18 170 and 22 220, respectively, and these tumors represent the eighth and fourth most common cancers worldwide. Despite many advances that have been made in the treatment of these cancers, the prognosis remains poor. Although surgery with lymph node dissection is the primary treatment in patients with resectable disease, for many patients, especially those with advanced and metastatic disease, surgery is not adequate therapy, and combined modalities including adjuvant therapy are needed.

ERBB2 (HER2/neu) is a proto-oncogene located on chromosome 17 that encodes a 185-kDa tyrosine kinase receptor belonging to the EGFR family, whose phosphorylation initiates signaling pathways that lead to cell division, proliferation, differentiation, and apoptosis. It is expressed in normal epithelial cells, and amplification and/or overexpression of this gene has been reported in up to 30% of breast cancers and in 9% to 27% of patients with gastric cancer. Overexpression of HER2/neu is slightly greater at the esophagogastrectomy junction in comparison to the stomach and overexpression in the stomach varies with histologic type (intestinal-type greater than diffuse type) and differentiation (moderately differentiated greater than poorly differentiated). ERBB2 (HER2/neu) appears to be an important prognostic factor in gastric cancer, but the literature is conflicting, as not all studies have shown an association between HER2/neu overexpression and poor prognosis.

In 2010, the results of an open-label, international, phase III randomized controlled clinical trial (Trastuzumab for Gastric Cancer, ToGA) showed that the anti-HER2/neu humanized monoclonal antibody trastuzumab (Herceptin, Genentech, San Francisco, California) was effective in prolonging survival compared with chemotherapy alone in patients with HER2/neu–positive adenocarcinoma of the stomach and the esophagogastrectomy junction, although overall survival was improved by less than 3 months. For patients with inoperable locally advanced, recurrent, or metastatic adenocarcinoma of the stomach or esophagogastrectomy junction for whom trastuzumab is being considered, assessment for tumor ERBB2 (HER2/neu) overexpression by IHC and amplification by fluorescence or other in situ hybridization is recommended by the NCCN.

Currently, ERBB2 (HER2/neu) testing is primarily being done on biopsy or surgical specimens that are formalin-fixed and paraffin-embedded. Immunohistochemistry permits evaluation of the membranous staining of the tumor cells, including intensity scored from 0 to 3+ and percentage of immunoreactive cells. Fluorescence in situ hybridization (FISH) is used to verify IHC equivocal cases, with results expressed as the ratio between the number of copies of the ERBB2 (HER2/neu) gene and the number of chromosome 17 centromeres (CEP17) in the nucleus of at least 20 cancer cells (HER2/neu:CEP17) with a cutoff point of 2.0.

No significant survival benefit was seen in the ToGA trial for patients who were IHC 0 or 1+ and FISH positive. For
treatment decisions, HER2/neu-positive gastric cancer has been defined as IHC 3+ and FISH positive in the United States and Japan, but IHC 3+ or 2+ with FISH positivity in Europe. In the United States, the US Food and Drug Administration (FDA) approved trastuzumab in association with chemotherapy for metastatic gastric cancer, using the eligibility criteria of the ToGA trial, limited to patients with a score of IHC 3+ or 2+ with FISH positivity, representing the European criteria.

In comparison to breast carcinomas, the heterogeneity of immunostaining is greater in gastric cancers, and the completeness of membrane staining required for positivity in mammary neoplastic cells is infrequent in gastric adenocarcinoma where expression often can be seen in a basolateral pattern. Hofmann et al developed a 4-tier HER2/neu scoring system, also used in the ToGA trial, for gastric cancer by applying an assessment area cutoff of at least 10% stained tumor cells for resection specimens and a small single cluster of cells for biopsy specimens. The NCCN guidelines recommend that assessment for ERBB2 (HER2/neu) status should be performed first using immunohistochemistry according to the modified scoring system used in the ToGA trial, as described above (3+ or 2+ IHC and FISH ratio ≥ 2), followed by FISH in 2+ tumors. Many institutions routinely perform both assays on all cases to shorten turnaround time, rather than using a 2-step approach. Computer-assisted image analysis can be used to improve the reproducibility of the assays.

