Molecular Characterization of Colorectal Cancers

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LEARNING OBJECTIVES

1. Describe the genetic mutations associated with hereditary colorectal cancer (CRC) including familial adenomatous polyposis, hamartomatous polyposis, mutant Y DNA glycosylase–associated polyposis, and hereditary nonpolyposis CRC (Lynch syndrome).
2. Describe the general mechanism through which tumor suppressor and protooncogene mutations promote cancer.
3. Summarize the guidelines on molecular biomarker testing for the evaluation of CRC prepared by the American Society for Clinical Pathology, College of American Pathologists, Association for Molecular Pathology, and American Society of Clinical Oncology related to mismatch repair testing, *RAS* mutational testing, and *BRAF* p. V600 testing.
4. Describe the preferred specimens for predictive biomarker testing for CRC including the requisite mutant allele frequency.

ABSTRACT

Characterization of the molecular mutations in colorectal cancers has made great progress since the initial report by Fearon and Vogelstein describing a series of histologic changes that correlate to a series of mutations. Since 1990, numerous mutational pathways have been associated with 1 or more actionable targets leading to more precise treatments in the development of colorectal cancers, including inherited and sporadic ones. The accumulation of mutations leads to the acquisition of cancer-promoting qualities such as increased cellular proliferation, immune evasion, and resistance to apoptosis. Mutations of tumor suppressor and protooncogenes such as *KRAS* and *BRAF* have an important role in the prognosis and therapeutic selection of treatment. Broadening the use of multigene next-generation sequencing panels in patients with colorectal cancer may allow earlier identification of more actionable targets in patients, providing more precise treatments.

ABBREVIATIONS: APC - adenomatous polyposis coli, CRC - colorectal cancer, EGFR - epidermal growth factor receptor, FAP - familial adenomatous polyposis, MLH1 - mutL homolog 1, MMR - mismatch repair, MSH - mutS homolog, MUTYH - mutant Y DNA glycosylase, PI3K - phosphatidylinositol 3-kinase, TGF-β - transforming growth factor-beta, TK - tyrosine kinase.

INDEX TERMS: colorectal cancer biomarkers, colorectal cancer mutational testing.

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EPIDEMIOLOGY OF COLORECTAL CANCER

Colorectal cancers (CRCs) are a group of heterogeneous diseases arising through various molecular pathways. Different types of CRCs are characterized by genetic mutations that determine responsiveness or resistance to therapy.[1] Seventy percent of CRCs are sporadic, with no clear association with inheritance patterns or family history.[2,3] These cancers typically are diagnosed over the age of 50 years and are generally associated with dietary and environmental factors causing the mutations that progress toward CRC.[4] An additional 25% of cases are termed "familial CRC," cancers that run in families but have no consistent mutations identified. Less than 10% of all CRCs show a clear inherited predisposition.[2] These inherited CRCs include familial adenomatous polyposis (FAP), hamartomatous polyposis, mutant Y DNA glycosylase (MUTYH)-associated polyposis, and hereditary nonpolyposis CRC (Lynch syndrome).[1,2]

COLON PHYSIOLOGY

Normally, the colon functions to reabsorb water, minerals, and nutrients that remain in the digestive tract. The colonic microbiome can digest the remaining proteins and nutrients, especially carbohydrates. Reabsorption is favored by a complex process of differentiation of the colonic epithelium and is regulated by a set of signaling pathways, including Wnt, bone morphogenetic protein, and transforming growth factor-beta (TGF-*β*).[1] Approximately 90% of colon cancers carry Wnt mutations.[5] Crypts and villi of the gastrointestinal epithelium house colon stem cells and progenitor cells deep in the crypts.

