

Chapter 2

Molecular Mechanisms of Colorectal Carcinogenesis

Jatin Roper and Kenneth E. Hung

Abstract Colorectal cancer (CRC) presents in three major forms: inherited, sporadic, and familial. Although the mechanisms underlying familial CRC are poorly understood, a large body of evidence suggests that inherited and sporadic CRC are caused by sequential genetic and molecular events. There are three distinct pathways of CRC pathogenesis: the chromosomal instability pathway (CIN), the microsatellite instability pathway (MSI), and the serrated pathway. The majority of CRCs arise from the CIN pathway, which is characterized by defects in chromosomal segregation, telomere stability, and the DNA damage response. Microsatellite instability derives from the loss of DNA mismatch repair and is found in about 15 % of all CRCs, 3 % of which are associated with Lynch syndrome. The serrated pathway, recognized only in the last 15 years, describes the progression of serrated polyps to CRC. The goal of this chapter is to discuss the key genetic and molecular elements of each pathway from a historical perspective and to describe the relevance of this knowledge to the care of patients with CRC.

Keywords Colorectal cancer • Chromosomal instability • Microsatellite instability • Mouse models • Serrated pathway

2.1 Introduction

Colorectal cancer (CRC) continues to be an enormous public health burden. It is the third most common cancer in men and second most common cancer in women worldwide, with nearly 1.2 million new cases yearly, and the third leading cause of

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cancer-related mortality, with approximately 600,000 deaths each year. The 5-year prognosis for patients with newly diagnosed metastatic colon cancer continues to be less than 20 % (Jemal et al. 2011). The underlying causes of CRC are complex and heterogeneous. Both environmental factors and genetic events contribute to CRC risk. Among the environmental risk factors for CRC are diets rich in unsaturated fats and red meat, total energy intake, excessive alcohol consumption, and reduced physical activity. Many studies have examined other exposures for their effects on CRC risk but have yielded ambiguous results (Chan and Giovannucci 2010). In contrast, there has been significant progress in identification of the specific genetic defects underlying the majority of CRCs. To develop effective CRC prevention, diagnosis, and treatment strategies, an understanding of the pathways and molecular events that drive CRC carcinogenesis is essential.

CRC presents in one of three patterns: inherited, familial, and sporadic. Inherited and familial CRC derive, at least in part, from germline mutations. Inherited CRC accounts for 10 % of cases and presents as well-characterized cancer predisposition syndromes including Lynch syndrome and familial adenomatous polyposis (FAP). Familial CRC accounts for 25 % of CRCs and presents without precisely defined Mendelian inheritance patterns or genetic etiology (Pino and Chung 2010). Sporadic CRC derives from somatic mutation, accounts for approximately 70 % of CRCs, and is not associated with family history. This chapter focuses on the genetic and molecular events underlying the three major pathways for sporadic and inherited colorectal carcinogenesis: chromosomal instability (CIN), microsatellite instability (MSI), and the CpG island methylator phenotype (CIMP) pathways.

2.2 CIN: Chromosomal Instability Pathway

2.2.1 *The Adenoma–Carcinoma Sequence*

By the mid-1970s, several pieces of indirect evidence suggested that colorectal adenocarcinomas may progress from adenomas (1) residual benign adenomatous tissue was found in carcinomas, (2) malignant foci were observed in larger polyps, and (3) there were rare observations of a benign-appearing polyp developing into an invasive carcinoma (Morson 1974). In 1987, Stryker and colleagues reported the natural history of unresected colonic polyps >1 cm in size in 226 patients who declined surgical resection. After 20-year follow-up, they found a 24 % risk of invasive adenocarcinoma at the site of the index polyp, and a 35 % risk of carcinoma at any colonic site (Stryker et al. 1987). Individuals affected by cancer predisposition syndromes, such as FAP, invariably develop CRC by the third or fourth decade of life if their colons are not removed (Lynch and de la Chapelle 2003). The National Polyp Study confirmed the hypothesis that colorectal carcinomas arise from adenomas through showing that polypectomy by colonoscopy reduces the

