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REVIEWS

Pathways of Colorectal Carcinogenesis



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Colorectal cancer is a heterogeneous disease that develops via stepwise accumulation of well-characterized genetic and epigenetic alterations. We review the genetic changes associated with the development of precancerous colorectal adenomas and their progression to tumors, as well as the effects of defective DNA repair, chromosome instability, microsatellite instability, and alterations in the serrated pathway and DNA methylation. We provide insights into the different molecular subgroups of colorectal tumors that develop via each of these different mechanisms and their associations with patient outcomes.

Keywords: CRC; DNA mismatch repair; genomic instability; serrated polyp.

Since Fearon and Vogelstein[1] proposed their multi-hit genetic model of colorectal carcinogenesis, accumulated insights have refined our understanding of the diverse genetic and epigenetic changes that underlie the initiation and progression of the adenoma–carcinoma sequence. A comprehensive analysis of mutations in 276 colorectal tumors by The Cancer Genome Atlas Network showed that among all malignancies, colorectal tumors have one of the highest mutational burdens; dozens of somatic mutations have been identified in multiple colorectal tumors. Colorectal tumors can be broadly categorized as hypermutated (>12 mutations per 10^6 bases) or nonhypermutated (<8.24 mutations per 10^6 bases).[2] Parallel efforts to subtype colorectal cancers (CRCs) based on gene expression profiles resulted in a new classification system, comprising 4 consensus molecular subtypes (CMS1–4). Each CMS has unique histopathologic features and correlations with progression and clinical outcomes.[3–6]

Although colorectal tumors are heterogenous at a genetic level, they appear to develop via several distinct pathways. The pathways of chromosome instability (CIN), microsatellite instability (MSI), and serrated neoplasia provide some information about their mechanisms of pathogenesis, although these pathways have some overlap. In addition, entirely new pathways continue to be recognized. Integrating data on gene expression signatures with tumor genotype has updated and refined these classifications.

We review the molecular changes associated with the transformation of normal colonic epithelium to histologically distinct precursor adenomatous lesions and, ultimately, malignant CRC. We discuss alterations in tumor suppressor genes, oncogenes, and DNA repair genes that contribute to colorectal carcinogenesis and review the correlations between these alterations and tumor progression.

Adenoma–Carcinoma Sequence

Most colorectal tumors arise from precancerous polyps that are broadly categorized as either traditional tubular adenomas or serrated polyps. Adenomas develop when normal mechanisms that regulate DNA repair and cell proliferation are altered. Constant epithelial renewal is required because of the continued loss of surface cells from the intestinal mucosa; proliferation occurs only at the crypt base. As mutant cells advance toward the colonic lumen, the typically predictable process of terminal differentiation and

Abbreviations used in this paper: **CIMP, CpG island methylator phenotype; CIN, chromosomal instability; CMS, consensus molecular subtype; COX, cyclooxygenase; CpG, cytosine/guanine; CRC, colorectal cancer; CVD, cardiovascular disease; EGFR, epidermal growth factor receptor; ERK, extracellular signal–regulated kinase; FIT, fecal immunohistochemical test; LOH, loss of heterozygosity; MMR, mismatch repair; MSI, microsatellite instability; MSS, microsatellite stable; PI3K, phosphoinositide 3 kinase; SCNA, somatic copy number alteration; TGFB, transforming growth factor β; TSA, traditional serrated adenoma.**

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eventual apoptosis is disrupted, and discrete adenomas form. Over time, adenomatous polyps increase in size, develop increasingly dysplastic features, and can eventually acquire invasive potential.

Sequential alterations in key growth regulatory genes mark the transition from normal to hyperproliferative epithelium. This stepwise progression, which couples specific genetic alterations with advancing histologic features, has served as a paradigm for solid tumorigenesis. Mutations in the adenomatous polyposis coli gene (*APC*), which encodes a tumor suppressor,[7] or the *BRAF* oncogene[8,9] are initiating events that give rise to traditional adenomas or serrated polyps, respectively. The subsequent events vary depending on the specific pathway engaged (Figure 1). Additional genetic alterations mark the progression from early to intermediate neoplasias, then to advanced polyps with high-grade dysplasia and, later, invasive tumors. However, not all adenomas advance to cancer—the accumulation of specific mutations in a particular order is essential for progression to malignancy. The timeline depends on the specific pathway of tumorigenesis. For example, tumorigenesis via the CIN pathway can take 10 years or more, whereas tumor development via the comparatively accelerated MSI pathway can occur in a few years.

CIN Pathway

The CIN pathway, observed in 65%–70% of sporadic colorectal tumors,[10,11] is characterized by chromosome changes that include somatic copy number alterations (SCNAs) caused by aneuploidy, deletions, insertions, amplifications, or loss of heterozygosity. Tumors that develop through this pathway are considered nonhypermutated because of the relative paucity of base pair mutations in coding sequences (Figure 1). Mechanisms that lead to CIN are usually characterized by defects in chromosomal segregation, such as those that control sister chromatid separation; disordered cell senescence, induced by telomere shortening and culminating in genomic reorganization; dysfunctional DNA damage-response machinery; and loss of heterozygosity (LOH) at a tumor suppressor gene.