Differentiated epithelial cells, such as Paneth, goblet, and enteroendocrine cells and enterocytes, migrate out of the crypts over a period of 14 days. They then undergo apoptosis (programmed cell death) and are shed in the stool.[1] This process of cell proliferation and differentiation is modified by mutations in the associated pathways and leads to transformation.[5]

ADENOMA CARCINOMA SEQUENCE

The adenoma carcinoma sequence describes the accumulation of genetic and epigenetic driver mutations (adenomatous polyposis coli [*APC*], *KRAS, SMAD4, TP53*) that result in the development of CRC.[6-9] In 1990, Fearon and Vogelstein described a multistep process of colorectal carcinogenesis that has served as a model for solid tumor carcinogenesis models.[10] This model has been modified by multiple alternate genetic pathways, including mutations in tumor suppressor genes, protooncogenes, DNA repair genes, cell cycle checkpoint genes, and apoptosis-related genes.[11] This adenoma–carcinoma sequence begins with the transformation of the normal glandular epithelial cells into benign neoplasms (adenomas). Subsequent progression and chromosome instability lead to the expansion of tubllovillous, tubular, and serrated invasive carcinomas. Chromosomal instability includes structural aberrations in chromosomes or changes in chromosome number, and these are found in 85% of all CRCs.[6] An example of chromosome alteration includes the loss of function of the *APC* tumor suppressor gene. *APC* encodes a tumor suppressor protein that acts as an antagonist of the Wnt signaling pathway. It is also involved in other processes, including cell migration and adhesion, transcriptional activation, and apoptosis.[12] Tumor suppressor genes arrest the mitotic cell and prevent cell division when abnormalities are detected. APC regulates spindle microtubules and is required to detect misaligned chromosomes in mitosis. APC also promotes the degradation of β-catenin. Loss of APC leads to an accumulation of β-catenin, which translocates to the nucleus to bind to DNA binding T-cell factor. This promotes the expression of MYC and cyclin D1, which leads to uncontrolled cell proliferation.[13] Germline mutations in the *APC* gene can lead to FAP. Loss of 1 copy of *APC* in the germline is not enough to progress to malignancy as there is still 1 normal copy of the gene that is sufficient to arrest the cell cycle. However, with every cell missing on copy of functional *APC*, a mutational event is more likely to cause the loss of the second APC gene and progress the cell toward adenoma development (2-hit hypothesis). At this point, chromosomal instability caused by environmental insult that would normally be arrested by APC may continue unchecked to dysplasia and carcinoma.[6,7,12-14]

MICROSATELLITE INSTABILITY AND MISMATCH REPAIR PROTEINS

Microsatellite instability is another cause of CRC.[15] Microsatellites are nucleotide sequences often found in noncoding regions, usually 1–6 base pairs in length repeated a variable number of times. They belong to a class of DNA nucleotide sequences known as variable number tandem repeats, which includes minisatellites, microsatellites, and short tandem repeats. These microsatellite sequences are present in the same locations on specific chromosomes but vary in the number of repeating units. These areas tend to be prone to mutational events and can be used in DNA profiling, cancer analysis, paternity testing, and population genetics.[16] Microsatellite instability occurs as a result of failure in the mismatch repair (MMR) proteins either through somatic mutation or aberrant methylation. MMR genes are essential for maintaining the integrity of the genome. Replication is normally extremely accurate, but mutations occur at roughly 1 in 10^9 to 10^{10} base pairs per cell division. Approximately 10^{-7} errors per genome occur due to the nucleotide selection when incorporating bases and the DNA polymerase proofreading step.[17] MMR failure drives a strong mutator phenotype that increases oncogenic potential.[17] MMR failure results in the inability to repair strand slippage and results in changes in the size of microsatellites. This is of particular importance when the microsatellite occurs in a gene coding region or promotor sequence. This failure of MMR and microsatellite instability is the cause of 15% of sporadic CRC cases.[16] Additionally, germline mutations of MMR genes are responsible for hereditary nonpolyposis CRC (Lynch Syndrome).[16] MMR gene mutations that frequently result in aberrant protein expression include mutL homolog 1 (*MLH1*) and mutS homolog (*MSH*) 2 genes. Additional genes implicated in faulty MMR include *MSH6*, *PMS2*, and epithelial cell adhesion molecule, which may have a role in inactivation of MSH2.[16,17]