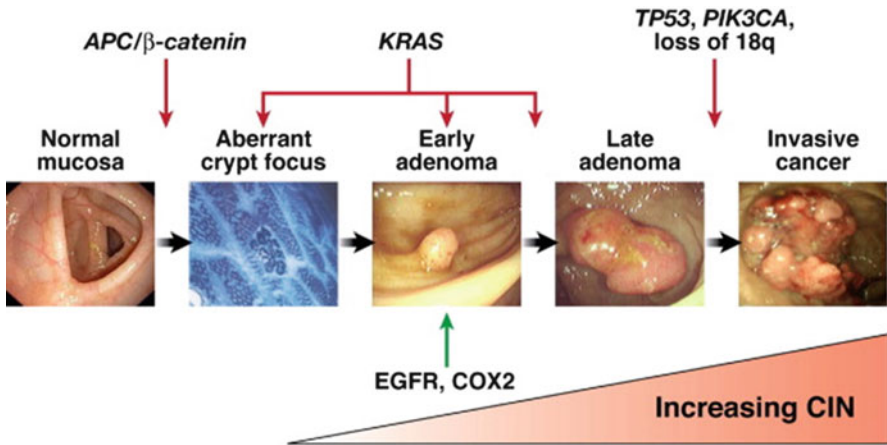


Fig. 2.1 The adenoma–carcinoma sequence. The initial step in colorectal carcinogenesis is thought to be the formation of aberrant crypt foci (ACF). Activation of the Wnt pathway occurs during this step as a result of inactivating mutations in the *APC* gene. Progression to adenoma and carcinoma is usually mediated by activating mutations in *KRAS* and loss of *TP53* expression, respectively. A subset of advanced adenomas may progress due to mutations in *PIK3CA* and loss of 18q. Reproduced with permission from: Pino MS, Chung DC (2010) The chromosomal instability pathway (CIN) in colon cancer. *Gastroenterology* 138(6):2059–2072

risk of subsequent CRC (Winawer et al. 1993). In 1990, Fearon and Vogelstein proposed a multistep genetic model of colorectal carcinogenesis in which inactivation of the adenomatous polyposis coli (*APC*) tumor suppressor gene occurs first in normal colonic mucosa, followed by activating mutations in the *KRAS* gene and subsequent additional mutations (e.g., *PIK3CA*, *TP53*, and *TGF-β* pathway genes) (Fig. 2.1 and Table 2.1) (Fearon and Vogelstein 1990; Vogelstein et al. 1988; Fearon 2011). Several key principles of the so-called adenoma–carcinoma sequence have been established (1) multiple genetic hits are required, (2) there are discrete intermediaries in the progression to cancer (Pino and Chung 2010; Haigis et al. 2008), and (3) adenomas arise from aberrant crypt foci in the colonic epithelium (Takayama et al. 1998).

2.2.2 Genomic Instability and Cancer

The mutation rate per nucleotide base pair is far too low (estimated to be approximately 10^{-9} per cell generation) to account for the multiple genetic mutations required for tumorigenesis (Albertini et al. 1990). Therefore, it has been proposed that cancer cells must acquire a “mutator phenotype” that increases the rate of spontaneous mutations (Loeb et al. 2003). 65–70 % of sporadic colorectal cancers exhibit

Table 2.1 Somatic mutations in oncogenes and tumor suppressor genes implicated in colorectal carcinogenesis