These karyotypic abnormalities are coupled with mutations in the tumor suppressor genes *APC* and *TP53* and activating mutations in *KRAS* and phosphatidylinositol-4,5-bisphosphonate 3-kinase catalytic subunit alpha (*PIK3CA*). Mutation of *APC* appears to be the earliest genetic event in colorectal tumorigenesis.[1,12] Loss of APC activity results in nuclear translocation of β-catenin and activation of the Wnt signaling pathway.

In the absence of Wnt signaling, APC, axin, and glycogen synthase kinase 3 beta (GSK3B) complex with β-catenin in the cytosol. This complex phosphorylates β-catenin, marking it for degradation by the ubiquitin-mediated proteasomal pathway. In the presence of Wnt signaling, β-catenin is stable. Mutant *APC* has decreased binding affinity for this multiprotein destruction complex, resulting in translocation of β-catenin to the nucleus, where it heterodimerizes with the TCF and LEF families of transcription factors. This results in constitutive activation of genes that are regulated by Wnt signaling and are associated with tumorigenesis, including *MYC*, the cyclin D1 gene (*CCND1*), vascular endothelial growth factor (VEGF) genes, and the peroxisome proliferator activated receptor delta PPARδ gene.[13] Mutations in other genes regulated by the Wnt pathway, such as *AXIN1*, *AXIN2*, or *CTNNB1*, can amplify Wnt signaling in the absence of an *APC* mutation.[14] The Wnt signaling pathway is therefore an important regulator of intestinal epithelial cell proliferation and provides an example for how the disruption of any component in a specific pathway can affect transcription of multiple genes to promote tumorigenesis.[15–17]

The Wnt pathway is activated in nearly all CIN tumors, and *APC* mutations have been identified in approximately 80% of these tumors.[2,3] In an effort to harmonize prior colorectal tumor functional profiles, which had only modest interstudy agreement,[4,6,18,19] the CRC Subtyping Consortium aggregated findings from more than 30 gene expression studies, performed with different platforms and sample preparation methods, into a single unified framework. Their efforts resulted in 4 classes of colorectal tumors based on robust, uniformly processed tumor gene expression profiles. CMS2 tumors are characterized by activation of the Wnt and MYC signaling pathways and frequent SCNAs—features consistent with the CIN phenotype.[3]

MYC is a component of a heterodimeric basic helix-loop-helix transcription factor complex that regulates cell cycle progression, metabolism, and apoptosis. Mutations in *MYC* are not found in most colorectal tumors, but amplifications of *MYC* have been found in colorectal and other tumor types.[20] Expression of MYC can be up-regulated via activation of the Wnt signaling pathway.[21] However, a meta-analysis found no clear association between tumor level of c-MYC protein and overall or disease-specific survival times.[22]

Autosomal dominant mutations in *APC* cause familial adenomatous polyposis, characterized by hundreds to thousands of adenomatous polyps.[23] Biallelic germline variants in the mutY DNA glycosylase gene (MUTYH), which encodes a base excision repair protein, cause MUTYH-associated polyposis. Colon adenomas that develop in patients with MUTYH-associated polyposis or familial adenomatous polyposis have mutations in *APC*.[24–26]

Activating mutations in *KRAS* often arise after mutations in *APC* and are found in nearly 40% of colorectal tumors.[27,28] KRAS is a component of several growth factor signaling pathways, including the epidermal growth factor receptor (EGFR) pathway. In this pathway, activation of KRAS results in constitutive activation of the Raf-MEK–extracellular signal–regulated kinase (ERK) pathway, phosphoinositide 3 kinase (PI3K) signaling via MTOR and the transcription factor nuclear factor κB (NF-kB). Proteins in the Raf family are serine/threonine kinases that activate MEK1 and MEK2, resulting in phosphorylation of ERK1 and ERK2 and then phosphorylation of enzymes that promote cell cycle progression.[29] This pathway is activated in many tumors, including colorectal tumors, and might be targeted by therapeutic agents—especially metastatic colorectal tumors.