MMR occurs when a nitrogenous base on 1 strand of DNA is changed and no longer pairs with the complementary base on the opposite strand following DNA replication in cell division. Normally the proofreading function of DNA polymerase recognizes this mismatch and repairs it during the replication process. When this system fails, the methyl-directed MMR system is activated.[17-19] This involves the recognition of the parent strand (the strand with the original and correct sequence of nucleotide bases, recognized through methylation), separation of the 2 strands, and removal of the erroneous sequence (often to include additional nitrogenous bases surrounding the mismatch). DNA polymerase can then return to the parent strand and produce the correct complementary sequence. The proteins MLH1, MSH2, MSH6, and PMS2 compromise various components of the MMR apparatus. When a mutational event occurs in these proteins, either through germline mutation (Lynch syndrome) or sporadic transformation, the result is colon epithelial cells with 1 intact gene.[20-22] A second hit on

the normal gene results in microsatellite instability and frameshift mutations in cell regulatory proteins, such as Bcl-2–associated X protein, histone deacetylase 2, TARBP2, and XPO5. This leads to uncontrolled proliferation, additional mutation, and progression through the adenoma–carcinoma continuum. Another pathway leading to loss of MMR involves aberrant DNA methylation.[23] This occurs in DNA to control unwanted expression of genes in certain cell types and has been associated with extrachromosomal inheritance of gene expression and epigenetics. Typically, hypermethylation silences gene expression when not warranted in the specific cell type, thereby facilitating the function of the cell and development of distinct tissue types. Hypermethylation of DNA most often occurs in sequences rich in cytosine–guanine repeats, known as CpG islands. Occasionally CpG islands occur in the promotor or coding sequence of MLH1, and hypermethylation of these sites creates silencing in this gene erroneously and failure of MMR. CRCs exhibiting loss of MMR due to hypermethylation of MLH1 are referred to as CpG island methylator phenotype. CRC screening is recommended for patients with Lynch syndrome as screening enables early detection and is thought to prevent advanced cancers.[24,25]

MYH-POLYPOSIS CRC

Mutations or silencing in MMR genes facilitates the development of CRC, but they are not the only mechanism for repairing DNA that may be compromised.[15] Base excision repair describes a similar mechanism of DNA proofreading that may be affected by mutation. Base excision repair occurs when a base not normally found in DNA is added erroneously. This includes the inclusion of uracil, or abnormally modified bases resulting from the effects of reactive oxygen species on the DNA.[26] The enzyme MYH glycosylase (a product of the *MUTYH* gene) recognizes abnormal bases in DNA and cleaves the bond between the base and the deoxyribose sugar, leaving the remainder of the nucleotide in place. Additional enzymes recognize the missing base, cleave the affected nucleotide, and add the correct base in its place. Germline mutations in both alleles of the *MUTYH* gene result in MYH-polyposis CRC.[26] The mechanism for this development includes subsequent inactivation of APC as described previously. Individuals with these germline mutations display an enhanced risk of CRC, up to 80% by the age range of 40–60 years.[15] Table 1 highlights selected hereditary CRC syndromes.

Table 1. Selected hereditary colorectal cancer

Selected Hereditary Colorectal Cancer Syndromes

Syndrome	Gene(s)	Mode of Inheritance
FAP	*APC*	Dominant
Hereditary nonpolyposis colorectal cancer	*MLH1*, *MSH2*, *MSH6*, *PMS2*, *TACSTD1*	Dominant
MYH-polyposis	*MUTYH*	Recessive

Discovery of germline mutations in these syndromes has been critical to understanding the molecular mechanisms of CRC. *(adapted from Ma et al. (2018) Pathology and genetics of hereditary colorectal cancer)*[16]