Gene	Chromosomal location	Type of mutation	Prevalence (%)	Function of gene product
Oncogenes				
<i>KRAS</i>	12p12	Point mutation (codons 12, 13 of exon 2)	40	Cell proliferation and survival
<i>PIK3CA</i>	3q26	Point mutations (E545K on exon 9, H1047R on exon 20)	15–30	Cell proliferation and survival
<i>CDK8</i>	13q12	Gene amplification	10–15	β -catenin activation
<i>EGFR</i>	7p12	Gene amplification	5–15	Cell proliferation and survival
<i>BRAF</i>	7q34	Point mutations activating kinase activity (most commonly V600E)	5–10	Cell proliferation and survival
<i>CMYC</i>	8q24	Gene amplification	5–10	Cell proliferation and survival
<i>CCNE1</i>	19q12	Gene amplification	5	
<i>NRAS</i>	1p13	Point mutation	<5	Cell proliferation and survival
<i>CTNNB1</i>	3p22	Stabilizing point mutations and in-frame deletions near N terminus	<5	Regulation of Wnt pathway target genes that promote tumor growth and invasion
<i>ERBB2 (HER2)</i>	17q21	Gene amplification	<5	Cell proliferation and survival
<i>MYB</i>	6q22-q23	Gene amplification	<5	Stimulates growth of intestinal stem cells
Tumor-suppressor genes				
<i>APC</i>	5q21	Frameshift, point mutation, deletion, allele loss leading to truncated protein	70–80	Inhibition of Wnt signaling
<i>TP53</i>	17q13	Point mutation (missense), allele loss	60–70	Cell cycle arrest, apoptosis and autophagy induction
<i>DCC</i>	18q21	Point mutation	50	Cell surface receptor for netrin-1, triggers tumor cell apoptosis
<i>TGFβRII (TGFBβR2)</i>	3p22	Frameshift, nonsense	25	Inhibition of cell growth
<i>SMAD4</i>	18q21	Nonsense, missense, allele loss	10–15	Intracellular mediator of the <i>TGF-β</i> pathway
<i>PTEN</i>	10q23	Nonsense, deletion	10	Inhibition of PI3K activity
<i>ACVR2A</i>	2q22	Frameshift	10	Cellular growth
<i>SMAD2</i>	18q21	Nonsense, deletion, allele loss	5–10	Intracellular mediator of the <i>TGF-β</i> pathway
<i>FBXW7</i>	4q31	Nonsense, missense, deletion	9	Targets oncoproteins for ubiquitin-mediated degradation
<i>SMAD3</i>	15q22	Nonsense, deletion	5	Intracellular mediator of the <i>TGF-β</i> pathway
<i>TCF7L2</i>	10q25	Frameshift, nonsense	5	Regulation of the Wnt signaling
<i>BAX</i>	19q13	Frameshift	5	Apoptotic activator
<i>LKB1 (STK11)</i>	19p13	Deletion	Rare (limited to PJS)	Regulation of cell polarity

Modified from Fearon ER. Molecular genetics of colorectal cancer. *Annu Rev Pathol* 2011;6:479–507

an accelerated rate of gains or losses of whole or large portions of chromosomes that result in karyotypic variability between cells. This chromosomal instability (CIN) appears to be a dominant trait (Lengauer et al. 1997, 1998). Consequences of CIN include an imbalance in chromosomal number (aneuploidy), subchromosomal genomic amplifications, and a high frequency of loss of heterozygosity (LOH). There are currently no standardized measures of chromosomal instability; hence, CIN-positive vs. CIN-negative tumors cannot be clearly defined (Pino and Chung 2010). One theory views cancers as clonal in origin, in that they arise from a single, genomically unstable cell, but develop genetic heterogeneity due to CIN. This explains observed heterogeneity within single tumors with regard to DNA content, chromosomal number, gene expression, metabolism, resistance to cytotoxic drugs, and metastatic potential (Duesberg et al. 2004).

