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Genomic and Epigenetic Instability in Colorectal Cancer Pathogenesis

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Abstract

Colorectal cancer arises as a consequence of the accumulation of genetic alterations (gene mutations, gene amplification, and so on) and epigenetic alterations (aberrant DNA methylation, chromatin modifications, and so on) that transform colonic epithelial cells into colon adenocarcinoma cells. The loss of genomic stability and resulting gene alterations are key molecular pathogenic steps that occur early in tumorigenesis; they permit the acquisition of a sufficient number of alterations in tumor suppressor genes and oncogenes that transform cells and promote tumor progression. Two predominant forms of genomic instability that have been identified in colon cancer are microsatellite instability and chromosome instability. Substantial progress has been made to identify causes of chromosomal instability in colorectal cells and to determine the effects of the different forms of genomic instability on the biological and clinical behavior of colon tumors. In addition to genomic instability, epigenetic instability results in the aberrant methylation of tumor suppressor genes. Determining the causes and roles of genomic and epigenomic instability in colon tumor formation has the potential to yield more effective prevention strategies and therapeutics for patients with colorectal cancer.

Colorectal cancer results from the progressive accumulation of genetic and epigenetic alterations that lead to the transformation of normal colonic epithelium to colon adenocarcinoma, which is called the polyp → cancer progression sequence (Figure 1). The sequential process of gene mutations and epigenetic alterations is widely believed to drive the initiation and progression of benign adenomas to malignant adenocarcinomas because these mutations affect signaling pathways that regulate hallmark behaviors of cancer.^{1,2} These mutations create a clonal growth advantage that leads to the outgrowth of progressively more malignant cells, which ultimately manifests itself as invasive adenocarcinoma.

Importantly, as suggested by Theodor Boveri almost 100 years ago, the acquisition of these mutations is facilitated by the loss of genomic stability, which appears to be a key molecular

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The authors express their regrets at not being able to note the outstanding work of many investigators studying genomic and epigenomic instability in cancer. Space limitations prevented inclusion of these studies in this review.

step in cancer formation.^{3–5} Indeed, the causative role of genomic instability in the pathogenesis of sporadic colon cancer formation is illustrated by the increased risk of colon cancer observed in the Lynch syndrome and mut Y homologue (MYH)-associated polyposis (MAP), which result, respectively, from germline mutations in genes that encode for DNA repair proteins: the mismatch repair (MMR) system and base excision repair (BER) system.^{6–8} Further evidence supporting the idea that genomic instability is central to cancer formation comes from recent data showing that germline mutations in genes that encode for other DNA repair proteins are associated with an increased risk of colon cancer, including *CHEK2*, *BRCA1*, *BRCA2*, and *BLM*, which all are involved in the regulation of double-strand DNA breaks.^{9–13}

It is notable that approximately 30% of the genes in the human genome encode for proteins that regulate DNA fidelity.¹⁴ This implies that not only is the maintenance of the integrity of genomic DNA important in eukaryotic cells but also that there are likely a variety of different mechanisms that can cause loss of genomic DNA stability. However, despite advances in our knowledge of the proteins that regulate DNA fidelity, the mechanism responsible for aneuploidy, a hallmark of chromosomal instability (CIN), in the majority of cancers is not known. This has led some investigators to propose that genomic instability can arise in the absence of genetic mutations and is self-propagating in cancer.⁹ However, in at least a subset of cancers, a specific genetic etiology has been found, which argues that specific, identifiable mechanisms that normally regulate DNA fidelity and are inactivated in cancer mediate the loss of genomic stability in colon cancer. In fact, in 15%–20% of colon cancers, inactivation of the MMR system either through the aberrant CpG island methylation of *MLH1* or point mutations in *MLH1*, *MSH2*, or other members of the MMR family leads to microsatellite instability (MSI). Furthermore, in some CIN colon cancers, mutations in the mitotic checkpoint regulators have been found.^{15,16} Other potential mechanisms that may play a role in genomic instability include telomere shortening/telomerase overexpression, impaired DNA replication checkpoint regulation, *APC* gene mutations, and abnormal centrosome number regulation.⁴ Recently, the ploidy status in yeast was shown to be primarily regulated by proteins that mediate homologous recombination, sister chromatid cohesion, and mitotic spindle function.¹⁷ The mechanisms responsible for causing genomic instability and the specific role that genomic instability appears to play in colon cancer and in driving different molecular pathways of colon cancer formation is discussed in more detail in the following text.

Timing of Genomic Instability in the Adenoma-Carcinoma Sequence

The evolution of normal epithelial cells to adenocarcinoma usually follows a predictable progression of histologic changes and concurrent genetic and epigenetic changes. These alterations provide a growth advantage and lead to clonal expansion of the altered cells. In a manner reminiscent of Darwinian evolution, subsequent alterations with waves of clonal expansion then occur as a consequence of progressive events that provide other growth advantages to the cells such as loss of cell contact inhibition. Predictably, the progressive accumulation of genetic alterations can be identified in more histologically advanced colon neoplasm (Figure 1).^{18,19} An unresolved issue related to genomic instability, which has been the subject of recent reviews, is whether genomic instability commonly initiates the adenoma-carcinoma sequence or whether it arises during this process and facilitates the formation of colon cancer.^{9,20,21}

The earliest identifiable lesion in colon cancer formation is the aberrant crypt focus (ACF). The true neoplastic potential of this lesion is still unclear, but it does appear that some of these lesions harbor mutations in *KRAS* or *APC* and can progress to frank adenocarcinoma. In particular, dysplastic ACF frequently carry mutations in *APC* and appear to have the highest potential for progressing to colon cancer. The increased likelihood of ACF with mutant *APC*

progressing to cancer may not only result from overactivation of the Wntless/Wnt signaling pathway but also from the induction of chromosome instability. APC has multiple protein-binding domains, including an EB1-binding domain in its C-terminal end. EB1 associates with the growing ends of cytoplasmic and spindle microtubules as well as centrosomes, and the yeast homologue of EB1 is required for a microtubule-dependent cytokinesis checkpoint.²² Fodde et al showed in mouse ES cells homozygous for the mutant *Apc*^{MIN} or *Apc*^{1638N} alleles CIN with whole-genome increment changes resulting in polyploidy. This CIN is a consequence of abnormal mitotic spindles that form in these cells because of an abundance of microtubules that inefficiently connect with kinetochores.²³ However, although these provocative findings suggest that truncating mutations in *APC* induce the genomic instability observed in colon adenomas and colon cancers, the significance of these observations is still unclear. The CIN seen in *Apc* mutant cells is polyploidy rather than gains or losses of individual chromosomes, which is what is seen in colon cancer.²⁴ A unifying model for these discrepancies is that APC inactivation may create a permissive state that allows the evolving tumor clone to tolerate the development of true aneuploidy.^{9,21,24}

Regardless of the role of mutant *APC* in the regulation of genomic stability in early colon neoplasms, further support for an active role of genomic instability in initiation of colon cancer comes from the observation that both CIN and MSI can be observed in adenomas.^{25–27} Stoler et al have demonstrated the presence of approximately 11,000 genomic alterations in colon adenomas using inter-(simple sequence repeat) polymerase chain reaction.²⁸ Thus, the timing of loss of genomic stability, either CIN or MSI, appears to be during initiation of adenoma but before progression to frank malignancy. Based on the observation of loss of heterozygosity events in adenomas and using mathematical modeling methods, it can be shown that genomic instability occurs very early in the colon tumorigenesis process and may even precede *APC* mutations.^{29–31} In addition to mathematical modeling showing that it is possible, if not likely, that genomic instability initiates tumor formation in the colon, Bartkova et al provided experimental evidence that suppression of DNA damage and subsequent genomic instability is one of the earliest antitumor mechanisms present in tissues. They have shown that an early event in the formation of several cancers, including bladder, lung, breast, and colon, is the activation of mechanisms involved with DNA damage regulation. In colon adenomas, phosphorylated Chk2 (pT68) can be detected, indicating the activation of double-strand DNA break repair in the tumors.³² Phosphorylated Chk2 is the activated state of Chk2, an effector kinase in the DNA damage network that is activated by ATM (ataxia telangiectasia mutated) when DNA double-strand damage occurs. In addition to phosphorylated Chk2, phosphorylated ATM (Ser1981) and γ -H2AX (Ser 139) can also be detected in colon adenomas, providing further evidence of activation of mechanisms involved in DNA damage regulation and the maintenance of genomic stability. These data suggest that early events in the tumorigenesis process activate mechanisms to maintain DNA stability via the ATR (ataxia telangiectasia mutated and Rad3 related)-ATM checkpoint and that deregulation of this DNA damage response is one of the first events that permit tumor progression.³²

MSI

The cause for MSI (sometimes referred to as MIN) was discovered with the observation that its presence is associated with loss of function of the DNA MMR system. The correlation between MSI and inactivation of the MMR system was initially recognized and studied in bacteria and yeast.^{6,33} With the recognition that MSI occurs in 15%–20% of sporadic human colorectal tumors and in >95% of colon cancers arising in patients with Lynch syndrome, a cancer family syndrome causing an increased risk of colon and extracolonic cancers, it was quickly shown that inactivation of members of the human DNA MMR gene family was the cause of microsatellite unstable colorectal cancer. In contrast, for most colorectal cancers, the

mechanism(s) responsible for causing CIN and CpG island methylator phenotype (CIMP) remain to be identified.