TUMOR SUPPRESSOR AND PROTOONCOGENE MUTATIONS

In addition to the molecular mechanisms described above that tend to be specific for CRCs, additional mutations in tumor suppressor genes and oncogenes are also implicated in many CRCs and should be examined in the cancer patient. Tumor suppressor genes control cell-cycle checkpoints and progression through mitosis. If errors are detected during mitosis, these proteins arrest the cell at the specific checkpoint until DNA repair mechanisms can be activated. If not, the cell will enter the apoptotic pathway and remove itself from the tissue, thereby protecting the organism as a whole. Tumor suppressor proteins are still functional when only 1 copy of the wild-type gene is present in the cell. The 2-hit hypothesis states that both copies of tumor suppressor genes (1 from each parent) must be inactivated in order to progress to neoplasia. In the case of Li–Fraumeni syndrome, patients are born with 1 deficient copy of the tumor suppressor gene *TP53*. Any additional mutations involving the remaining functional *TP53* gene result in advancement to neoplasia within the cell. Patients with Li–Fraumeni syndrome are therefore much more likely to develop diverse cancers throughout their lifetimes. Additional examples of tumor suppressor genes in CRCs include *APC* (the most common) and *TGF-β*. Oncogenes arise from mutations in protooncogenes, which also have a role in cell growth and development. These genes are different than tumor suppressor genes in that typically only 1 of the 2 alleles is mutated. This results in a gain-of-function mutation that leads to uncontrolled proliferation. Oncogenes associated with CRCs include epidermal growth factor receptor (*EGFR*), *BRAF,* mitogen-activated protein kinase, and phosphatidylinositol 3-kinase (*PI3K*).[7]

Upregulation of growth factor receptors is particularly problematic for CRCs in that the growth factor signaling drives growth and proliferation of the tumor.[15,27] The EGFR exists as a homodimer that responds to growth factor signaling. This causes EGFR to dimerize, thereby activating its tyrosine kinase (TK) activity within the cell. In tumor cells, the EGFR-TK signal is inappropriately turned on either through upregulation or faulty signaling mechanisms. The downstream effects of EGFR-TK activity include activation of RAS/RAF, MEK, extracellular signal-regulated kinase, and PI3K. These pathways are associated with proliferation and cell growth and in the tumor cell can lead to invasion, angiogenesis, and the inhibition of apoptosis. Current therapies for tumors exhibiting upregulation of EGFR include tyrosine

kinase inhibitors and anti-EGFR antibodies. These therapies should only be used in patients with tumors in which EGFR is upregulated, which is another important reason for molecular testing of these tumors.

OTHER CRC ONCOGENIC MECHANISMS

Approximately 40% of CRCs harbor *KRAS* mutations.[28,29] KRAS is a small oncogenic GTPase that represents the most common mutation in human cancers.[29] It is also important to note that the discovery of *KRAS* mutations that prevent the production of wild-type RAS is a predictor of resistance to anti-EGFR antibodies. Another mutation detected in molecular testing includes the *BRAF* V600E mutation that activates MEK and drives cell proliferation. BRAF inhibitor therapy (PLX4032 and PLX4720) is ineffective in slowing growth in tumors exhibiting this mutation.[27,30] Chromosomal translocations are also powerful oncogenic drivers, and several Food and Drug Administration–approved therapies exist as inhibitors of anaplastic lymphoma kinase, ROS1, RET, and others. Oncogenic gene fusions occur in approximately 0.9%–1.8% of CRCs. Receptor tyrosine kinase fusion products are the target in these therapies.[31] Copy number variations, mutations in noncoding regions, dysregulation of microRNA, epigenetic changes, and mutations in chromatin modifications may all act as selections that promote tumor formation and progression.[32]

GUIDELINES ON MOLECULAR MARKER SELECTION

Guidelines on molecular biomarker testing for the evaluation of CRC from the American Society for Clinical Pathology, College of American Pathologists, Association for Molecular Pathology, and American Society of Clinical Oncology include the following recommendations. Clinicians should order MMR status testing in patients with CRC to identify patients at high risk for Lynch syndrome and/or for prognostic stratification.[33] Patients with CRC who are being considered for anti-EGFR therapy must receive *RAS* mutational testing, which should include *KRAS* and *NRAS* codons 12 and 13 of exon 2, codons 59 and 61 of exon 3, and codons 117 and 146 of exon 4 ("expanded" or "extended" RAS). Additionally, *BRAF* p.V600 (*BRAF* c.1799 [p.V600]) position mutational analysis should be performed in CRC tissue in selected patients for prognostic stratification. *BRAF* p.V600 mutational analysis should be performed in deficient MMR tumors with loss of *MLH1* to evaluate for Lynch syndrome risk. Presence of a *BRAF* mutation strongly favors a sporadic pathogenesis. The absence of BRAF mutation does not exclude risk of Lynch syndrome.[33,34]