FUTURE BIOMARKERS IN ESOPHAGEAL AND GASTRIC ADENOCARCINOMA

Currently, numerous phase II and III clinical trials are in the process of evaluating other biomarkers in esophageal, esophagogastroduodenal junction, and gastric adenocarcinomas. Epidermal growth factor receptor is currently being studied in advanced esophageal and esophagogastroduodenal junction cancer. The role for the EGFR pathway in esophageal carcinoma is leading to the study of the activity of EGFR-targeted agents. The presence of activating mutations in EGFR in esophageal adenocarcinomas and Barrett esophagus has been described. As well, overexpression of EGFR has been reported in the progression from Barrett mucosa to adenocarcinoma, with one study reporting a 13-fold increase in EGFR in adenocarcinoma compared to tissues with Barrett esophagus. Expression of EGFR in esophageal adenocarcinoma has been associated with poor response to therapy, poor overall survival, and development of cytotoxic drug resistance.

The role of genotype-directed tyrosine kinase therapy is being tested currently in several phase II clinical trials. Loss of PTEN has been detected in a subset of esophageal adenocarcinomas and shown to be associated with shorter overall survival and disease-free survival and may have a negative predictive role in anti-EGFR treatment.

The Met receptor is a tyrosine kinase that acts as a receptor for hepatocyte growth factor. Activation of the Met/hepatocyte growth factor pathway plays a role in gastrointestinal development, cell migration, and adhesion. Met has been found to be overexpressed in esophageal adenocarcinoma in surgical specimens and in cell lines, and appears to be an early event found at stages in dysplasia in Barrett esophagus. Others found that the overexpression of the Met receptor and hepatocyte growth factor shows increased expression along the dysplasia to adenocarcinoma sequence. Met receptor activation in esophageal cells lines is associated with β-catenin nuclear signaling (seen in esophageal tumorigenesis), preceding a reduced expression of E-cadherin; the expression of Met in esophageal adenocarcinoma is associated with a poorer prognosis in vivo. Met inhibitors may have therapeutic implications in the future and are currently being studied in phase II clinical trials.

Other biomarkers being investigated in esophageal and gastric adenocarcinoma include MEK, ERK, MAPK, PI3K, VEGFR, RAS, RAF, NF-kB, transforming growth factor β, Wnt, and numerous microRNAs. It is most likely that a panel of biomarkers with consideration of relevant targets for agents, associated pathways with cross-talk, and mucosal and submucosal environmental factors, such as inflammation and epithelial-mesenchymal transition, will be key to providing the optimal prognostic and predictive tools for decisions on therapies for patients.

BIOMARKERS IN GASTROINTESTINAL STROMAL TUMORS

Gastrointestinal stromal tumors (GISTs) that have differentiation characteristics of the interstitial cells of Cajal are the most common mesenchymal neoplasms arising in the gastrointestinal tract, but represent less than 1% of all gastrointestinal tumors. Although the true incidence of these tumors is not known, there are 4000 to 5000 new GIST cases diagnosed in the United States each year. They occur most commonly in the stomach (approximately 60%), small intestine (approximately 30%), and esophagus and colorectum (approximately 5% each). Microscopically, GISTs are classified into 3 main histologic subtypes: spindle cell, epithelioid, and mixed. Although most GISTs are benign, they can behave aggressively with metastases to the abdominal cavity, liver, and rarely, the lungs. The pathologic risk stratification for GISTs is based on size, number of mitoses, and anatomic site, with gastric tumors generally behaving in a more benign fashion than the other sites.