Thus, MSI is a marker for loss of DNA MMR activity. In the analysis of tumor DNA, it has become clear that in some tumors, genetic sequences with short repetitive nucleotide sequences such as A_n or CA_n (termed microsatellites, where n is the number of repeats) can become shorter or longer in length when compared with nontumor DNA from the same individual. A microsatellite may lengthen in a daughter cell if there is nucleotide-pairing slippage (looping) along the newly synthesized strand during DNA synthesis or may shorten if the template strand microsatellite has slippage during DNA replication. This alteration in microsatellite length in genomic DNA defines MSI. In the laboratory, MSI is identified by the electrophoretic resolution of amplified microsatellite DNA sequences and the determination of >30% of markers examined having length changes compared with normal DNA from the same individual.³³ Although the DNA MMR system recognizes and directs repair of single nucleotide mispairs (see the following text), this aspect is not usually tested when examining tumors for MSI. Multiple point mutations as well as MSI are observed in these tumors, providing the basis for the term “hypermutable” phenotype, and this increase in the spontaneous mutation rate appears to be the mechanism for the apparent rapid neoplastic progression in Lynch syndrome and sporadic tumors that exhibit MSI^{34,35} (see Figure 1 for Lynch syndrome). If the mismatch occurs in the coding region for a particular gene, the newly introduced point mutation may affect the expression and/or function of that particular gene.^{36,37} The process of DNA mutations is initially random in MSI cancers affecting any susceptible microsatellite repeat; however, ultimately clones that acquire mutations in key regulatory genes gain a growth advantage that promotes the formation of the carcinoma (see the following text).

The DNA MMR System

The human DNA MMR system is an evolutionarily conserved system that involves a number of proteins that recognize and direct repair of nucleotide mismatches and is responsible for maintaining replicative fidelity of DNA.^{38,39} The DNA sequences repaired by the MMR system are residual errors that have apparently escaped the normal editing function of DNA polymerase. Base mispairs, if not corrected by the MMR system, may cause nucleotide transitions or transversions, allowing a novel base to alter the authentic genetic sequence.³⁸ Such point mutations in genes that regulate cell growth can accumulate in cells with defective MMR and may promote neoplastic growth. The MMR system replaces the mispair on the newly synthesized daughter strand because these proteins can recognize the methylation pattern (in bacteria) and the nicks present (in higher organisms) in the newly synthesized strand.^{40,41} The proteins that comprise the MMR system bind to nucleotide base-base mismatches of double-stranded DNA or loops of inaccurately replicated repetitive sequences (microsatellites) and target the DNA area for excision, resynthesis, and ligation.⁴² Most knowledge of the biochemical function and recognition fidelity of the DNA MMR system comes from studies in *Escherichia coli* and *Saccharomyces cerevisiae*. In fact, the names of the human proteins are derived from bacteria and yeast. Mutated S and mutated L proteins are found in bacteria that display MSI; in yeast, the proteins have been named mutS and mutL, and in humans, the proteins are named hMutL and hMutS.

Defects in the MMR genes *hMSH2* and *hMSH6* (human Mut S homologues), *hMLH1* (human Mut L homologue), and *hPMS2* (human postmitotic segregation, a Mut L homologue) in humans have been linked to Lynch syndrome (formerly known as hereditary nonpolyposis colorectal cancer), an autosomal dominant condition in which one of the DNA MMR genes is mutated in germline cells^{43–50} (Table 1). No germline mutation in *hMSH3* has been identified in families with this syndrome, perhaps due to redundancy of function of the hMutS homologues.⁵¹ In contrast to the germline inactivation of the MMR genes seen in Lynch

syndrome, the 15%–20% of sporadic colorectal tumors with MSI inactivate the MMR system by a somatic epigenetic mechanism: the aberrant methylation of the 5' untranslated region of the *hMLH1* gene.^{52–54} The hypermethylation silences gene transcription of *hMLH1*, thus inactivating DNA MMR and allowing the phenotype of MSI.

One wild-type allele of an MMR gene is generally sufficient to maintain normal MMR function. For colon cancer to develop in patients with Lynch syndrome, a second somatic event (in addition to the vertically transmitted mutant allele) must occur in the wild-type allele of a colonocyte. This makes both copies of an MMR gene completely inactive⁵⁵ and causes the hypermutable phenotype seen in tumors in patients with Lynch syndrome. Similarly, both alleles in sporadic colorectal tumor with MSI are inactivated by biallelic hypermethylation of *hMLH1*.^{52–54} When a physical deformation remains in the newly replicated DNA double helix (caused by the mispairing of nucleotides or due to slippage and looping at microsatellite loci), a complex termed hMutS α (a heterodimer of hMSH2 and hMSH6 proteins) or hMutS β (a heterodimer of hMSH2 and hMSH3) identifies the error and binds the DNA at this site.^{56–59} In particular, based on studies of the bacterial and yeast MMR proteins, hMutS α appears to recognize and bind single-base pair mismatches and loops of DNA containing <2 looped nucleotides, while hMutS β recognizes loops of DNA containing >2 or more nucleotides^{60,61} and may repair DNA loops up to 16 nucleotides in length,⁶² with larger loops repaired by a non-MMR mechanism.⁶³ Subsequently, hMutS α or hMutS β recruit the hMutL α complex, a heterodimer of hMLH1 and hPMS2 proteins, which targets the newly synthesized, daughter DNA strand for “long patch” excision repair (Figure 2). In vitro nicks in the DNA direct the strand specificity of repair, which can proceed in either the 5' $\rightarrow$ 3' or 3' $\rightarrow$ 5' direction, depending on the location of the nick relative to the mispair.^{40,41,62,64} The MMR proteins hydrolyze adenosine triphosphate (ATP) during the recognition and repair process.⁶⁴ The hydrolysis of ATP is believed to act as a “molecular switch” in that the MMR proteins are turned “on” when in the adenosine diphosphate (ADP)-bound form and bound to the DNA double strand and “off” when in the ATP-bound form as a mode to dissociate from the strand.⁶⁵ Initially based on biochemical studies and later corroborated by the crystallographic structure of the bacterial “S” MMR protein, Gradia et al proposed a “sliding” clamp model in which hMutS α slides along the DNA double strand for finite distances until it exchanges ADP for ATP or encounters a mismatched nucleotide.⁶⁴ Loss of any of the components of the MMR system will inactivate or attenuate repair, and the heterodimers help stabilize the individual partnered proteins. For instance, when hMSH2 is missing, hMSH6 and hMSH3 become unstable as individual proteins.⁵¹ When either hMSH6 or hMSH3 is absent, the other protein increases its expression for interaction with hMSH2.⁵¹ It remains unclear how hPMS1 (binds to hMLH1 to form hMutL β) or hMLH3 (binds to hMLH1 to form hMutL γ) interacts within the entire complex to effect repair, although hMutL γ may play a repair role during meiosis.^{66,67}

DNA MMR requires the presence of a number of proteins that aid in its function. In *E coli*, in addition to the MMR proteins MutS and MutL, repair involves MutH (aids in strand discrimination), UvrD (a DNA helicase), ExoI, ExoVII, ExoX or RecJ (involved in DNA excision), Pol III holoenzyme (for DNA resynthesis), SSB (single-stranded DNA binding and protection), and DNA ligase (for nick ligation).⁶⁶ Similarly, the human DNA MMR system has a number of components in addition to the core MMR proteins that effect repair. These proteins have been primarily identified by homology to *E coli*, although there is no currently identified human homologue for MutH or UvrD. Exonuclease 1, a 5' $\rightarrow$ 3' exonuclease, is involved in both 5' and 3' directed MMR and excises mismatched DNA.^{68–70} Human pol δ directs DNA resynthesis, and proliferating cellular nuclear antigen interacts with hMSH2 and hMLH1 (as well as hMSH6 and hMSH3) to help localize hMutS α and hMutS β to newly replicated DNA for initiation of MMR and DNA resynthesis.^{71–76} hMutL α possesses a proliferating cellular nuclear antigen/replication factor C-dependent endonuclease activity that

plays a key role in 3' nick-directed MMR that involves exonuclease 1.⁷⁷ The single-strand DNA binding replication protein A (RPA) is involved in all stages of MMR. RPA binds to nicked heteroduplex DNA before hMutS α and hMutS β , stimulates mismatched-provoked excision, protects single-strand DNA gapped regions generated during excision, and facilitates DNA resynthesis.⁷⁸ RPA also becomes phosphorylated after pol δ becomes active to close DNA gaps, which reduces the affinity of RPA for DNA.⁷⁹ The high mobility group box 1 protein can interact with hMSH2 and hMSH6, binds to mismatches, and has DNA unwinding activity to provoke mismatched excision.⁸⁰ Human DNA ligase 1 directs nicked DNA ligation.⁷³ Overall, the MMR process is highly conserved from *E coli* to humans to include the components of MMR, substrate specificity, nick-directed strand specificity, and the bidirectionality for repair.