2.2.3 Mechanisms Leading to Chromosomal Instability

2.2.3.1 Defects in Chromosomal Segregation

The CIN phenotype can result from defects in pathways that regulate chromosomal segregation. The mitotic or spindle checkpoint ensures proper chromosome segregation by delaying the metaphase-to-anaphase transition until all pairs of duplicated chromatids are properly aligned on the spindle. Genes that encode proteins operating as spindle checkpoint regulators include *mitotic arrest-deficient* (*MAD1L1* and *MAD2L1*), *budding uninhibited by benzimidazoles 1* (*BUB1*), and *kinesin family member 11* (*KIF11*). Mutations in *BUB1* result in abnormal spindle checkpoint and CIN in chromosomally stable cell lines (Bardelli et al. 2001). Cells from dominant-negative mBub1 mutant mice demonstrate escape from apoptosis, continued cell cycle progression, and disrupted spindles (Taylor and McKeon 1997). Kinesin spindle protein, also known as Eg5, is a motor protein responsible for mitotic spindle formation and chromosomal separation during mitosis. Overexpression of *Eg5* in mice leads to spindle defects, CIN, and solid tumor formation (Castillo et al. 2007). Mutations in the hZw10, hZw1ch/FLJ10036, and hROD/KNTC genes, which encode kinetochore proteins, have been reported in CRC (Wang et al. 2004).

Chromosomal missegregation due to defects in the mitotic checkpoint may lead to aneuploidy, a concept first proposed by Theodor Boveri in 1902—well before the advent of chromosomal karyotyping (Boveri 2008). The aneuploidy hypothesis proposes a two-step mechanism for tumor initiation. The first step is an event (i.e., a defect in spindle formation) that promotes chromosomal missegregation and aneuploidy. In the second step, aneuploidy destabilizes the genome, gives rise to polyclonal mutations, and results in heterogeneous karyotypes. Aneuploidy therefore stimulates tumorigenesis either by increasing the chances of LOH of a tumor-suppressor gene or by amplifying an oncogene through chromosomal duplication (Duesberg et al. 2004; Castillo et al. 2007).

2.2.3.2 Centromere Dysfunction

Another proposed cause of CIN is abnormal centromere number and function. Centrosomes serve to anchor cytoplasmic microtubules as they are arranged into a mitotic spindle apparatus. Extra centrosomes in cancer cell lines may lead to the formation of multiple spindle poles during mitosis, resulting in unequal distribution of chromosomes and CIN (Ganem et al. 2009). Polo-like kinases (Plk) are serine/threonine kinases, which regulate centrosome duplication. Elevated expression of Plk1 has been observed in 73 % of CRCs and correlate with tumor invasion, lymph node involvement, and stage (Takahashi et al. 2003). The centrosome-associated Aurora A protein is amplified and positively associated with CIN in CRC, but metastatic CRC patients with increased Aurora A gene copy number have longer overall and progression-free survival, particularly in *KRAS* wild-type tumors (Dotan et al. 2002; Herz et al. 2011). The related Aurora B protein regulates chromatid segregation, and its expression is correlated with advanced stages of CRC (Katayama et al. 1999a).

2.2.3.3 Telomere Dysfunction

CIN may also be driven by telomere dysfunction. Telomeres are hexameric DNA repeats (TTAGGG in humans) that protect the ends of eukaryotic chromosomes from fusing and breaking during segregation. A portion of telomeric DNA is lost after each round of DNA replication due the inability of DNA polymerase to completely synthesize the 3' end of chromosomes. Cells with sufficiently shortened telomeres are targeted for senescence and apoptosis by DNA damage checkpoints. Cells that survive the checkpoint activate telomerase, which elongates telomeres. In mice deficient in the RNA component of telomerase (*Terc* $-/-$), telomere shortening results in aberrant crypt foci, adenomas, and gastrointestinal tumors (Rudolph et al. 2001; Plentz et al. 2003). 77–90 % of CRCs harbor shorter telomeres, compared to adjacent normal tissue, but increased telomerase activity has also been reported (Engelhardt et al. 1997; Takagi et al. 1999; Katayama et al. 1999b; Nakamura et al. 2000; Gertler et al. 2004; Chadeneau et al. 1995; Tatsumoto et al. 2000). These findings suggest that telomere shortening promotes CIN that initiates carcinogenesis, whereas telomerase activation in established carcinomas leads to immortality of cancer cells.