By immunohistochemistry, most GISTs are strongly and diffusely positive for KIT (CD117), mainly in the cytoplasm and less commonly in a membranous or dotlike pattern. Because only 5% of GISTs have been found to be negative for KIT, this stain is very useful in differentiating these tumors from other mesenchymal tumors in the gastrointestinal tract. CD34 in a membranous pattern also shows positivity in 60% to 70% of GISTs, depending on location; however, its expression may be seen in a number of other sarcomas and is not required for the diagnosis of GISTs. Discovered on GIST-1 (DOG1) is a chloride channel protein that may also be helpful to confirm the diagnosis of GIST, in both KIT-positive and in a subset of KIT-negative GISTs. The expression is both membranous and cytoplasmic, and may show higher sensitivity than KIT in these tumors.

c-KIT is a 145-kDa glycoprotein growth factor receptor for stem cell factor (SCF) belonging to the type III transmembrane receptor tyrosine kinase family. It is composed of a SCF-binding extracellular domain, a juxtamembrane domain, and 2 cytoplasmic tyrosine kinase domains. The binding of SCF results in homodimerization and triggers the phosphorylation of downstream signaling, causing activation of cell signaling, proliferation, differentiation, adhesion, and apoptosis. KIT mutations are detectable in 75% to 80% of GISTs and lead to constitutive activation of c-KIT tyrosine kinase function. Most mutations of KIT in sporadic...
GISTs cluster in exons 11, 9, 13, and 17. Exon 11 encodes the intracellular juxtamembrane portion of the KIT receptor, and exon 9 encodes the C-terminal extracellular domain of the KIT receptor. Mutations of these exons lead to constitutive kinase activation. Exons 13 and 17 encode the adenosine triphosphate (ATP)–binding pocket of the tyrosine kinase I domain and kinase activation loop of the tyrosine kinase II domain, respectively. Although mutations are heterogeneous and include insertions/deletions, duplications, and single-nucleotide polymorphisms, the most common mutations in KIT are in-frame deletions, mostly in exon 11, and duplications in exon 9.

Platelet-derived growth factor receptor α (PDGFRα) is a 170-kDa receptor tyrosine kinase that is important in cell proliferation, differentiation, growth, and development. Mutations in PDGFRα are detectable in around 35% of GISTs lacking KIT mutations. The most common mutations occur in exons 18, 12, and 14. Exon 18 encodes the activation loop of the distal kinase domain, with the most common mutation being a missense mutation leading to substitution of aspartate by valine at codon 842 (D842V). Exon 12 encodes the juxtamembrane domain of the receptor, and exon 14 encodes the proximal kinase domain. Single-nucleotide polymorphisms are the most common mutations in PDGFRα-mutated GISTs. Between 10% and 15% of GISTs that do not have KIT or PDGFRα mutations are designated as KIT-PDGFRα “wild-type” GISTs and show poor response to tyrosine kinase inhibitors. A small percentage of these “wild-type” GISTs have been shown to carry the exon 15 BRAF V600E mutation and mutations in genes encoding subunits of succinate dehydrogenase.

Imatinib is a potent tyrosine kinase inhibitor that targets both KIT and PDGFRα. Its mechanism of action involves binding to the ATP-binding pocket of tyrosine kinase and inhibiting phosphorylation of the receptor, blocking the activation of downstream signaling cascades that lead to cell proliferation. The mutational status of GISTs has been shown to correlate with response to imatinib, and molecular testing has become important in individualizing patient treatment. For example, GISTs with single-nucleotide substitutions in KIT exon 11 have been shown to be associated with less aggressive behavior than those with deletions in the same exon. Alternatively, GISTs with deletions in exon 11 affecting codons 557 through 558 are associated with poorer relapse-free survival and this may be a time-dependent prognostic factor limited to the first 4 years after surgery. Other retrospective analyses found that patients with exon 11 mutations experienced a higher response rate, progression-free survival, median time to tumor progression, and overall survival than patients with KIT exon 9 mutant or “wild-type” GISTs. Further, activating mutations in KIT exon 9 are the strongest predictor of poor response to imatinib, with significantly increased relative rate of progression and death when compared to exon 11 mutations.

Most mutations in exon 11 are sensitive to 400 mg daily of oral imatinib; however, a higher dose (400 mg twice a day) has been recommended for patients with exon 9 mutations.

Although up to one-third of GISTs with a PDGFRA mutation are responsive to imatinib, those with the most common mutation in PDGFRA, ie D842V, are generally associated with poor response to that drug. “Wild-type” GISTs, and those with mutations in BRAF or one of the succinate dehydrogenase subunit genes, generally indicate poor response to imatinib. Despite the overall efficacy of imatinib, resistance to treatment eventually develops. The most common genotypes leading to early (innate) failure include KIT exon 9 mutations; PDGFRA mutations, particularly the D842V mutation; and “wild-type” GISTs. Acquired resistance is frequently caused by clonal evolution via secondary mechanisms. Mutations in KIT exons 13, 14, and 17 have been found to cause structural changes to the imatinib binding site, leading to decreased drug affinity.