Tumorigenesis With Loss of DNA MMR

It is believed that certain key tumor suppressors drive the pathogenesis of MSI tumors and that these genes include a unique complement of genes that are commonly mutated in MSI tumors but not in CIN tumors. Rarely, microsatellite sequences are present in the coding region (exons) of critical growth regulatory genes, and because of the tumor-promoting effects of their mutational inactivation, these genes are the ones selected for when mutation in MSI tumors. Mutation of the coding microsatellite occurs with defective MMR, resulting in length changes in the microsatellite within the gene producing a frameshift mutation that renders the protein inactive. An analysis of repetitive sequences in tumor tissue led to the identification of inactivated genes in these tumors. For example, a repeat of 10 adenines in the *TGFBR2* gene undergoes frameshift mutation in ~85% of colorectal tumors with MSI, inactivating this receptor^{36,81} and causing the tumor cells to escape the growth-suppressive effects of the ligand transforming growth factor (TGF)- β 1. It is interesting to note that 15% of tumors with MSI carry monoallelic frameshift mutations of *IGF2R* at its polyguanine sequence, which may uncouple TGF- β 1 growth suppression because the ligand cannot be activated by cleavage from its latent peptide.⁸² Another targeted gene is *BAX*, a member of the *BCL2* gene family. *BAX* heterodimerizes with *BCL2* within the cell, and the relative amounts of the heterodimer determine the commitment of the cell to programmed cell death. *BAX* contains a polyguanine (G)₈ tract that shows monoallelic frameshift mutations in 50% of colorectal cancers with MSI.^{83,84} Mutation of *BAX* (by diminishing the ratio of *BAX* to *BCL2*) prevents programmed cell death, helping to immortalize cells.^{83,84} Mutation of *TGFBR2* and *BAX* appear to be late in the adenoma to carcinoma progression because these mutations occur in high-grade dysplasia at the interface of defining malignancy.^{26,85} Additionally, mutations in *TGFBR2* from MSI tumors of patients with stage III colon cancer are associated with improved survival.⁸⁶ Other genetic targets identified include the MMR genes *hMSH3* and *hMSH6*, which may broaden and accelerate the accumulation of mutations⁸⁷ (Figure 3 and Table 2). A comprehensive search using a genetic database for coding microsatellites and subsequent colorectal tumor analysis revealed 9 genes that were mutated in more than 20% of the tumors.⁸⁸ In addition to *TGFBR2*, *BAX*, and *hMSH3*, these include *ACVR2* (the gene for the activin receptor type 2), *SEC63* (human homologue of a yeast DnaJ-like endoplasmic reticulum translocon component protein gene), *AIM2* (an interferon-inducible gene), reduced nicotinamide adenine dinucleotide-ubiquinone oxidoreductase B14.5B subunit gene, *KIAA0977* (probable human homologue of the mouse embryonal protein *cordon-bleu*), and *PA2G4/EBP1* (homologue of the mouse cell cycle protein p38-2G4).⁸⁸ Subsequently, *ACVR2* has been shown to be mutated at an exon 10 polyadenine tract in the majority of microsatellite unstable colon cancers with loss of *ACVR2* protein,³⁷ and the loss of *ACVR2* expression correlates with an increase in local tumor growth.⁸⁹ Further, reconstitution of mutated *ACVR2* leads to restitution of growth inhibitory signaling in colon cancer cells.⁹⁰ The role of the other frequently mutated genes in the pathogenesis of tumors with MSI is not currently known.

The *BRAF* gene encoding a downstream component from KRAS in the RAS/RAF/MAPK pathway is also often mutated in sporadic MSI tumors,⁹¹ although the mechanism is unknown because *BRAF* does not contain a coding microsatellite sequence. Mutations in *BRAF* are usually V600E missense mutations, which activate the protein to become an incessant signaling molecule.⁹¹

A number of clinicopathologic features of MSI colorectal tumors segregate it from tumors with CIN (although many of these features match CIMP tumors due to the overlap with hypermethylated *hMLH1*). These include correlation with the tumor's location in the proximal (right) colon and an inverse relation to allelic loss.^{34,92,93} In addition, MSI colorectal tumors tend to be diploid, tend to possess a mucinous histology, have a better overall survival compared with microsatellite stable tumors,⁹⁴ and have a lymphocytic reaction surrounding the tumor. The mechanisms responsible for the immune cell histologic differences are not fully understood but may involve truncated immunogenic proteins resulting from frameshift mutations.⁹⁵ In comparison, CIN colorectal tumors are aneuploid and have an overall worse prognosis compared with MSI tumors.^{96,97}

The Relationship Between Chemotherapy Responsiveness and DNA MMR Activity

In addition to its role in recognizing and directing repair of polymerase mistakes within DNA, the human MMR system is also capable of recognizing certain DNA adducts caused by exogenous alkylation damage.^{98–100} O⁶-methylguanine adducts are recognized by the MMR system and result in a G₂/M cell cycle arrest and cell death.⁹⁸ Similarly, when 6-thioguanine is incorporated into the DNA of MMR-proficient cells, a G₂/M cell cycle arrest is induced.⁹⁹ Both O⁶-methylguanine and 6-thioguanine are believed to be recognized by the MMR system because of mispairing with thymidine (or cytosine) with the altered nucleotide on the newly synthesized DNA strand. It is unclear how the selectivity of recognition of adducts occurs, although some of the selectivity may be mediated through effects on interstrand hydrogen bonding. For instance, 8-azoguanine does not interfere with classic Watson–Crick hydrogen bonding, which is in contrast to 6-thioguanine and O⁶-methylguanine, and thus it may escape recognition because of this. Furthermore, the MMR proteins can recognize specific intrastrand and interstrand cross-links induced by cisplatin or carboplatin and not those by its analogues. Because both cisplatin and its substituted amine analogues would be expected to cause distortion of double-stranded DNA, it has been suggested that lack of recognition of the cisplatin analogues by the MMR system might be due to steric hindrance of binding by the hMSH2 protein to the altered DNA.^{100,101}

Notably, 5-fluorouracil (5-FU), the central agent used for the medical treatment of patients with advanced colorectal cancer, is recognized by the MMR system despite its inability to deform DNA.^{102,103} Because of the ability of 5-FU to inhibit thymidylate synthase, the enzyme required for thymidine triphosphate synthesis, DNA is subsequently synthesized with uridine triphosphate or 5-fluorodeoxyuridine triphosphate when thymidine triphosphate is depleted, allowing incorporation of the fluoropyrimidine into DNA.¹⁰⁴ 5-FU will selectively kill cells with intact MMR compared with cells with deficient MMR,¹⁰² and MMR-deficient cells resulting from hypermethylated *hMLH1* that are 5-FU resistant become sensitive to 5-FU when *hMLH1* is demethylated in vitro.¹⁰⁵ Biochemical experiments have confirmed that human MMR proteins, particularly hMutS α , bind to 5-FU incorporated into DNA.^{103,106} Some studies of patient survival have shown a similar effect of 5-FU when separated by MSI status. Patients with stage II and III sporadic colorectal cancer with MSI tumors do not show a survival benefit with 5-FU therapy as compared with patients with MMR-proficient tumors in retrospective and prospective studies.^{107–109} Similarly, stage III patients with Lynch syndrome do not demonstrate a 5-year survival benefit with 5-FU treatment over untreated patients.¹¹⁰ These

observations raise the possibility that MSI may be useful as a predictive marker for 5-FU treatment as well as a prognostic marker for the natural history of this molecular class of tumors.

It is not known how MMR signals the demise of the cancer cell after recognition of 5-FU incorporated into DNA. Data from O⁶-methylguanine–induced MMR recognition indicate p53 becomes phosphorylated at its serine residues 15 and 392,¹¹¹ and this modulation may be one mechanism for p53 activation. The observed G₂/M cell cycle arrest involves the ATM and Rad3-related (ATR) kinase, as demonstrated by the ablation of ATR (or inhibition of the ATR-activated checkpoint kinase CHK1) attenuating the cell cycle arrest.¹¹² ATR can phosphorylate serine 15 of p53 after gamma irradiation or UV exposure.¹¹³ ATR-induced CHK1 kinase (or the ATM-induced CHK2 kinase) can phosphorylate cdc25A, a cell cycle phosphatase that is degraded upon its phosphorylation. Cdc25A controls the activation of CDK1 and CDK2 kinases that regulate the G₁, intra-S, and G₂ phases of the cell cycle.¹¹⁴ The G₂ checkpoint activation was accompanied by the formation of nuclear foci containing the repair protein ATR as well as RPA.¹¹² Taken together, ATR activation after O⁶-methylguanine formation is dependent on the presence of DNA MMR and posttranslationally modifies by phosphorylation the downstream targets CHK1 (and perhaps p53) to effect the proteasome-assisted destruction of cdc25A to cause cell cycle arrest. Apoptotic signaling by MMR recognition of O⁶-methylguanine lesions appears to be mediated by mitochondrial signaling and eventual caspase activation.¹¹⁵ The activation of cell death after the recognition of O⁶-methylguanine (1) is dependent on the presence of hMutS α , (2) can temporarily be blocked by overexpression of bcl-2 and bcl-X_L, negative regulators of mitochondrial-regulated apoptosis, and (3) is not dependent on death receptor signaling.¹¹⁵ Neither the cell cycle arrest nor the cell death events demonstrate how DNA MMR responds to the O⁶-methylguanine lesion but highlight potential downstream effectors to prevent replication of damaged DNA.