2.2.3.4 Loss of Heterozygosity

LOH is a key feature of CIN-positive tumors and distinguishes tumors arising from the CIN pathway from tumors arising from the MSI pathway. Approximately 25–30 % of alleles are lost in tumors (Lengauer et al. 1998). Mitotic nondisjunction, recombination between homologous chromosomes, and chromosomal deletion are among the implicated mechanisms. One study found that the majority of losses on chromosome 18 involved the whole chromosome and were caused by mitotic

nondisjunction. Losses limited to a part of a chromosome were thought to be due to interchromosomal recombinations and deletions associated with DNA double-strand breaks (Thiagalingam et al. 2001).

2.2.3.5 Deficiencies in DNA Damage Response

Deficiencies in DNA damage response have been linked to human cancer. Inactivating mutations in ataxia telangiectasia mutated (*ATM*) and ataxia telangiectasia and Rad3-related (*ATR*) protein kinases lead to the ataxia telangiectasia and Seckel syndromes, respectively (Khanna and Jackson 2001). Other syndromes linked to impaired DNA damage response include Li–Fraumeni (*TP53* mutations) and hereditary breast–ovarian cancer (*BRCA1* and *BRCA2* mutations). Of these genes, only *TP53* has been directly implicated in human colorectal cancer. Haploinsufficiency of histone H2AX, an *ATM* and *ATR* substrate, leads to genomic instability and tumor susceptibility in a p53-deficient background, and mouse embryonic fibroblasts derived from *ATM*- and H2Ax-deficient mice show severe genomic instability (Bassing et al. 2003; Celeste et al. 2003; Zha et al. 2008). Deficiency in *Chk1*, a DNA damage checkpoint protein, causes mitotic defects and disrupts Aurora B during mitosis, resulting in failure of cytokinesis and multinucleation (Peddibhotla et al. 2009).

2.2.4 Genetic Abnormalities Implicated in the Chromosomal Instability Pathway

Recent comprehensive sequencing studies have identified over 80 somatic mutations in exons of colorectal tumors. However, a limited number of these mutations are found in a significant percentage of tumors. Wood et al. predicted that perhaps 15 or fewer of these mutations in any given CRC are critical drivers of tumor initiation, progression, and/or maintenance (Wood et al. 2007). Many of the genes identified by sequencing analysis were already well known to be somatically mutated in CRC (e.g., *APC*, *KRAS*, and *TP53*). Table 2.1 describes data on oncogenes and tumor suppressor genes that are somatically mutated in CRC.

2.2.4.1 APC and the Wnt Pathway

The earliest genetic event in colorectal carcinogenesis is activation of the Wnt pathway, typically via disruption of *APC* on 5q21 (Powell et al. 1992). The *APC* gene product is an approximately 300-kDa protein with multiple functional domains that regulates differentiation, adhesion, polarity, migration, development, apoptosis, and chromosomal segregation (Fig. 2.2). Restoration of *APC* protein expression in CRC cells that lack endogenous *APC* expression promotes apoptosis. In the absence of