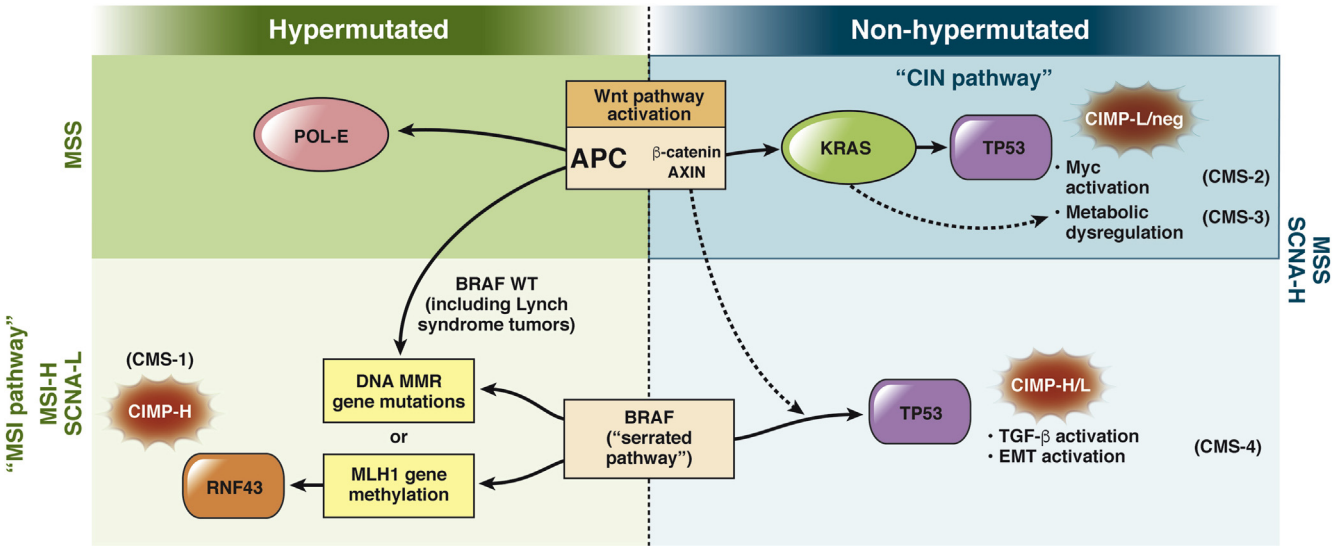

Figure 1. Pathways of colorectal carcinogenesis. Activation of the Wnt pathway (primarily via *APC* mutation) or a mutation in *BRAF* can initiate colorectal tumorigenesis. *BRAF* mutations promote tumorigenesis via the serrated neoplasia pathway, leading to MSI with hypermutation or MSS without hypermutation (indicated in the figure). Colorectal tumor classifications include CIN, MSI, and the serrated pathway (see CMS). EMT, epithelial to mesenchymal transition; H, high; L, low; neg, negative.

Inhibitors of EGFR are used in the treatment of metastatic tumors, but they are not effective in colorectal tumors with mutations in *KRAS* or *BRAF* because these tumors maintain activity of this pathway.[30–32]

Analyses of gene expression patterns in colorectal tumors have identified a relationship between *KRAS* mutations and CMS3 (also called the metabolic phenotype). CMS3 tumors are characterized by CIN, but with fewer SCNAs than the CMS2 subtype. Gene set messenger RNA enrichment analysis of CMS3 tumors found evidence for dysregulation of metabolic pathways, including those that involve sugars (such as glucose and fructose), amino acids (such as glutamine), lysophospholipids, and fatty acids. These metabolic aberrations might support tumor growth[3] and are consistent with reports that activation of KRAS affects glucose metabolism and hypoxia.[33,34]

Many colorectal tumors with mutations in *KRAS* also contain mutations in genes encoding catalytic subunits of PI3K. PI3K is a heterodimeric lipid kinase that phosphorylates phosphatidylinositol, a cell signaling molecule. Increased activity of PI3K can increase prostaglandin synthesis to inhibit apoptosis in colorectal tumor cells.[35] Activating mutations in *PIK3CA* arise late in the adenoma-carcinoma sequence and are found in 10%–20% of colorectal tumors, as well as in breast, brain, ovarian, liver, and lung tumors.[36,37] PIK3CA regulates cell proliferation and survival, inactivating proteins that promote apoptosis such as forkhead box protein O1 (FOXO1) and the Rac family of GTPases. Gain-of-function mutations in *PIK3CA* activate AKT signaling via MTOR to promote cell proliferation. Mutations in *PIK3CA* have been associated with tumors in female patients, with proximal location, and with MSI and mutations in *KRAS*.[38–42] Interestingly, the ratio of mutational burden at exons 9 and 20 in tumors is associated with their tissue of origin (colorectal, breast, gastric, or endometrial) and with survival times of patients with CRC.[36,43]

Prostaglandin-endoperoxide synthase 2 (PTGS2, also called cyclooxygenase 2 [COX-2]) is also involved in CRC development. COX-2 is an immediate to early response enzyme located on the luminal side of the endoplasmic reticulum and nuclear membrane.[44] It can be activated by cytokines and other stimuli and is overexpressed in adenomas and colorectal tumors,[45] although no somatic mutations in *COX2* have been found in colorectal tumors. During colorectal carcinogenesis, COX-2 might convert free arachidonic acid into prostanoids, including prostaglandins such as PGE_2, which regulate proliferation of colorectal tumor cells.[46] Mice with disruption of *Ptgs2* have a decreased incidence of small- and large-bowel neoplasia.[47,48] COX-2 also regulates angiogenesis and promotes tumor vascularization.[49,50]