Overall, the MMR system and other repair mechanisms such as BER have been implicated in mediating the response of cancer cells to chemotherapy. This has raised the possibility that the assessment of expression or activity of these proteins in colorectal cancer might be useful as a predictive marker for the response of patients with colorectal cancer to specific therapies and as a guide to the selection of optimal therapy.

CIN

Overview of CIN and Colon Cancer

CIN is the most common type of genomic instability observed in colon cancer and occurs in 80%–85% of colorectal tumors. Several forms of CIN can be observed in cancer in general. Recently proposed categories of genomic instability include the following: (1) subtle sequence changes, including base substitutions, deletions, or insertions as well as MSI; (2) alterations in chromosome number (aneuploidy, also termed CIN), (3) chromosome rearrangements, and (4) gene amplification.^{3,116} The genome of colon cancers, like many solid tumors, is often marked by a complex pattern of chromosome translocations that appears nearly random and is mediated at least in part by inactivation of proteins involved in homologous recombination–mediated events (eg, sister chromatid exchange and homologous recombination between nonallelic repetitive DNA sequences) and in nonhomologous end-joining events.^{116,117} This form of genomic instability, “translocation instability,” is likely mediated by proteins involved in double-strand DNA break repair, single-strand DNA break repair, and DNA replication, as well as by mechanisms that cause aneuploidy, which include mitotic spindle checkpoint regulators as well as proteins that regulate mitotic chromosome transmission.¹¹⁶ However, the role of chromosome rearrangements in solid tumors, like colon cancer, is still being defined.^{3,117,118} Finally, gene amplification occurs infrequently in colon cancer but does appear to play a role in driving stage transitions in at least some colon cancers.^{119,120}

Despite the high frequency of CIN in colon cancer and the fact that aneuploidy is recognized as a hallmark of cancer, our understanding of the specific molecular events that result in this form of genomic instability is remarkably incomplete. Although there are clear examples of specific alterations of proteins that maintain DNA fidelity, causing CIN and predisposing to tumor formation, it is still debatable as to whether aneuploidy is merely a tolerated state rather than a fundamental pathogenic process of tumorigenesis. The identification of specific patterns of chromosome gains and losses that occur during the colon adenoma to carcinoma sequence and the demonstration that CIN is an early event in tumor formation that increases with tumor progression is consistent with the idea that CIN is a pathogenetic phenomenon in colon cancer (Figure 4).^{28,121,122} Furthermore, studies of mice heterozygous for the centromere-linked, kinesis-like motor protein CENP-E show that the cells from these mice missegregate chromosomes during mitosis due to a weakened mitotic checkpoint and develop lymphomas and lung adenomas at increased rates. Importantly, these mice have an intact DNA damage response, which implicates the occurrence of aneuploidy itself as the main factor leading to the predisposition to neoplasms.¹²³

In addition and importantly for our understanding of CIN in colon cancer, Lengauer et al have shown that aneuploidy is indeed the result of an abnormally high rate of CIN that persists throughout the life of the tumor.³ Using cell fusion and chromosome transfer techniques, Lengauer et al also proved that the aneuploid state does not cause CIN and that CIN is an autosomal dominant trait, suggesting it can arise from gain-of-function mutations. Despite this insight into CIN, the mechanism(s) responsible for CIN has been identified in only a subset of colon cancers. *TP53* mutations were initially suspected to be the leading cause of genomic instability in colon cancer, but it appears more likely that only specific *TP53* mutations cause CIN and the remainder of the mutant forms of *TP53* likely affect other processes such as apoptosis and create a permissive state that allows CIN to occur but does not actually cause the CIN.¹²⁴

Mechanisms for CIN

In light of the appreciation of CIN as being a hallmark feature of the majority of colon cancers and of the likely contributory role of genomic instability in colon carcinogenesis, there has been intense interest in identifying the mechanisms responsible for the CIN. In an analogous approach to that used to determine the basis for MSI through the study of unicellular organisms that are MMR deficient, the mechanisms responsible for CIN are being first unraveled through the study of yeast that show CIN in order to identify proteins that regulate DNA fidelity during DNA replication and in response to genotoxic stress. These studies have proven to be extremely challenging because of the vast number of genes that regulate the fidelity of chromosomes. In fact, it is already appreciated that the list of candidate genes numbers more than 100 and includes genes used in kinetochore structure and function, centrosome and microtubule formation and behavior, chromosome condensation, sister chromatid cohesion, and cell cycle checkpoint control.²⁴ Aneuploid cancers tend to show cytologic abnormalities during mitosis, including abnormal centrosome numbers, multipolar spindles, and lagging chromosomes, implicating deregulation of the processes that mediate the mitotic spindle checkpoint as a cause of CIN.^{24,125–127} In addition to inactivation of proteins that regulate the mitotic spindle checkpoint, inactivation of proteins that regulate DNA damage checkpoints, chromosome metabolism, and centrosome function have been directly shown in *in vitro* systems to influence chromosomal stability. Consequently, proteins involved in the regulation of the mitotic spindle checkpoints and DNA replication checkpoints have been the most closely scrutinized as potential causes of CIN in cancer (Figure 5).

In light of the common occurrence of mutations in *APC*, *KRAS*, *PIK3CA*, *SMAD4*, *TP53*, and other CIN genes, assessment of the role of mutations in these genes as contributing factors to

CIN in colorectal cancers has been performed.^{128,129} To date, there are no convincing data that mutations in any of these genes provide more than a permissive role for CIN, although there is a tight association between mutant *APC* and CIN mutant *TP53* and CIN.^{24,130–132} Interestingly, although *TP53* mutations have been implicated as the cause of some forms of genomic instability, no simple correlation between p53 mutation and the CIN phenotype was shown by Lengauer et al or by others.^{122,130} It remains to be determined if any of the other genes recently found to be mutated in colorectal cancer may play a more causal role in CIN.¹²⁸

An important implication of gaining an improved understanding of the mechanisms responsible for mediating different forms of CIN is that there appears to be mechanism-specific susceptibilities to different classes of chemotherapy. Thus, identifying the mechanisms responsible for CIN in specific colorectal cancers is predicted to lead to a molecular typing strategy in which determination of the mechanism altered in the cancer will ultimately lead to the selection of chemotherapeutic agents that will be optimal for an individual's specific tumor (ie, "personalized medicine"). The majority of the discussion of CIN in colorectal cancer that follows will focus on the possible mechanisms responsible for CIN in colorectal cancer.

BER defects and CIN—Inactivation of a second "DNA caretaker" mechanism, the BER system, is found in a subset of colorectal cancer cell lines and appears to lead to a predisposition to point mutations that contribute to colon cancer formation by causing genomic instability at the level of the base pair. Inactivation of one of the BER genes, *MYH*, is a cause of an autosomal recessive form of adenomatous polyposis, called the MAP syndrome.¹³³ Germline mutations in *MYH*, which encodes for a protein involved in BER, is the cause of adenomatous polyposis in up to 5%–10% of individuals who have an adenomatous polyposis syndrome. *MYH* germline mutations were discovered as a cause of adenomatous polyposis when investigators identified an excessive number of somatic G:C → A:T mutations in neoplasms of people with adenomatous polyposis but who had no detectable germline mutations in *APC*.^{8,134,135} This type of mutation is commonly a consequence of oxidative damage to DNA that results in 8-oxo-7,8-dihydro-2'-deoxyguanosine, which is one of the most stable deleterious products of oxidative DNA damage.^{133,136} The BER system is responsible for repairing this form of DNA damage, which led these investigators to assess candidate genes involved in this process: *OGG1*, *MTHF1*, and *MYH*. This assessment revealed biallelic germline *MYH* mutations in a subset of people with adenomatous polyposis but who did not have germline mutations in *APC*. The most common mutations are Tyr165Cys and Gly382Asp, which account for 82% of the mutant alleles detected to date.¹³⁵ Despite the role of germline mutations in *MYH* as a cause of a colorectal cancer family syndrome, somatic *MYH* mutations are not common in sporadic colorectal cancer. A study of 1042 unselected patients with colorectal cancer in Finland revealed no somatic *MYH* mutations.^{133,137} Of interest, the tumors arising in the setting of biallelic *MYH* germline mutations do not show differences in the frequency of *TP53*, *SMAD4*, or *TGFBR2* mutations but do appear to show CIN, suggesting they have a unique molecular pathogenesis compared with sporadic colorectal cancer.^{138,139} The role of *MYH* in causing CIN is not clear, but an analysis of adenomas arising in individuals with MAP compared with people with familial adenomatous polyposis (FAP) with array comparative genomic hybridization revealed chromosomal gains and losses in MAP and FAP adenomas. Cardoso et al found in 80% and 60% of MAP and FAP adenomas, respectively, that there were aneuploid changes, with frequent losses at chromosome 1p, 17, 19, and 22 and gains affecting chromosomes 7 and 13 in both groups of tumors.¹³⁸ The discovery of *MYH* germline mutations in people with a hereditary colorectal cancer syndrome provides more evidence for the importance of genomic instability in cancer formation.