Sunitinib is a tyrosine kinase inhibitor with broader spectrum tyrosine kinase inhibition and also targets vascular endothelial growth factor receptor (VEGFR1), VEGFR2, FLT3, and RET. It is approved for the treatment of advanced GISTs after imatinib resistance or intolerance. With treatment, overall survival and median progression-free survival have been shown to be longer for patients with primary KIT exon 9 mutations or a wild-type genotype than for those with KIT exon 11, and longer for those with secondary KIT exon 13 or 14 mutations than those with secondary exon 17 or 18 mutations.

Laboratory testing for KIT and PDGFRA mutations usually involves the use of formalin-fixed, paraffin-embedded tissue from either a biopsy or surgical specimen. The relevant exons of each gene are amplified by using PCR, and the amplicons are sequenced. These mutations are mutually exclusive in GISTs.

FUTURE BIOMARKERS IN GASTROINTESTINAL STROMAL TUMORS

In addition to numerous ongoing phase II and III trials involving GISTs and imatinib and sunitinib, there are several other tyrosine kinase inhibitors currently being studied in trials. Most of these agents target KIT and PDGFRA, while others also target VEGFR. Crenolanib is a selective inhibitor of PDGFRα and PDGFRβ that has been reported to inhibit the PDGFRA D842V mutant kinase and to be 100- to 150-fold more potent than imatinib against that mutation. A phase II trial is ongoing. Regorafenib blocks VEGFR2-3, c-KIT, TIE2, PDGFRβ, FGFR1, RET, RAF, and p38 mitogen-activated protein kinase and had significant activity in patients with advanced GISTs previously treated with imatinib and sunitinib. An international phase III trial is currently underway.

Heat shock protein 90 (Hsp90) is a chaperone protein that is involved in protein folding, stabilization, and degradation. There are several HSP90 inhibitors in phase II clinical trials for the treatment of tyrosine kinase inhibitor-resistant GISTs. One such agent, AT13387, has been shown to inhibit the proliferation of imatinib-sensitive and imatinib-resistant cell lines in vitro and in vivo. Evidence that the PI3K–mammalian target of rapamycin (PI3K–mTOR) signaling pathway is activated in primary GISTs with KIT and PDGFRA mutations has led to the study of AKT Inhibitors in imatinib-resistant GISTs. Although minimal activity in such inhibitors has been shown to date, ongoing studies are in development.

The Notch signaling pathway has been shown to play a critical role in the development of several cancers, and a tumor suppressor role of the Notch pathway in GISTs via negative feedback with KIT has been described. A Notch signaling pathway inhibitor is now in phase Ib/II trial for patients with advanced sarcomas, including GISTs. Finally, protein kinase C θ is activated in GISTs, irrespective of KIT and PDGFRA mutational status, and can serve as a
diagnostic marker for GISTs; observations suggest that a protein kinase C θ inhibitor may lead to increased GIST apoptosis.\(^{131}\)

**CURRENT BIOMARKERS IN PANCREATIC ADENOCARCINOMA**

Pancreatic cancer is the fourth leading cause of cancer deaths in the United States with estimated new cases in 2014 numbering 46,420 and deaths, 39,590.\(^{132}\) Pancreatic ductal adenocarcinoma continues to be associated with a poor prognosis, despite ongoing efforts. Surgical resection remains the primary treatment modality; however, approximately 80% of patients have unresectable or metastatic disease at the time of diagnosis.

Chemotherapy for locally advanced pancreatic adenocarcinoma includes gemcitabine, 5-fluorouracil, or capecitabine with radiation therapy, with the addition of erlotinib, docetaxel, and FOLFIRINOX (5-fluorouracil, leucovorin, oxaliplatin, and irinotecan) for advanced or metastatic disease, but with marginal to no benefit.\(^{133}\) Understanding of the molecular mechanisms underlying the development of this cancer is imperative to develop better treatment options to improve outcomes. To date, there are no FDA-approved molecular biomarkers to aid in the treatment of pancreatic adenocarcinoma. However, research on the underlying genetic alterations leading to this disease is ongoing.