COX-2 might contribute to invasive and migratory activities of CRC cells. During the early stages of colorectal tumorigenesis, expression of COX-2 is increased in the stroma and connective tissue, rather than the colonic epithelium.[48,51,52] These observations might account for the association between use of COX inhibitors such as nonsteroidal anti-inflammatory drugs (including aspirin and selective COX-2 inhibitors) and reduced risk of lesions in the adenoma–carcinoma sequence, including metastatic CRC.[53–58] Supported by large-scale epidemiology studies, as well as data from primary prevention trials for cardiovascular disease (CVD), the US Preventive Services Task Force recommends low-dose aspirin therapy for primary prevention of CVD and CRC in adults 50–59 years old with an increased risk of CVD, no increased risk for bleeding complications, a life expectancy of at least 10 years, and a willingness to adhere to regular, long-term use.[59]

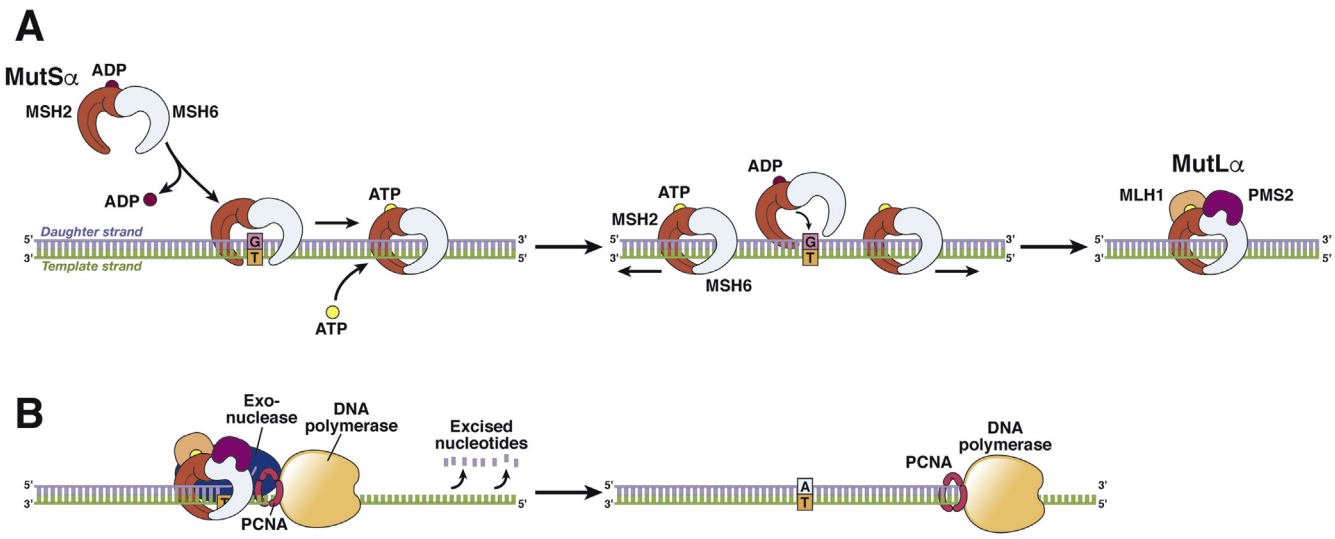

Figure 2. MMR of single-pair DNA mismatches and insertion/deletion loops. (*A*) Upon detection of a mismatch, MSH2 will heterodimerize with either MSH6 or MSH3 to form MutSα (to correct single base-base or short IDL mismatches) or MutSβ (principally to correct larger IDLs), respectively. Then MutSα or MutSβ will complex with MutL α, β, or γ (MLH1 coupled to either PMS2, PMS1, or MLH3) to direct exonuclease-1 and proliferating cell nuclear antigen (PCNA) to remove the misincorporated nucleotide(s). (*B*) This allows DNA polymerase and DNA ligase to re-synthesize, ligate, and anneal the corrected daughter strand. The MSI phenotype is a consequence of dysfunctional proteins typically tasked with the recognition and repair of these common mismatch errors in DNA replication.

The *TP53* gene, on human chromosome arm 17p, is the most commonly mutated gene in cancer. Its product, P53, regulates the transcription of genes that regulate DNA repair and cell responses to oxidative stress. Loss-of-function alterations in P53 are detected more frequently in colorectal tumors than adenomas with microscopic foci of invasive cancer and more frequently in these invasive adenomas than benign adenomas.[60] This association between the frequency of *TP53* mutations and lesion stage indicates that mutant forms of P53 promote tumor development at a late stage of tumorigenesis.[61,62] Approximately 60% of CIN tumors contain inactivating mutations in *TP53*.[2] Patients with the Li–Fraumeni syndrome, characterized by germline mutations in *TP53,* have a modest increase in risk of CRC compared with the overall population. Analysis of data from patient registries found an association between germline alterations of *TP53* and young-onset CRC (diagnosed in patients younger than 50 years).[63] This observation is important because cases of young-onset CRC have increased greatly in the United States and parts of Europe and Asia, in contrast to reported decreases in CRC incidence in other age groups. Inherited genetic factors might contribute to a subset of these cases.[64–69]