Mitotic spindle checkpoint genes and CIN—The proteins that regulate spindle-kinetochore interactions during mitosis are the best demonstrated to date to have a role in CIN

in human cancers. Studies in model organisms have shown that the proteins encoded by the mitotic spindle checkpoint genes that control sister chromatid separation at the metaphase-anaphase transition can cause CIN. These genes are highly conserved, suggesting that they are a plausible functional class of genes that will be shown to be a common cause of CIN in human cancers.^{15,139} The *mad* and *bub* genes, in particular, are highly conserved key components that regulate the mitotic spindle checkpoint, and these genes have already been shown to be deregulated in a subset of colon cancers. The *MAD* and *BUB* gene products appear to operate as checkpoint sensors and signal transducers.¹⁵ Activation of *MAD* and *BUB* causes cell cycle arrest through the inhibition of the anaphase promoting complex/C (APC/C), a large ubiquitin-protein ligase. The APC/C normally becomes active at the metaphase-anaphase transition, resulting in the degradation of securin, a protein that inhibits anaphase. Securin interacts with separins, a class of caspase-related proteases that regulate cohesins, which create physical links between sister chromatids. *MAD2* directly binds to the APC/C and blocks the APC/C-dependent ubiquitin-mediated degradation of securin. There is also a second *MAD2*-independent mechanism that involves *BUBR1* and *BUB3*.¹⁴⁰ In addition to *MAD* and *BUB*, there are additional, incompletely characterized factors involved in the regulation of the APC/C. For instance, studies to date indicate that the securin-separin complex is regulated by an autofeedback loop that prevents premature degradation of securin and that other proteins such as polo kinases (PLK) mediate the activity of this complex.¹⁴¹ All of these mechanisms serve to delay the progression to anaphase to allow enough time for the appropriate alignment of chromosomes on the metaphase plate so that the chromosomes appropriately sort during cell division. Thus, all of these mechanisms are candidates for mediating CIN in colorectal cancer.

Data showing mitotic checkpoint gene deregulation could cause CIN in colon cancer came from a landmark study by Cahill et al in 1998. These investigators found mutations in 2 members of this class of genes after recognizing that aneuploid colon cancer cell lines behave in a pattern consistent with having a spindle checkpoint defect.¹⁵ Mutations in 2 genes that control the human mitotic checkpoint, *BUB1* and *BUBR1*, were found in a subset of CIN colon cancer cell lines. The mutant *BUB1* functions in a dominant negative fashion, as would be predicted from previously published studies involving cell fusion experiments with CIN cancer cell lines.¹²² Mutations in other human mitotic checkpoint genes, *MAD2* and *MAD1*, have been identified in breast cancer and leukemia, respectively, suggesting mutations in other genes that control chromosomal stability remain to be found in colorectal cancers.^{142,143} Furthermore, *PTTG*, the “pituitary tumor-transforming gene” and the human homologue of securin, is overexpressed in a variety of tumor types, including colon cancer.^{144–146} Importantly, *MAD2*, *BUB1*, and *BUBR1* mutations generate the types of chromosome loss seen in naturally occurring human cancers, which has not been the case in studies of other candidate CIN-regulating genes such as components of the anaphase-promoting complex.^{15,147} Other genes involved in kinetochore function that have been found to be mutated in colon cancer include *CDC4*, *hROD*, *hZW10*, *hZWILCH*, and *DING*.^{125,148} Furthermore, some proteins that regulate kinetochore function have been found to be overexpressed in colon cancer, including CENP-A, CENP-H, HEC1, Aurora-B (AIM1), and INCENP.^{125,149–152} Of note, despite the initial success of this candidate approach, mutation analysis of other mitotic spindle checkpoint genes, *MAD1L1*, *MAD2*, *MAD2B*, *BUB3*, *MPS1L1*, and *CDC20*, has not revealed any mutations in a panel of 19 aneuploid colorectal cancer cell lines.¹⁵

Centrosome regulation and CIN—In addition to the deregulation of proteins that directly govern the mitotic spindle checkpoint being a candidate mechanism for CIN, abnormal centrosome number and function have been proposed as a candidate mechanism for aneuploidy since Boveri first observed centrosomes more than 100 years ago.¹⁵³ Most solid cancers are characterized by centrosome amplification as well as by aneuploidy, and there is a strong association between abnormal centrosome number and aneuploidy in cancers.^{154–156} Because the centrosome plays a central role in chromosome segregation during mitosis, the centrosome

amplification observed in cancers is postulated to increase the frequency of abnormal mitoses and chromosome missegregation.^{157–160} More concrete evidence of abnormal centrosome function causing CIN in colon cancer became available as the result of assessment of the genes in a locus on chromosome 20q13, which is frequently amplified in a variety of cancers including breast, colon, and pancreatic cancer.¹⁶¹ Sen et al cloned *STK15/BTAK/ARK1/aurora 2*, a centrosome-associated serine-threonine kinase, from this region and detected overexpression in breast cancer cell lines.¹⁶² Subsequent studies revealed *STK15* amplification and overexpression in colon cancer cell lines as well and demonstrated that overexpression of this gene can induce aneuploidy in NIH3T3 cells.^{161,163} Studies in *Drosophila* and *Saccharomyces cerevisiae* indicate that *STK15* has a role in centrosome maturation, spindle assembly, and chromosome segregation and that its over-expression may lead to abnormal mitotic spindle assembly and CIN.^{153,164,165} Furthermore, studies in prostate and breast cancer have shown an association between aneuploidy, abnormal centrosome number, and clinically aggressive disease.^{158,159} Thus, *STK15* amplification appears to be a promising potential cause of CIN. In addition to *STK15* playing a role in abnormal centrosome function in colon cancer, 2 other cell cycle-regulated proteins, polo-like kinase 2 and 4 (PLK2 and PLK4), are overexpressed in some cases of colon cancer and have been implicated in aneuploidy and abnormal centrosome function.¹⁶⁶ Beyond these proteins that directly interact with centrosomes, many proteins that directly or indirectly regulate the number and/or function of centromeres are abnormal in colon cancer. Some proteins that are involved with regulating centrosome number, including nucleophosmin, MPS1, and mortalin, are targets of transcriptional or posttranslational modification by cdk2/cyclin E and are overexpressed in cancer.^{157,167,168} It is also predicted that overactivation of CDK4/CDK6 and cyclin D contributes to tumorigenesis by favoring the generation of amplified centrosomes.¹⁶⁹ The results of these studies create a strong argument that the abnormal regulation of centrosomes is a cause of CIN in colon cancer, but further studies are needed to develop a better understanding of the role of centrosome amplification and centrosome-associated kinase activity in CIN in primary human colon cancer.

DNA checkpoint genes and CIN—Genomic stability is maintained not only by checkpoints that regulate centrosome function and number, mitosis, and sister chromatid separation but also by checkpoints that regulate the maintenance of DNA fidelity in response to genotoxic stress. In fact, cell cycle checkpoints were originally recognized as pathways that mediate cell cycle progression in response to DNA damage. Mutations that disrupt replication checkpoint genes, such as *rfc5-1*, *dpb11-1*, *mec1Δ*, *ddc2Δ*, and *dun1Δ*, substantially increase the rate of genome rearrangements.¹⁷⁰ These DNA damage checkpoints are traditionally considered in 2 classes: (1) the DNA damage checkpoints that recognize and respond to DNA damage from genotoxic agents and (2) the DNA replication checkpoints that monitor the fidelity of replicated DNA.¹⁷¹ The proteins involved in the DNA checkpoints act in one or more of the following roles: (1) sensors of DNA damage, (2) transducers of the DNA damage signal to the effector proteins, or (3) effectors that regulate cell cycle arrest/progression and DNA repair. The proteins are also commonly characterized based on the type of DNA damage they most commonly repair.¹⁷² A number of these DNA repair proteins have been identified in yeast, and some have already been shown to have a role in human cancer, including *TP53*, *ATM*, the ATM-related gene *ATR*, *BRCA1* and *BRCA2*, which interact with the human Rad51 homologue, and *hRAD17*^{173–177} (Table 3). In yeast, *Mec1* and *Tel1*, which are homologous to *ATM* and *ATR*, appear to operate as the transducer/effector components of an important DNA damage-signaling network that regulates the cellular response to a variety of DNA damage events. These proteins belong to a family of protein kinases termed PIKKs (phosphatidylinositol 3-kinase-like protein kinase), and they phosphorylate several key effector proteins (eg, Rad53p/hCHK2, Chk1p/hCHK1) in response to DNA damage. These effector proteins require additional factors to form a pentameric replication factor C-like

complex that can associate with sites of DNA damage and elicit the appropriate cellular response.¹⁷⁸ These DNA replication checkpoints operate during S phase to either arrest or slow replication in response to DNA damage and consist of both an S-phase checkpoint as well as an intra-S-phase checkpoint. These checkpoints are redundant, but if both are inactivated there is a synergistic increase in the rate of genomic rearrangements. The kinds of rearrangements observed in primary human cancer are similar to those seen when these checkpoint proteins are inactivated in yeast, providing indirect evidence for a role of the replication checkpoints in the genomic instability found in colon cancer.¹⁷⁹ Of all these checkpoint proteins, *TP53* has been most clearly shown to have a role in human colon cancer and to at least play a permissive role for the development of CIN.