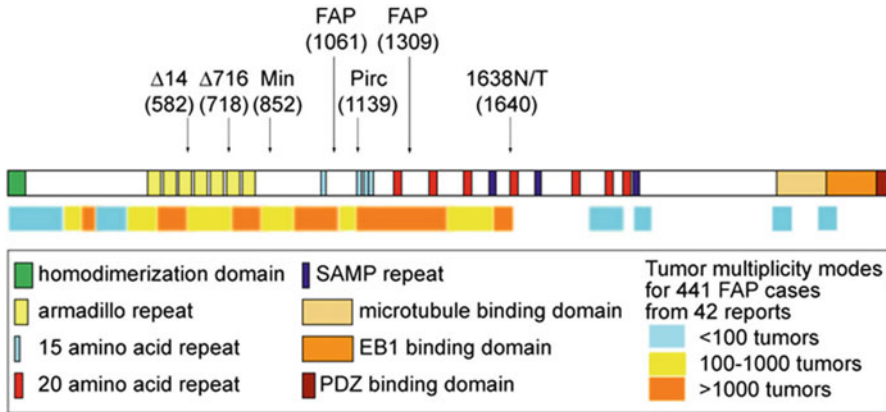


Fig. 2.2 The Wnt signaling pathway. (a) In the absence of Wnt ligand, the complex containing APC, glycogen synthase kinase 3 β (GSK3 β), casein kinase 1 α (CK1 α), and an Axin scaffold targets cytoplasmic β -catenin for proteasomal degradation. In the nucleus, Wnt target genes are silenced by Groucho. (b) In the presence of Wnt ligand, the receptors Frizzled (Fz) and low-density lipoprotein receptor-related protein (LRP) trigger the phosphorylation of the cytoplasmic tail of LRP by GSK3 β . Disheveled (Dsh) recruits Axin to the phosphorylated tail of LRP. Phosphorylation of β -catenin does not occur; β -catenin accumulates in the cytoplasm and translocates into the nucleus, where it activates the transcription of multiple target genes by interacting with the TCF family of transcription factors. Reproduced with permission from: Pino MS, Chung DC (2010) The CIN in colon cancer. *Gastroenterology* 138(6):2059–2072

Wnt ligand signaling, APC binds to the scaffold protein Axin to promote sequential phosphorylation of the N-terminus region of β -catenin by casein kinase 1 and glycogen synthase kinase-3 β (GSK3 β), thus targeting phosphorylated β -catenin for ubiquitination and subsequent proteasomal degradation. In the setting of CRC in which both *APC* alleles are mutated, loss of APC protein allows for cytoplasmic accumulation of β -catenin, which then complexes with DNA-binding proteins of the TCF/LEF (T-cell factor/lymphoid enhancer family) family, and translocates to the nucleus, where it drives transcription of multiple genes with TCF DNA-binding sites involved in tumor growth and invasion (Fig. 2.3) (Mann et al. 1999).

In sporadic CRC, *APC* mutations are present in microscopic adenomas, 50–60 % of small (<0.5 cm) adenomas and are found at similar frequency in advanced adenomas and carcinomas, indicating that inactivating mutations in *APC* are an early event in colorectal carcinogenesis (Powell et al. 1992; Miyaki et al. 1994; Cottrell et al. 1992). Kinzler and Vogelstein argue that APC is a “gatekeeper gene” which is “responsible for maintaining a constant cell number in renewing cell populations.” (Kinzler and Vogelstein 1996) Nearly all somatic mutations lead to premature truncation of the APC protein. Both *APC* alleles are inactivated in adenomas and carcinomas that arise in FAP patients as well as in sporadic disease. While germline-inactivating mutations in *APC* are located throughout the gene, somatic mutations are clustered between codons 1286 and 1513 (Miyoshi et al. 1992).