LOH at chromosome arm 18q is found in more than 70% of colorectal tumors (only of advanced stages).[1] This observation indicates the presence of colorectal tumor suppressor genes at this region. Candidates include the Deleted in Colorectal Cancer (*DCC*) and genes that encode proteins in the transforming growth factor β (TGFB) pathway SMAD2 and SMAD4.[70–72] However, mutations in these genes are rare in human colorectal tumors, and deletions of any of these genes in experimental models have not been consistently associated with colorectal tumorigenesis. LOH at 18q has been associated with shorter survival times, although this result might be confounded by the association of 18q LOH with other negative prognostic factors, including high body mass index, higher tumor grade, and hypomethylation at long interspersed nucleotide element 1 (LINE-1) elements of genomic DNA.[73–75]

MSI Pathway

In contrast to the CIN pathway, characterized by a high frequency of genomic copy number alterations, colorectal tumors can also develop through hypermutable pathways, characterized by frequent somatic DNA base pair mutations. The MSI pathway is the primary mechanism for this hypermutable phenotype. Broadly, mutations in DNA mismatch repair (MMR) genes (*MLH1*, *MSH2*, *MSH6*, *PMS2*) or *EPCAM* (encodes a protein that regulates *MSH2*) cause instability within microsatellite regions. DNA microsatellites are repeated tandem sequences consisting of mononucleotide, dinucleotide, or even higher-order nucleotide repeats. These areas accumulate errors, as DNA polymerase struggles to efficiently bind these repeating genome sequences.

Failure to correctly assess the number of bases to insert into these invariable areas or even slippage during new strand synthesis (which generates a temporary insertion-deletion loop) can produce a frameshift mutation that results in a truncated or nonfunctional protein. Ordinarily, in the absence of deleterious MMR gene mutations, the mismatch repair system recognizes these mistakes and performs DNA excision repair before daughter strand replication (see Figure 2). Cells in tumors with the MSI phenotype do not properly detect and repair mismatched

DNA, allowing them to maintain and replicate their mutations and acquire additional mutations (a hypermutation phenotype).

MSI is observed in nearly 15% of sporadic colorectal tumors and nearly all colorectal tumors that develop in patients with Lynch syndrome, the most common hereditary colon cancer syndrome, caused by germline mutations in DNA MMR genes.[76] However, most colorectal tumors with MSI are sporadic. The most common cause of the MSI phenotype is epigenetic silencing of the *MLH1* gene through promoter hypermethylation. Colorectal tumors of the MSI phenotype usually have high levels of methylation at regulatory regions throughout the genome, including the cytosine/guanine (CpG) island methylator phenotype (CIMP). MSI tumors also have an increased frequency of a mutation that encodes a V to E substitution in amino acid 600 of BRAF (in $BRAF^{V600E}$) but a low frequency of *APC* and *TP53* mutations compared with colorectal tumors that develop via the CIN pathway.[8,77,78] In patients with Lynch syndrome, colorectal tumors that arise via a germline mutation in a DNA MMR gene also have MSI but do not usually have mutations in *BRAF*.[79]

MSI mutations include those in the TGFB receptor-2 gene (*TGFBR2*), which encodes a protein that prevents colon epithelial cell proliferation. *TGFBR2* is mutated in more than 90% of MSI colorectal tumors.[80] Mutations in *TGFBR2* accumulate within a specific polyadenine tract to inactivate the receptor, so it can no longer signal to prevent proliferation. Other genes disrupted by MSI include those that encode proteins that regulate proliferation (*GRB1*, *TCF4*, *WISP3*, *ACVR2*, *IGF2R*, *AXIN2*, and *CDX*), cell cycle arrest or apoptosis (CASP5, *PRDM2*, *BCL10*, *PTEN*, *PA2G4*, and *FAS*), and DNA repair (*MBD4*, *BLM*, *CHK1*, *MLH3*, *RAD50*, *MSH3*, and *MSH6*).[81,82]

The events that initiate tumorigenesis via the MSI pathway vary. *APC* mutations are found in 35%–50% of MSI tumors,[2,3] so the initiating event of adenoma formation might be shared by MSI and CIN tumors. However, a distinct set of MSI tumors can develop via an initiating *BRAF* mutation (Figure 1).[8,9] These tumors share traits of the MSI and serrated pathways. Once MSI occurs, colorectal tumors develop more rapidly than via the CIN pathway. Tumors with high levels of MSI (MSI-high) are associated with shorter times of progression, attributed to the hypermutable environment; CRC develops within 1–3 years as opposed to the decades required by CIN tumors.[83]

MSI status is determined by using a standard panel of microsatellite markers defined by the National Cancer Institute/Bethesda consensus guidelines. Tumors with frameshifts in 30% or more of marker genes are classified as MSI-high tumors. Tumors with frameshift mutations in fewer than 30% of marker genes are classified as MSI-low. Finally, those without instability are deemed microsatellite stable (MSS).[84] Polymerase chain reaction can reliably detect the presence of MSI, although immunohistochemical analysis of MMR proteins is the most common method. Tumor cells that lack MMR proteins, based on immunohistochemical analysis, are categorized as MSI-high. Genotypes of patients' colorectal tumors are routinely analyzed to identify factors that might be targeted therapeutically. MSI status can also be determined by DNA sequencing.[85]