In addition, mutations or amplification of other DNA checkpoint genes known to cause experimental forms of CIN have been identified in cancer, including *hBUB1*, *ATM*, *ATR*, *BRCA1*, *BRCA2*, *STK15*, *PLK1*, and *CDC4*.^{15,161,163,174–176,180} More recently, Wang et al used computational analysis to identify >1000 possible genes that could cause genomic instability based on their homology to genes in yeast and *Drosophila melanogaster*. They performed mutation analysis of 100 of these genes, which were selected based on the strength of the phenotypes observed in the model systems and on sequence similarity to the human homologue, in 24 early-passage colon cancer cell lines and found 19 somatic mutations in 5 genes.¹⁶ *MRE11*, which is involved in double-strand DNA break repair, is one of the genes that was identified in this screen for regulators of CIN in human cancers. *MRE11* is commonly mutated in MSI colon cancers, although its functional role in colon cancer is a subject of debate.¹⁸¹

Cell cycle proteins and CIN—Recently, evidence has accrued that has implicated cycle proteins in the maintenance of chromosome fidelity in human cancers. Somatic mutations in *hCDC4*, which is also known as *Fbw7* or *Archipelago*, were found in 11.5% of human colon cancers (n = 22/190) and in 4 of 58 adenomas.¹⁸⁰ *CDC4* is an evolutionarily conserved E3 ubiquitin ligase that regulates the G₁-S checkpoint by targeting proteins for destruction by the SCF complex of proteins. After identifying *CDC4* mutations in a subset of colon cancers, Rajagopalan et al assessed the functional consequence of *CDC4* inactivation by disrupting both alleles through homologous recombination and in the karyotypically stable colon cancer cell lines HCT116 and DLD1. They observed nuclear atypia, an increased frequency of multipolar spindles, and chromosome instability. Notably, this effect was dependent on an increase in cyclin E, which is a substrate of *CDC4*.¹⁸⁰ The significance of deregulation of the *CDC4*/cyclin E pathway has been substantiated by other investigators, who have shown that inhibition of degradation of cyclin E in mice leads to tumors that display genomic instability and that Fbxw7/h*CDC4* cooperates with p53 to suppress tumor formation.^{182,183} It appears that Cdc4 is regulated by p53 and is one of the downstream effectors of p53-mediated regulation of genome stability, especially in the setting of genomic stress. Notably, Cdc4 may affect genome stability not only through the regulation of cyclin E but also through effects on Notch and/or c-Jun.¹⁸³ Yoon et al have also shown that KLF4 is a p53-regulated protein that can prevent centrosome amplification after γ -irradiation-induced DNA damage.¹⁸⁴ Thus, KLF4 may be one of the ultimate effectors of CIN that results from Cdc4 deregulation. In aggregate, these data provide further support for an underlying deregulation of cell cycle proteins as being a basis for CIN and for the hypothesis that deregulation of mechanisms that mediate DNA fidelity lead to complex consequences that ultimately induce CIN.

Telomeres and telomerase—Telomeres are special chromatin structures at the ends of linear chromosomes that are believed to play a fundamentally important role in protecting these regions from degradation and recombination. The regulation of telomeres is tightly associated with the regulation of senescence. In vertebrates, telomeres are composed of tandem repeats of the TTAGGG sequence and are bound by specific proteins.¹⁸⁵ Conventional DNA

polymerases are not able to completely synthesize chromosomal ends, resulting in the gradual shortening of telomeres with successive rounds of cell divisions until a critical short length is reached that elicits the activation of cellular checkpoints similar to those activated by DNA damage.¹⁸⁶ In human cells, this telomere shortening culminates in activation of the Hayflick limit and the cessation of cell divisions. However, if p53 or Rb is inactivated, then the cells can continue to divide and will go through a period of massive cell death termed “cellular crisis.” Cells that survive crisis appear to activate mechanisms to maintain the telomeres and do this most frequently by increasing the expression of telomerase, a specialized ribonucleoprotein complex that consists of a catalytic telomerase reverse transcriptase (TERT) and an RNA subunit encoded by *TERC*. Most human cancers express telomerase, and it appears that during the evolution of normal cells to cancer cells the cells progress through a period of severe telomere dysfunction before regaining mechanisms to maintain telomere length.¹⁸⁷ Now data from experiments from *Terc*^{-/-} mice suggest that this period of telomere dysfunction may cause marked CIN that promotes the formation of cancer cells.¹⁸⁶ Consistent with this model, human colon cancers show a peak in the anaphase bridge index, which is a measure of the number of metaphases that contain anaphase bridges, in early high-grade dysplastic lesions and less in more advanced carcinoma stages.¹⁸⁸ However, the identification of chromosomal abnormalities in early colon adenomas, at a stage that precedes telomere dysfunction, suggests that telomere dysfunction cannot be the sole mechanism that induces genomic instability in colon cancer and that its role in tumor formation may depend on the nature of concurrent genetic alterations in the tumor cells. In support of this model, *mTerc*^{-/-}/*Min* (*Apc*^{+/+}), mice form fewer macroadenomas than mice with wild-type *mTerc*, but *mTerc*^{-/-}/*TP53*^{-/-} mice show a high level of genomic instability similar to that seen in human cancers and reduced tumor latency in late-generation mice.^{186,189,190} Thus, it appears that telomere dysfunction may also contribute to genomic instability seen in cancer, although more definitive evidence of its role in human cancer remains to be shown.

Other factors that may participate in the development of CIN in colon cancer—

Other factors than genetic inactivation of the proteins controlling the replication checkpoints have also been proposed to play a role in inducing genomic instability in colon cancer through effects on cell cycle regulation and DNA replication. These mechanisms include TGF- β resistance, JC virus infection, and *C-MYC* overexpression.^{191,192} For example, *MYC* overexpression has been implicated in attenuating the DNA damage checkpoint at G₁-S phase and contributing to tumorigenesis through the induction of aneuploidy by this mechanism.¹⁹³ Nonetheless, the data supporting these mechanisms having a role in genomic instability are confined to results from in vitro systems and have not been generated from in vivo models or in primary human cancers to date.

Epigenetic Alterations and Epigenetic Instability

In addition to genomic DNA instability being a common phenomenon in colorectal cancer, epigenetic instability also appears to be common in colorectal neoplasms. Although recognized more than 2 decades ago, epigenetic alterations, specifically aberrant DNA methylation, have not been intensively studied until recently because of controversy surrounding the significance of these alterations to the pathogenesis of colorectal cancer. DNA methylation is present throughout the majority of the human genome and is maintained in relatively stable patterns that are established during development.¹⁹⁴ In humans, approximately 70% of CpG dinucleotides carry this epigenetic modification. However, there are regions that are enriched for CpG dinucleotides, called CpG islands, that are present in the 5' region of approximately 50%–60% of genes and are normally maintained in an unmethylated state. In cancers, many of these CpG islands become aberrantly methylated, and this aberrant methylation can be accompanied by transcriptional repression^{195,196} (examples in Table 4).

As mentioned previously, the significance of these epigenetic alterations in the pathogenesis of cancer has been a point of substantial controversy.^{197,198} Nonetheless, there are now sufficient data to show that the aberrant methylation of at least some of these genes, such as *MLH1*, *MGMT*, and *HIC1*, can be pathogenetic in cancer^{52–54,199,200} (Figure 6). The aberrant methylation of *MLH1* occurs in >80% of sporadic MSI colorectal cancers, and the restoration of *MLH1* expression and function by demethylating the *MLH1* promoter in MSI colorectal cancer cell lines strongly suggests that such aberrant methylation is a cause rather than a consequence of colorectal carcinogenesis.^{52–54} Moreover, it is likely that the aberrant hypermethylation of 5' CpG dinucleotides that has been shown to silence a variety of tumor suppressor genes in colorectal cancer, including *CDKN2A/p16*, *MGMT*, *p14^{ARF}*, and *HLTF*, may be similarly pathogenetic in colorectal cancer.^{52–54,195,201–203} Of specific note, methylation of *CDKN2A/p16*, a canonical tumor suppressor gene, is detected in 40% of colorectal cancers²⁰² and has been found in not only colorectal cancer but also in colorectal adenomas, as have other aberrantly methylated genes.^{204,205} This observation, as well as the detection of aberrantly methylated genes *HLTF*, *SLC5A8*, *MGMT*, *MINT1*, and *MINT31* in ACF, shows that aberrant promoter methylation is occurring early in the adenoma sequence, although it does not confirm that the aberrant methylation is a primary rather than a secondary event in the tumorigenesis process^{206–208} (Figure 6).