Figure 1 shows an image of a next-generation sequencing (NGS) platform. Tissues from metastatic or recurrent CRC tumors are the preferred specimens for treatment-predictive biomarker testing and should be used if such specimens are available and adequate. In their absence, primary tumor tissue is an acceptable alternative and should be used. Formalin-fixed, paraffin-embedded tissue is an acceptable specimen for molecular biomarker mutational testing in colorectal carcinoma.[33] Use of other specimens (eg, cytology speci mens) will require additional adequate validation, as would any changes in tissue- processing protocols. Laboratories should use CRC molecular biomarker testing methods that are able to detect mutations in specimens with at least 5% mutant allele frequency, considering the analytical sensitivity of the assay (limit of detection) and tumor enrichment (eg, microdissection).[33]

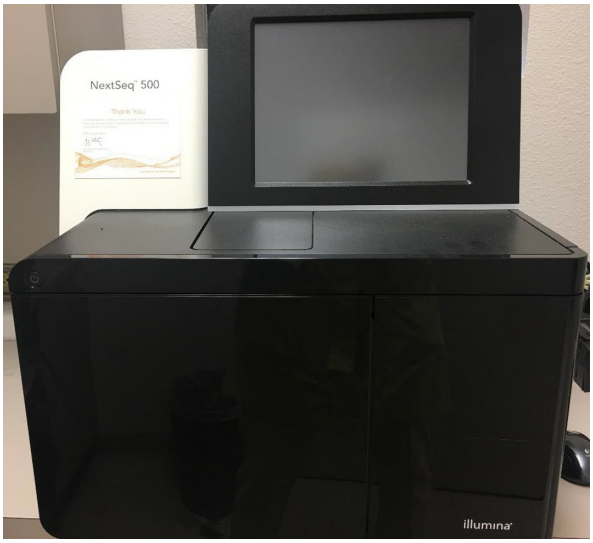

Figure 1. NextGen Sequencing. Next-generation sequencing (NGS) is a powerful platform that enables the sequencing of millions of DNA molecules from multiple patients simultaneously.

CONSENSUS MOLECULAR SUBTYPES AND EXPANDED MULTIGENE PANELS

Numerous groups have sought to classify molecular signature subtypes of CRC into consensus groups. In 2021, Guinney et al led an international consortium to demonstrate interconnectivity between 6 independent classification systems.[35] These 6 subtypes were distilled into 4 consensus molecular groups with distinguishing features, named CMSI 1–CMSI 4 (Table 2). A mixed group is proposed to exist as a transition group or represent tumor heterogeneity. These groups may be used or modified in the future for subtyping based on their molecular characteristics and may influence precision therapy.[35] Emerging data from a study by Coughlin indicate that perhaps it is time to broaden the use of germline multigene panel testing in CRC patients. Expanded multigene panel use has the potential to identify pathogenic and actionable variants.[36,37]

Table 2. Consensus molecular subtypes of CRC

Consensus Molecular Subtypes of CRC

Subtype	Features	Percent of Total
CMS1 (MSI immune)	Hypermutated Microsatellite instability Strong immune activation	14%
CMS2 (canonical)	Epithelial Chromosomally unstable Marker Wnt and MYC activation	37%
CMS1 (metabolic)	Metabolic Evident metabolic dysregulation	13%
CMS1 (mesenchymal)	Prominent TGF-β activation Stromal invasion Angiogenesis	23%

An international consortium led by Guinney at Fred Hutchinson Cancer Research Center and other institutions has grouped molecular subtypes of CRC into a classification as the future basis for subtype-based targeted interventions.[35]

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