Within the CMS framework, CMS1 tumors are characterized by hypermethylation of DNA, MSI-high, and infiltration by immune cells.[3] Hypermethylation of promoter regions of the *MLH1* gene, in particular, results in its silencing, accumulation of DNA mutations, and expression of many mutant forms of proteins. These are immunogenic, resulting in tumor infiltration by cytotoxic T cells and natural killer cells,[86–88] followed by expression of checkpoint molecules that inhibit T-cell activation and cytokine production, such as programmed cell death ligand 1. MSI-high tumors are therefore susceptible to treatment with PD-1 inhibitors,[89,90] resulting in a paradigm shift in the treatment of CRC.

A high proportion of CMS1 tumors have the BRAF V600E mutation and CIMP-high status. However, patients with these tumors have a better prognosis than patients with other CRC subtypes.[91] These tumors have distinctive clinical and histopathologic features in that they tend to arise in the proximal colon, contain mucin, and be poorly differentiated. There is growing consensus that adjuvant, single-agent, fluoropyrimidine-based chemotherapy is not effective, and might even be harmful, for patients with MSI-high colorectal tumors.[92–96] Although these tumors have characteristics such as poor differentiation, the presence of MSI indicates a good prognosis, so determining MSI status is now routinely recommended for the staging of incident CRC.[97] Among patients with localized tumors, those with MSI-high tumors had longer survival times than patients with MSS or MSI-low tumors of a similar stage.[88,96,98–100]

Serrated Neoplasia Pathway

Adenomas with intestinal-type dysplasia are not the only precursor lesions to CRC. Advances in endoscopic technology have improved detection of serrated polyps—saw-toothed lesions with a varied spectrum of histopathologic correlates. Serrated polyps are believed to give rise to nearly 15% of CRCs, via the serrated neoplasia pathway.[101]

Serrated polyps are a heterogeneous group of lesions characterized by a stellate pattern of crypt in-folding and include benign hyperplastic polyps, precancerous sessile serrated adenomas or polyps, or traditional serrated adenomas (TSAs).[102] A large proportion of interval CRCs (those that develop within recommended surveillance periods, typically 3–5 years) are believed to arise via the serrated pathway.[103–105]

Hyperplastic polyps are the most frequently occurring subtype of serrated polyps, accounting for nearly two thirds of all serrated lesions, and rarely undergo malignant transformation.[106–108] Hyperplastic polyps have narrow crypt bases with serration confined to the upper portion and may be further subdivided by predominant mucin type: microvesicular, goblet cell–rich, and mucin-poor hyperplastic polyps.

The second most common form of serrated lesions is sessile serrated adenomas and polyps, which are believed to

account for one third of all serrated lesions, although precise overall prevalence estimates have proven elusive.[106,107] Features that may make their identification challenging include a flat or sessile morphology that is easily missed by white-light imaging without chromoendoscopy and subtle distinguishing histopathologic traits that confound interobserver reliability, even among expert gastrointestinal pathologists.[109–111] Nuanced attributes such as abnormal proliferation, branching and dilated T- or L-shaped crypts, or subtle cellular dysplasia contribute to the heterogeneity of risk estimates.

TSAs account for a smaller proportion of serrated lesions. TSAs are histologically defined by protuberant growth patterns with villiform projections. These defining factors make distinctions between TSAs and tubulovillous adenomas more challenging. Cytologic dysplasia manifests as nuclear atypia with reduced differentiation and eosinophilic cytoplasm.[112]

The serrated pathway is a distinct mechanism of colorectal carcinogenesis. The pace of tumor progression is not fully characterized, but the subset of serrated tumors that acquire MSI are associated with a comparatively accelerated progression from precancerous lesion to carcinoma, like all MSI-high tumors. A distinguishing trait of the serrated pathway is the activating V600E mutation in BRAF, a component of the mitogen-activated protein kinase pathway.[8] A large proportion of microvesicular hyperplastic polyps contain *BRAF* mutations, so this mutation is believed to occur early in the serrated pathway, causing constitutive activation of the mitogen-activated protein kinase–ERK pathway and uncontrolled cell division.[8,78,113–117] *BRAF* is mutated in most sessile serrated adenomas but rarely in conventional adenomas, supporting the concept that the serrated pathway is an alternative route to CRC.[117]