In addition, some studies have suggested that there is a subgroup of colorectal cancers that hypermethylate a high proportion of genes that may belong to a distinct subclass of colorectal cancers, termed CIMP. The criteria for defining CIMP colorectal cancers is in evolution, which has contributed to some of the controversies in this field of study.^{209–211} If definitively proven to be a unique molecular subgroup of colorectal cancers, these tumors will be defined by a high proportion of aberrantly methylated gene promoters that arise by distinct and unique mechanisms compared with non-CIMP tumors.^{202,203,210,212} The concept of CIMP tumors has been surrounded by substantial controversy because it is not clear whether tumors that display the CIMP molecular phenotype are truly a unique molecular subgroup of tumors or a group of tumors that are on the extreme part of a normal distribution with regard to aberrant DNA methylation.^{209,211} The mechanism responsible for causing CIMP tumors is not known at this time, but it is known that this class of tumors commonly possesses mutant *BRAF* V600E.²¹⁰ Furthermore, the existence of other classes of CIMP tumors (eg, CIMP2, CIMP-low, CIMP-high) has been suggested recently, raising the possibility that in the future tumors may be able to be classified by both the type of genomic instability and the type of epigenomic instability they display.^{211,213} There has also been considerable research activity regarding markers that will accurately identify CIMP tumors (Table 4). These studies have led to different classification schemes and to the identification of different panels of markers that associate with excessive DNA methylation in colorectal cancer.^{210,211} It is not clear at this time which scheme and marker panel will ultimately be the most accurate for identifying CIMP tumors, assuming that this class of colorectal cancers is ultimately shown to arise via a process that is distinct from non-CIMP tumors.

Also worthy of note is recent progress in our understanding of mechanisms through which DNA methylation may affect transcription, which is tightly allied with mechanisms that regulate chromatin structure. DNA methylation may impair transcription by direct inhibition between methylated promoters and transcription factors, such as AP-2, CREB, E2F, CBF, and nuclear factor κ B.^{194,214} CpG island methylation also can mediate transcriptional silencing by recruiting methyl binding proteins MeCP2, MBD2, and MBD3, which recognize methylated sequence and recruit histone deacetylases. The histone deacetylases then induce changes in chromatin structure that impede the access of transcription factors to the promoter.^{195,214} Indeed, the posttranslational modification state of the histones, which includes modifications such as acetylation of histone 3 (H3) at lysines 9 (K9) and 18 (K18), acetylation of histone 4 at lysine 12 (K12), dimethylation of histone 4 at arginine 3 (di-me R3), and dimethylation of

histone 3 at lysine 4, among others, appears to regulate the chromatin state in a transcriptionally active (euchromatin) or transcriptionally repressed state (heterochromatin) through a “histone code.”²¹⁵ This histone code is altered from the normal state in cancer and appears to cooperate with aberrant methylation to alter the expression of tumor suppressor genes in cancer.^{216,217} It is noteworthy that the relationship between DNA methylation and posttranslational modification of histones is complex and only partially understood at this time. Studies have shown changes in the methylation state of H3/lysine 9 and H3/lysine 4 precede changes in DNA methylation, suggesting that the histone modification state and chromatin structure may cause the DNA methylation changes as opposed to the DNA methylation directing alterations in the histones as noted previously.¹⁹⁴

Thus, alterations in both the epigenome and genome occur commonly in colorectal cancer and likely drive the tumorigenesis process through activating oncogenes and inactivating tumor suppressor genes. The specifics of the process(es) through which genomic and epigenomic instability contribute to gene-specific alterations that drive colorectal cancer formation are under intense investigation. It is widely believed that the instability will ultimately be shown to play an important role in cancer formation through the creation of a permissive state that leads to gene mutations and epigenetic alterations that create opportunities for neoplastic clone progression.

Conclusions

The study of colon cancer genetics and epigenetics has yielded a host of new insights and paradigms that have broadly informed the studies of most solid tumors and the role that genomic instability plays in carcinogenesis in general. Recent progress in the investigation of genomic instability in colorectal cancer has provided support for the following: (1) the role of genomic instability early in the cancer formation process, even in the premalignant phase of the adenoma-carcinoma sequence, (2) the genetic and epigenetic basis for genomic instability in at least a subset of colon cancer (eg, inactivation of DNA repair genes, such as *MLH1* and *MYH*), and (3) the complex mechanisms through which genomic instability arises as the result of impaired function of genes involved with the maintenance of DNA fidelity. Nonetheless, many challenges remain. A more complete understanding of the basis of CIN, aneuploidy, and aberrant methylation of the cancer genome has yet to be achieved. Furthermore, the translation of molecular genetics to new diagnostic, prognostic, and therapeutic modalities remains a challenge but carries the promise of more effective medical treatment strategies for colon cancer.

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Abbreviations used in this paper

ACF	aberrant crypt focus
APC/C	anaphase promoting complex/C
ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia mutated and Rad3 related
BER	base excision repair
CIMP	CpG island methylator phenotype
CIN	chromosomal instability

FAP	familial adenomatous polyposis
5-FU	5-fluorouracil
MAP	mut Y homologue-associated polyposis
MMR	mismatch repair
MSI	microsatellite instability
MYH	mut Y homologue
RPA	replication protein A
TGF	transforming growth factor

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Figure 1.

Depiction of colorectal tumor progression in sporadic and high-risk genetic syndromes. The general paradigm is that a tumor is initiated from a normal colonocyte stem cell that has sustained genetic damage over time due to the local environment and any germline genetic mutation that has been inherited. The damaged DNA provides a growth advantage that drives tumor progression as successive clonal outgrowths are generated, ultimately forming carcinoma. In FAP, tumor initiation is accelerated with the inheritance of a germline *APC* mutation; in Lynch syndrome, tumor initiation might be normal to slightly accelerated, but tumor progression is greatly accelerated due to the hypermutable phenotype that occurs with loss of DNA MMR. Photomicrographs depict, in order, normal colon, tubular adenoma, high-grade dysplasia, and cancer.

Figure 2.

Schematic of DNA MMR. The MMR heterodimer hMSH2-hMSH6 (hMutS α) recognizes single nucleotide mispairs and will bind to DNA as a sliding clamp. The heterodimer hMLH1-hPMS2 (hMutL α) then binds to hMutS α to eventually guide an exonuclease to remove several bases from the newly synthesized DNA strand, with eventual resynthesis of DNA with the correct base pairing. At microsatellite sequences, insertion-deletion loops of one nucleotide are typically recognized by hMutS β (*upper panel*), but larger insertion-deletion loops are recognized by the heterodimer hMSH2-hMSH3 (hMutS β) (*lower panel*), followed by binding by hMutL α , excision, and resynthesis as described previously. The roles of hMLH1-hPMS1 and hMLH1-hMLH3 in human MMR function are not entirely clear. When the MutS complexes bind DNA, they exchange ADP for ATP. For the MMR proteins to be released from DNA, ATP is hydrolyzed to ADP.

BRAF mutations occur in sporadic MSI-H tumors, and are rarely seen in Lynch syndrome tumors.

Figure 3.

Progression of colorectal tumors with MSI. MSI tumors, whether sporadic or from patients with Lynch syndrome, lose MMR function early in the polyp → cancer progression sequence. Sporadic tumors almost uniformly lose MMR function due to hypermethylation of the promoter of *hMLH1*, whereas patients with Lynch syndrome have a germline mutation in one of the MMR genes. It is generally believed that Wnt signaling, the gatekeeper for colorectal neoplasia, is affected, but the full mechanisms are not clear and include mutation of *CTNNB1*, the gene encoding β -catenin in some cases of Lynch syndrome. The serrated adenoma may be one histologic form for MSI colorectal tumors. In the milieu of MMR absence, a hypermutable phenotype develops in which multiple mutations occur in DNA. Although most mutations occur in noncoding sequences such as intronic DNA microsatellites, certain genes such as *TGFR2*, *ACVR2*, *BAX*, *hMSH3*, *hMSH6*, and others that have coding microsatellite sequences become frameshifted in the absence of DNA MMR. These mutations help drive the progression of the tumor. *BRAF* mutations are principally found in sporadic tumors with MSI and not tumors from patients with Lynch syndrome and can be used to differentiate these 2 groups of tumors. Notably, in MSI and non-MSI tumors, signaling through the TGF- β superfamily is altered to favor tumor promotion in a not yet fully understood fashion.

Figure 4.

Progression of colorectal tumors with CIN. The hallmark of the CIN pathway is aneuploidy. Initiation of neoplasia in this pathway occurs with interruption of components of the Wnt signaling pathway, including somatic mutation in one allele and loss of heterozygosity of the second normal allele of the *APC* gene, and is seen in dysplastic ACF histologically. Progression is then driven by successive waves of cellular clonal expansion that acquire enhanced growth characteristics and include mutational activation of the proto-oncogene *KRAS* and mutation of *TP53* with subsequent loss of heterozygosity of the normal remaining *TP53* allele to allow carcinoma formation. In some colorectal tumors, mutational activation of *PIK3CA* occurs late in the adenoma-carcinoma sequence. Several TGF- β signaling molecules are also affected in CIN progression, including mutations of the kinase domain of *TGFBR2* and loss of heterozygosity at chromosome 18q, the site of *SMAD4* and *SMAD2*. Several genes are believed to be involved in metastasis and include gene amplification of *PRL3*.

Figure 5.

Schematic diagram of cell cycle checkpoints in relation to cell cycle phases. The DNA damage, DNA replication, and mitotic/spindle-kinetochore checkpoints are shown with a corresponding list of a subset of human and *S cerevisiae* genes that function at each of the checkpoints. The sensor proteins and downstream signal transduction and effector proteins are listed for the DNA replication checkpoints in S phase. The proteins listed at the other checkpoints operate as sensor, transducers, or effector proteins.

Figure 6.