Whole-exome sequencing performed on pancreatic ductal adenocarcinomas revealed an average of 63 genomic alterations, with most consisting of point mutations.\(^{134}\) Four important frequently mutated genes contributing to pancreatic carcinogenesis include KRAS, SMAD4 (DPC4), TP53, and CDKN2A/p16.\(^{135}\) Activating mutations detected at codon 12, and less commonly at codons 13 and 61, of the KRAS gene are identified in up to 90% of pancreatic adenocarcinomas. KRAS mutations found in precursor pancreatic adenocarcinoma (pancreatic intraepithelial neoplasia) have led to their use as a potential biomarker of the disease.\(^{136}\) However, KRAS mutations are far from exclusive to malignancy and have been found in chronic pancreatitis and nonmalignant lesions. The most frequently identified mutation, KRAS G12D, has been shown to be important for all stages of pancreatic carcinogenesis, including progression and metastasis, and its inactivation leads to regression of the tumor.\(^{136}\) Unfortunately, targeting KRAS is difficult, and strategies directed at downstream targets are in use.

Antisense oligonucleotides directed against KRAS G12D have been developed to inhibit expression of the KRAS G12D protein. A phase I trial is currently recruiting to study their effects.\(^{137}\) Also of interest, mutant KRAS peptides are being studied as a tumor-specific antigen to stimulate the immune system against pancreatic adenocarcinoma. Three of 5 patients with resected pancreatic adenocarcinoma who received subcutaneous vaccination with mutant KRAS did not have progression of disease and were without acute or delayed systemic side effects, while 2 patients had progression of disease with lung metastases.\(^{138}\) Other phase II studies are examining the influence of EGFR and KRAS mutations on the efficacy of treatment with and without erlotinib in pancreatic cancer.\(^{139}\) Further study of possible adjuvant therapies is needed.

The tumor suppressor genes TP53, CDKN2A/p16, and SMAD4/DPC4 are also found to be altered in pancreatic adenocarcinoma. TP53 regulates the cell cycle at the G1/S interface that leads to cell cycle arrest and apoptosis. Although mutations involving TP53 are found in 50% to 75% of pancreatic adenocarcinomas,\(^{140}\) the actual clinical significance of TP53 in these lesions is not clear, with some studies showing poor survival but others showing that mutations of TP53 did not serve as a negative prognostic factor.\(^{141}\) Loss of expression of CDKN2A/p16 results in uncontrolled cell proliferation and has been described in up to 95% of pancreatic cancers.\(^{140}\) Deletions, point mutations, and promoter hypermethylation have been described. Loss of CDKN2A/p16 expression has been correlated with poor differentiation, metastasis, and shorter overall survival,\(^{142}\) but contradictory results have been reported. It has also been shown that combined biallelic p16 inactivation and KRAS activation promotes pancreatic tumor progression and metastasis.\(^{143}\)

SMAD4 (DPC4) encodes a member of the Smad family of signal transduction proteins, which act in signal transduction through activation of transforming growth factor β (TGF-β). SMAD4 has been found to be inactivated in up to 55% of pancreatic adenocarcinomas.\(^{140}\) Results in the literature from studies on patient outcomes and SMAD4 expression vary. An older study\(^{144}\) found that operative resection was associated with longer survival in those patients with loss of SMAD4 expression, and that survival in those with tumor expression was no different with resection compared to operative biopsy. Neither CDKN2A nor TP53 expression in this study cosegregated with patient outcome or each other.