After mutation of *BRAF*, serrated tumors develop via 2 different routes (Figure 1). One route converges with the MSI pathway—mutations in an MMR gene result in the MSI-high phenotype. These tumors typically develop from sessile serrated adenomas and share features with CMS1 tumors. Alternatively, tumors with mutations in *BRAF* can acquire *TP53* mutations and activate several oncogenic pathways, including Wnt signaling, TGFB signaling, and the epithelial-to-mesenchymal transition; these do not result in MSI-high but rather MSS tumors (Figure 1).[3] These tumors typically develop through the traditional serrated adenoma as an intermediate lesion and have features of CMS4 (mesenchymal subtype) tumors. Unlike CMS1 tumors, CMS4 tumors have MSS with CIN, low levels of hypermutation, and high SCNA. CMS4 tumors have activation of pathways that facilitate an immunosuppressive microenvironment and permit stromal inflammation and tumor invasion, such as the angiogenic pathway. These factors may contribute to the abilities of CMS4 tumors to evade the immune response, resulting in the lowest survival rates of the CMSs.[118,119] Compared with classic CIN tumors (MSS and no mutations in *BRAF*), patients with MSS and mutant *BRAF* tumors had shorter times of overall survival (hazard ratio, 2.16) and disease-specific survival (hazard ratio, 2.59). In contrast, patients with MSI and mutant *BRAF* tumors have longer times of overall survival (hazard ratio, 0.74) and disease-specific survival (hazard ratio, 0.74).[119]

Although *BRAF* mutations are the first detected event in the serrated pathway, it is not uncommon for the Wnt pathway to also become activated.[120] In this scenario, the Wnt pathway is activated not by truncating mutations in *APC* but, rather, by alternate routes including missense *APC* mutations or *RNF43* mutations (Figure 1).[121,122] RNF43 is an E3 ubiquitin ligase that can inhibit Wnt signaling through R-spondin. Up to 85% of MSI-high tumors with methylation at *MLH1* have a somatic mutation in RNF43.[122]

Despite successes in other tumor types that carry *BRAF* mutations, such as melanoma, use of single-agent BRAF inhibitors in *BRAF*-mutant CRCs has been disappointing.[123,124] This has been attributed to the frequent development of resistance mutations in compensatory molecular pathways, such as up-regulation of EGFR.[125] Recently, a strategy that combines inhibition of BRAF with inhibitors of MEK and EGFR has shown promising results in *BRAF*-mutant colon tumors, and this multidrug approach is currently recommended by the National Comprehensive Cancer Network.[126]

Tumors that develop through either arm of the serrated neoplasia pathway also typically exhibit high levels of CpG island methylation. CpG islands are dense clusters of cytosine/guanine (CpG) dinucleotides linked by a phosphodiester bond that are particularly enriched in gene promoter regions.[127] Hypermethylation of these promoter islands, particularly those upstream from tumor suppressor genes, abrogates their transcription, resulting in gene silencing and eventual tumor formation. The full spectrum of molecular mechanisms underlying the hypermethylation phenotype are not fully elucidated. However, one important mechanism depends on the transcriptional repressor MAFG, which recruits BACH1, CHD8, and DNMT3B to bind and hypermethylate specific gene promoters, including *MLH1*.[128] Importantly, mutant BRAF up-regulates levels of MAFG to enhance its binding to promoters.

CIMP has multiple definitions. Tumors were initially classified as CIMP-high ($\geq$3) or CIMP-low ($<$2) based on the number of positive methylation markers found at locations of 5 genes (*MINT1*, *MINT2*, *MINT31*, *CDKN2A*, and *hMLH1*).[129] Since then, multiple other panels have been proposed that include different genes (*CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3*, and *SOCS1*).[130] In addition, assessment of CIMP can now be made through genome-wide methylation analyses.[131] This lack of consistency has made it challenging to compare independent studies. Nevertheless, CIMP-high status is identified in approximately 20% of colorectal tumors, and this most often occurs in combination with a *BRAF* mutation and hypermethylation of *MLH1*, features that describe a large fraction of MSI-H tumors.[130,131] Although CIMP can be found in most sporadic MSI-H tumors, it is not restricted to MSI-H tumors. In fact, half of all tumors with CIMP do not have methylation of *MLH1* or MSI.[132,133]

The CIMP can be first observed in the early stages of tumorigenesis. For example, microvesicular hyperplastic polyps can be CIMP-high, and the frequency of the CIMP

increases compared with sessile serrated adenomas, TSAs, and other more advanced lesions.[130,134,135] There is also an association between the CIMP in normal tissue and the occurrence of serrated polyps, indicating a field effect.[136] Meta-analyses found that CIMP status correlated with mucinous or poorly differentiated tumors that contain *BRAF* mutations and MSI, often in the right colon, predominantly in older and female patients.[137,138] However, there does not appear to be a clear and reproducible prognostic role for the CIMP.