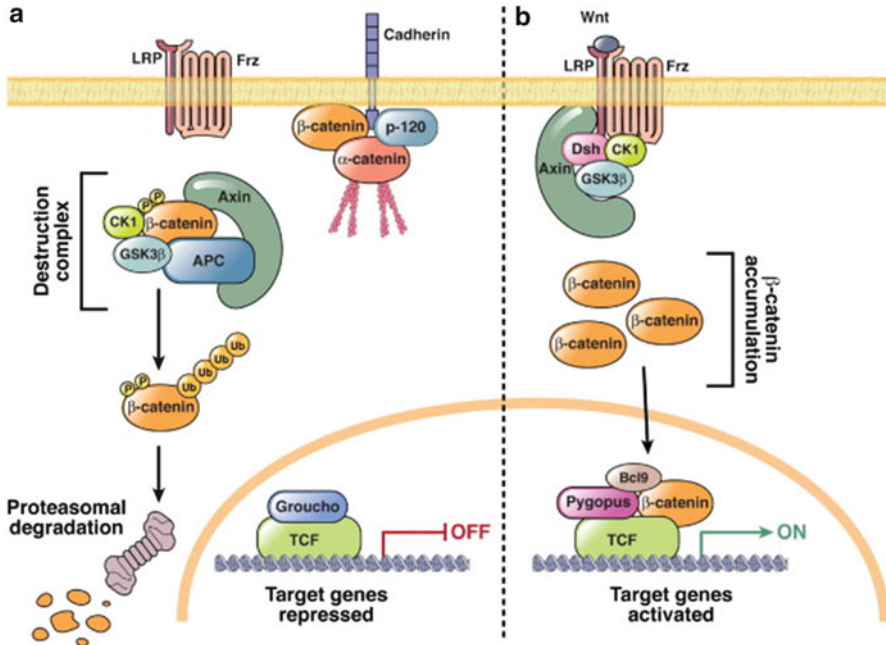


Fig. 2.3 The APC protein. Cartoon of the 2,843 amino acid adenomatous polyposis coli (APC) protein with selected sequence motifs and interaction partners. The N-terminus has a domain which regulates homodimerization. Repeated sequences with homology to *Drosophila* armadillo protein are in the N-terminus third of APC ("armadillo repeat"). Multiple 20-amino acid repeats mediate binding to β -catenin and Axin in the central third of APC. The C-terminal third of APC has a basic region that is involved in microtubule binding and interactions with the protein EB1. Arrows indicate orthologous locations of mouse and rat model mutations and the most common FAP mutation sites. Reproduced with permission from William Dove (<http://www.mcardle.wisc.edu/dove/Data/Apc.htm>)

An alternative mechanism for *APC* gene inactivation may be hypermethylation of the *APC* promoter, which has been reported in 18 % of colorectal adenomas and carcinomas (Esteller et al. 2000).

2.2.4.2 Other Mutations in Wnt Pathway Components

Gain-of-function somatic mutations in β -catenin (*CTNNB1*) that affect key amino acids in β -catenin's N-terminal phosphorylation and ubiquitination motifs have been identified in a subset of CRCs, although they are common in other cancer types. However, these mutations have been found in 50 % of CRC with wild-type *APC*, which underscores the importance of the Wnt pathway in CRC (Sparks et al. 1998). A germline mutation in *AXIN2* was identified in a family with familial CRC and tooth agenesis, which suggests that the mutation may have interfered with the function of Axin in regulating β -catenin (Lammi et al. 2004).

2.2.4.3 KRAS

The RAS family of small G-proteins consists of K-RAS4A, K-RAS4B, H-RAS, and N-RAS, which are molecular switches downstream of growth factor receptors such as the epidermal growth factor receptor (EGFR) (Malumbres and Barbacid 2003). EGFR is affected by somatic mutations (e.g., point mutations or gene amplification) in fewer than 5 % of CRCs. In contrast, the *KRAS* oncogene is mutated in 40 % of CRC. Single nucleotide point mutations in codons 12 and 13 of exon 2, codon 146 in exon 4, and rarely in codon 61 of exon 3, lock the enzyme in the guanosine triphosphate bound (GTP), activated form, which leads to constitutive activation of RAS. A small number of CRCs have *NRAS* mutations at codon 12, 13, or 61. *KRAS* mutations are frequently found in aberrant crypt foci but are not required for adenoma initiation (Pretlow and Pretlow 2005). *KRAS* mutations are demonstrated in 10 % of adenomas smaller than 1 cm and 40–50 % of adenomas >1 cm, suggesting that *KRAS* plays a role in colorectal adenoma progression (Vogelstein et al. 1988). Targeted disruption of mutant *KRAS* alleles in CRC cell lines reduced cell growth, and activating *Kras*^{G12D} mutation accelerated tumor growth in a mouse model for sporadic CRC (Shirasawa et al. 1993; Hung et al. 2010).