Conceptual progression of colorectal tumors with CpG island Methylator Phenotype. The hallmark of the CIMP pathway is abnormal methylation of several promoter sequences of tumor suppressor genes. This pathway in part overlaps with sporadic MSI tumors because the MMR gene *hMLH1* is targeted for hypermethylation, which causes loss of MMR function. Although a full understanding of the CIMP is not clear, including the mechanism for hypermethylation of genes that is observed, we propose the following adenoma-carcinoma sequence based on studies published to date. Instead of dysplastic ACF with interrupted Wnt signaling, hyperplastic ACF may be the initial lesion in this pathway. Methylated promoters of *MGMT*, *EVL*, *HLTF*, *SFRP2*, *SLC5A8*, and *MINT1* develop during the initiation phase of colorectal tumor development. *hMLH1* promoter hypermethylation might correspond to the development of a serrated adenoma, with methylated *TSP1* and *TIMP3* helping to drive the progression of the CIMP tumor.

Table 1

Genes Involved in DNA MMR

MMR gene	Chromosomal location	MMR partners	MMR fidelity	Lynch syndrome germline mutation (%)
<i>hMSH2</i>	2p15	hMSH6, hMSH3	Complete loss	31–45
<i>hMSH6</i>	2p15	hMSH2	Single base pairs, single insertion/deletion loops	5–8
<i>hMSH3</i>	5q11-q13	hMSH2	Larger insertion/deletion loops	0
<i>hMLH1</i>	3p21	hPMS2 (principally), hMLH3, hPMS1	Complete loss	32–49
<i>hPMS1</i>	2p32	hMLH1	Unclear	0
<i>hPMS2</i>	7p22	hMLH1	Complete loss?	0–2
<i>hMLH3</i>	7p22	hMLH1	Unclear	0

Table 2

Some Targeted Genes in MMR-Deficient (or MSI) Colorectal Cancers and Their Reported Mutation Frequency

Gene	Chromosome	Microsatellite tract	MSI colorectal cancer mutation frequency (%)	Normal function
<i>TGFBR2</i>	3p22	A ₁₀	90	TGF- β signaling
<i>ACVR2</i>	2q22-23	A ₈ and A ₈	86	Activin signaling
<i>IGF1R</i>	6q26-27	G ₈	10	Insulin-like growth factor and TGF- β signaling
<i>BAX</i>	19q13.3-q13.4	G ₈	50	Apoptosis
<i>hMSH3</i>	5q11-q12	A ₈	50	DNA MMR
<i>hMSH6</i>	2p16	C ₈	33	DNA MMR
<i>E2F-4</i>	16q21-q22	(CAG) ₁₃	65	Cell cycle control
<i>PTEN</i>	10q23.3	A ₆ and A ₆	19–34	Growth regulation
<i>MBD4 (MED1)</i>	3q21-q22	A ₁₀	40	DNA repair and binding to methylated DNA
<i>TCF4</i>	10q25.3	A ₉	39	Growth regulation
<i>CHK1</i>	11q22-q23	A ₉	10	G ₂ cell cycle checkpoint
<i>STK11 (LKB1)</i>	19p13.3	C ₆	<2	Signal transduction
<i>BLM</i>	15q26.1	A ₉	<18	Chromosome stability/DNA repair; helicase
<i>Caspase-5 (ICErel-III)</i>	11q22.2-22.3	A ₁₀	62	Apoptosis
<i>CDX2</i>	13q12.3	C ₆ and G ₆	<2	Homeobox protein
<i>TBP</i>	6q27	(CAG) ₁₉ and (CAG) ₁₆	83	TATA binding protein
<i>RIZ</i>	1p36	A ₈ and A ₉	26	Interacts with RB
<i>hRAD50</i>	5q31	A ₉ and A ₈	31	DNA repair
<i>SEC63</i>	6q21	A ₁₀	49	ER chaperone protein
<i>AIM2</i>	1q22	A ₁₀	48	Interferon-inducible protein

Table 3**DNA Damage Repair Genes and CIN**

Human gene	Protein function	Gene alteration	Syndrome
<i>BRCA1</i>	Sensor: BRCT containing	BRCA1: germline mutation BRCA2: germline mutation	Hereditary breast-ovarian cancer
<i>MRE11</i>	Sensor: DSB recognition/repair	Germline mutation Somatic mutation in MSI colon cancer	Ataxia-telangiectasia-like disorder
<i>BLM</i>	Sensor: replication proteins	BLM ^{Ash} : increased risk of colon cancer	Bloom syndrome
<i>WRN</i>		Aberrant DNA methylation	Werner syndrome
<i>RTS</i>			Rothmund-Thomson syndrome
<i>ATM</i>	Transducer: PI3-kinase (PIKK)	Germline mutation	Ataxia-telangiectasia
<i>TP53</i>	Transducer: effector kinase	TP53: germline mutation Somatic mutations	Li-Fraumeni syndrome
<i>CHK2</i>	Transducer: effector kinase	Germline mutation	Li-Fraumeni-like syndrome
<i>MYH</i>	BER	MYH: germline mutation	MAP
<i>MGMT</i>	DNA damage repair	Aberrant DNA methylation	
<i>BUB1</i>	Kinetochore regulation	Somatic mutation	
<i>BUBR1</i>		Somatic mutation	
<i>MAD2</i>		Abnormal expression	
<i>hRod</i>		Rare somatic mutation	
<i>hZw10</i>		Rare somatic mutation	
<i>hZwilch</i>		Rare somatic mutation	
<i>CENP-A</i>		Gene overexpression	
<i>CENP-H</i>		Gene overexpression	
<i>HEC1</i>		Gene overexpression	
<i>AIM1</i>		Gene overexpression	
<i>INCENP</i>		Gene overexpression	
<i>Ku70</i>	Nonhomologous end-joining	Loss of expression	
<i>Ku86</i>		Loss of expression in rectal cancer	
<i>AURKA</i>	Chromosome segregation defects	Gene amplification and overexpression	
<i>TERC</i>	Telomere regulation	Overexpression	
<i>CCNE1</i>	Cell cycle regulation	Overexpression	
<i>FBXW7/CDC4</i>			
<i>CDC25A ?</i>			
<i>CDKN2A</i>		Aberrant DNA methylation	

NOTE. Some genes that have been identified to cause cancer family syndromes or to be deregulated in human colon cancer. BRCT, Brcal carboxy terminus; DSB, double-strand break.

Table 4

Selected Genes Affected by Aberrant Methylation in Colorectal Cancer

Gene	CIMP association	Age-related methylation vs cancer-related methylation	Concurrent mutations	Methylation frequency in colorectal cancer (%)
<i>MLH1</i>	CIMP1	C-type	<i>BRAF V600E</i> MSI	10–20
<i>TIMP3</i>	CIMP1	C-type	<i>BRAF V600E</i> MSI	20–30
<i>MINT17</i>	CIMP1		<i>BRAF V600E</i> MSI	—
<i>P14</i>	CIMP1	C-type	<i>BRAF V600E</i> MSI	10–20
<i>RIZ1</i>	CIMP1	C-type	<i>BRAF V600E</i> MSI	10–20
<i>CDKN2A</i>	CIMP1	C-type	<i>BRAF V600E</i> MSI	20–30
<i>MINT12</i>	CIMP1		<i>BRAF V600E</i> MSI	20–40
<i>RUNX3</i>	CIMP1 and “New”/Laird	C-type	<i>BRAF V600E</i> MSI	>20
<i>SOCS1</i>	CIMP1 and “New”/Laird	C-type	<i>BRAF V600E</i> MSI	20
<i>MINT1</i>	CIMP1 and CIMP2			0–20
<i>MINT31</i>	CIMP1 and CIMP2			0–20
<i>MINT27</i>	CIMP2		<i>KRAS</i> mutant MSS/MSI-L	—
<i>MINT2</i>	CIMP2		<i>KRAS</i> mutant MSS/MSI-L	0–20
<i>MEGALIN</i>	CIMP2	C-type	<i>KRAS</i> mutant MSS/MSI-L	10–20
<i>NEUROG1</i>	CIMP2 and “New”/Laird	C-type	<i>BRAF V600E</i>	20
<i>CACNA1G</i>	“New”/Laird	C-type	<i>BRAF V600E</i>	20
<i>IGF2</i>	“New”/Laird	A-type	<i>BRAF V600E</i>	100
<i>APC</i>		C-type		10–20
<i>CALCA</i>		C-type		50
<i>MIR342/EVL</i>		C-type		70–80
<i>TSP1</i>		C-type		20–30
<i>HIC1</i>		C-type		50
<i>HLTF</i>		C-type		50
<i>VIM</i>		C-type		60–80
<i>MGMT</i>		C-type		30–40
<i>SFRP2</i>		C-type		60–80
<i>SLC5A8</i>		C-type		40–60
<i>ESR1</i>		A-type		>90
<i>MYOD1</i>		A-type		>90
<i>N33</i>		A-type		80–90
<i>PAX6</i>		A-type		60–70

Gene	CIMP association	Age-related methylation vs cancer-related methylation	Concurrent mutations	Methylation frequency in colorectal cancer (%)
<i>RASSF1</i>		A-type		50
<i>RARβ2</i>		A-type		10–40

A-type, age-related methylation; C-type, cancer-related methylation.