Other Pathways

Efforts to genotype large panels of colorectal tumors in an unbiased manner have revealed recurrent mutations in genes not previously associated with CRC pathogenesis. For example, The Cancer Genome Atlas project completed a comprehensive molecular analysis of 276 colorectal tumors, and, in addition to expected mutations in *APC*, *TP53*, and *KRAS*, frequent mutations were identified in *POLE*, *ARID1A*, *SOX9*, and *FAM123B*.[2] The identification of DNA polymerase protein mutations has specifically led to the characterization of a new molecular pathway. POLE, a catalytic subunit of DNA polymerase epsilon, and POLD1, its lagging-strand equivalent for DNA polymerase δ1, each provide proofreading capabilities to their respective enzyme complexes. Mutations in *POLE* generate a hypermutated phenotype with high frequency of single-nucleotide variants in the absence of aneuploidy or MSI (Figure 1).[139] *APC* mutations appear to be the initiating event in this pathway, but the full spectrum of downstream mutations that develop in the context of this hypermutated environment is not yet described. Although these tumors do not have MSI, the hypermutated milieu nevertheless makes these tumors promising candidates for immunotherapy.[140,141]

A new subgroup of colorectal tumors has been proposed; these tumors appear to have stable genomes, without significant levels of aneuploidy or hypermutation. These tumors also are initiated through *APC*, have modest levels of DNA hypermethylation (CIMP-low), and acquire mutations in *KRAS*, *PIK3CA*, *SOX9*, and *PCBP1*.[139] The gene expression pattern of this subset of tumors most closely resembles the pattern of the CMS3 metabolic subtype, consistent with the frequent activation of *KRAS*. *TP53* mutations were less frequently observed, which might account for the low rates of aneuploidy in these tumors. The outcomes of patients with this group of tumors have not been defined. More detailed characterizations of these subtypes are underway.

Implications for Diagnostics

The identification of molecular features of colorectal tumors could lead to new diagnostic tools. Multitarget stool-based tests have been developed to noninvasively screen for CRC and its precursors. These tests detect methylation patterns and genetic mutations in stool samples that are associated with the adenoma–carcinoma sequence.[142] Heralded as the first commercialized CRC-based screening assay, the Cologuard (Exact Sciences Corp, Madison, WI) test detects mutant *KRAS*, aberrantly methylated promoter regions of *BMP3* and *NDRG4,* and fecal hemoglobin through an immunologic assay. Combined results from Cologuard and the fecal immunohistochemical test (FIT) identified patients with CRC with 92% sensitivity, compared with 74% sensitivity for the FIT alone, but with lower specificity (87% for the combined tests vs 95% for the FIT alone).

In 2016, the US Food and Drug Administration approved a second-generation blood assay for a tumor-associated DNA marker for CRC screening. The septin 9 gene (*SEPT9*) encodes an eponymous guanosine triphosphate–binding protein involved in apoptosis, pseudopod protrusion, tumor cell migration, and invasion.[143] *SEPT9* is hypermethylated in CRC tissue compared with normal colon.[144–146] Tests to detect methylated *SEPT9* in plasma samples (Epi proColon 2.0; Epigenomics AG, Berlin, Germany) detect CRC with only 48% sensitivity and 92% specificity.[147] Although the performance characteristics are inferior to those of the FIT, the *SEPT9* testing remains an option for patients unwilling to pursue other CRC screening strategies.

Quantification of circulating tumor cells (CTCs) or circulating cell-free DNA derived from tumor cells (circulating tumor DNA) can be used to monitor for CRC recurrence and response to treatment and is a promising area of investigation. Apart from the *SEPT9* assay, the utility of so-called liquid biopsies has not been firmly established for CRC screening. Rather, the amount of circulating tumor cells or circulating tumor DNA detected in blood might be measured as a marker of disease recurrence after curative treatment.[148] Serial, noninvasive surveillance of patients undergoing treatment for CRC might allow for more timely identification of resistant clonal evolution. There is evidence for the rapid emergence of cancer cells with mutations that mediate resistance to EGFR inhibitors that can be detected in circulating DNA, even before recurrence is detected by radiology.[149–152]

Future Directions

A wide variety of genetic and molecular changes mediate colorectal carcinogenesis. CRC is no longer considered to be a single disease entity. Advancement of care for patients with CRC will depend on continued characterization of the different subtypes of colorectal tumors and development of therapeutic strategies based on these differences. In an effort to evaluate and summarize this progress, in 2017 an expert panel, represented by the American Society for Clinical Oncology, American Society for Clinical Pathology, Association for Molecular Pathology, and College of American Pathologists, offered 21 guideline statements for use in the analysis of colorectal tumors and care of patients.[153]

Identification of the colorectal tumor subtypes leads to important areas for future studies. For example, although the CIMP is well described, we do not know how aberrations in DNA methylation occur or how to reverse or overcome them. As we elucidate the effects of modifiable lifestyle and environmental factors on CRC risk, we will have to learn how features of the diet, obesity, and sedentary behavior

contribute to colorectal tumor development. The association between *Fusobacterium* species and other intestinal microbes with CRC risk will lead to many studies of the effects of the composition of the microbiome on intestinal epithelial cell transformation and tumorigenesis. There have been accomplishments, such as the discoveries that immune checkpoint inhibitors are effective against MSI tumors, that nonsteroidal anti-inflammatory and COX-2 inhibitors reduce risk of CRC, and that EGFR inhibitors are effective against tumors without mutations in *KRAS.* As we learn more about the mechanisms of CRC pathogenesis and the different types of colorectal tumors, new therapeutic and diagnostic approaches will arise.

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Conflicts of interest
The author disclose no conflicts.

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