Another study found that SMAD4 gene inactivation was significantly associated with shorter overall survival (median, 2.7 months shorter). The investigators\(^{145}\) also found no correlation between survival and mutations of CDKN2A or TP53. Oshima et al\(^{146}\) also found that loss of SMAD4 expression was an independent and significant poor prognostic factor for overall and disease-free survival. They also found that abnormal immunolabeling of p53 was associated with tumor dedifferentiation and presence of locoregional recurrence, and that loss of CDKN2A/p16 was associated with lymphatic invasion and postoperative widespread metastases.\(^{146}\) Another more recent study\(^{147}\) found that tumor SMAD4 expression was not a predictor of recurrence pattern, nor was it associated with early death. There was a trend toward worse survival with a loss of SMAD4 expression that did not reach statistical significance. Finally, Herman et al\(^{148}\) found that SMAD4 loss was associated with inferior recurrence-free survival but was not associated with overall survival.

**FUTURE BIOMARKERS IN PANCREATIC ADENOCARCINOMA**

In addition to the biomarkers that were investigated as described above, there are numerous other phase I and II trials currently ongoing in pancreatic adenocarcinoma and addressing biomarkers. Targets downstream from RAS include RAF, MEK, and ERK, which are intracellular protein serine/threonine kinases that act as downstream mediators of various signals. Activation of RAF leads to phosphorylation of RAF, which phosphorylates MEK, and then ERK. Diep et al\(^{149}\) found a synergistic effect between erlotinib and 2 MEK inhibitors, compared to monotherapy, in pancreatic cell lines with wild-type KRAS. Others\(^{150}\) found that MEK inhibition in pancreatic cancer leads to EGFR-mediated PI3K activation and sensitivity to combinations of MEK and
EGFR inhibitors in pancreatic adenocarcinoma cell lines. Phase I trials examining MEK inhibitors in combination with PI3K/mTOR inhibitors or irinotecan are ongoing.\textsuperscript{151,152} The serine/threonine kinase Akt lies at a critical signaling node downstream of phosphatidylinositol 3-kinase (PI3 kinase) and is important in promoting cell survival and inhibiting apoptosis. Akt and its expression has been reported in pancreatic adenocarcinoma. Prognostic significance of activated Akt expression was reported in one study\textsuperscript{153} that found that patients with low intensity p-Akt expression showed a significantly better 5-year survival rate than those with high expression, and univariate analysis revealed that p-Akt expression was significant in overall survival. Another study\textsuperscript{154} found similar results, with expression of p-Akt correlating with survival both on univariate and multivariate analysis. Two phase I and II trials are examining AKT inhibitors in combination with chemotherapy in pancreatic cancer.\textsuperscript{155,156}

The Notch pathway is important in cell signaling. Notch-1 is a downstream mediator of KRAS and has been reported to promote pancreatic neoplasia\textsuperscript{157}; nuclear expression of Notch-3 has been associated with more aggressive disease and reported to indicate poor prognosis in pancreatic cancer.\textsuperscript{158} Inhibition of Notch signaling has been shown to block the activity of γ-secretase that in turn blocks subsequent signaling in pancreatic cancer. A γ-secretase inhibitor PF-03084014 in combination with gemcitabine was shown to be highly effective in producing durable tumor regression, compared to gemcitabine alone, in pancreatic xenografts.\textsuperscript{159} Targeted therapy against γ-secretase is currently being investigated in several phase I and II clinical trials in combination with gemcitabine as first-line therapy in advanced pancreatic cancer.\textsuperscript{160,161}

CONCLUDING COMMENTS

The molecular biology of gastrointestinal tract cancers has been studied extensively for more than 3 decades, including in The Cancer Genome Atlas and other recent comprehensive genomic projects. As is apparent in this review, numerous biomarkers have been identified in basic and translational discovery studies, but few have achieved analytic validation of their assays and even fewer have shown evidence of clinical utility in the management of patients with a gastrointestinal cancer. For the most promising biomarkers, inconsistent results plague the literature, and the frequency of discordant findings dampens enthusiasm for regulatory approval, standard-of-care usage, and reimbursement. Nonetheless, the potential is evident for biomarkers to improve outcomes for patients with gastrointestinal cancer by making precision oncology a reality. Increasing emphasis on high-quality studies and continued advances in the technology and processes used in clinical molecular diagnostics laboratories will increase the number and types of validated biomarkers with documented clinical utility that will be provided for cancer patients in this rapidly evolving area of pathology and laboratory medicine.

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