The best characterized effector of *KRAS* is the Raf-mitogen-activated protein kinase (MEK)-extracellular signal-regulated kinase (ERK) pathway. The Raf family includes three serine–threonine kinases (A-RAF, B-RAF, and C-RAF) that phosphorylate MEK1 and MEK2, which then activate ERK1 and ERK2. ERK in turn activates substrates such as JUN and ELK1, transcription factors that regulate genes such as cyclin D1, which is involved in cell cycle control (Pruitt and Der 2001). RAS is linked to nuclear factor- κ B (NF- κ B), a transcription factor that regulates immune response and cell survival. TBK1 can activate NF- κ B by phosphorylating its inhibitor I κ B. TBK1 and NF- κ B signaling are essential in *KRAS*-mediated tumors; suppression of TBK1 induced apoptosis specifically in *KRAS*-transformed cancer cell lines, whereas inhibition of NF- κ B blocked RAS-induced formation of lung tumors in mice (Barbie et al. 2009; Meylan et al. 2009).

2.2.4.4 PIK3CA and PTEN

PIK3CA, the gene encoding the catalytic p110 α subunit of type I PI3Ks, is somatically mutated in 15–30 % of CRCs, most commonly in exons 9 (E532K, E545K) and 20 (H1047R) (Samuels et al. 2004). These *PIK3CA* mutations are oncogenic in CRC cell lines (Samuels et al. 2005). *PIK3CA* mutations predict reduced progression free survival in response to EGFR-inhibitor therapy (Souglakos et al. 2009). The PTEN protein is a phospholipid phosphatase that mediates dephosphorylation from PIP₃ to PIP₂. Germline mutations in the *PTEN* tumor suppressor gene are found in patients with Cowden syndrome, who demonstrate benign GI tumors but not an increased risk for CRC. However, approximately 10 % of sporadic CRCs exhibit somatic *PTEN* mutations, and loss of PTEN likely enhances PIP3-mediated

activation of AKT, which in turn acts on downstream antiapoptotic factors and the mTOR pathway. However, the significance of PTEN mutations in sporadic CRC is still unclear (Chalhoub and Baker 2009).

2.2.4.5 TP53

TP53 is located on chromosome 17p and encodes a transcription factor that is a tumor suppressor and master regulator of hundreds of genes involved in DNA metabolism, apoptosis, autophagy, cell cycle regulation, senescence, angiogenesis, immune response, cell differentiation, motility, and migration. P53 dysfunction is almost universal in human tumors, and loss of p53 function is reported in 4–26 % of adenomas, 50 % of adenomas with foci of carcinoma, and 50–75 % of CRC, which suggests that mutation and LOH of *TP53* plays a major role in the transition from adenoma to carcinoma (Leslie et al. 2002). Selection for *TP53* defects at the adenoma–carcinoma transition may reflect the fact that stresses such as DNA-strand breakage, telomere erosion, and hypoxia may activate apoptotic and cell-cycle arrest pathways in tumor cells with wild-type *TP53* function. As such, mutations in *TP53* may facilitate continued growth and invasion in the setting of stresses that might otherwise hinder tumor cell survival at the adenoma–carcinoma transition. Approximately 80 % of *TP53* mutations are missense mutations, which lead to the synthesis of a partially inactive protein. *TP53* is induced by oncogenic proteins such as c-Myc, RAS, and adenovirus E1A. *TP53* is normally negatively regulated by MDM2, E3-ubiquitin ligase, and MDM4, which target *TP53* for ubiquitination, while in stress situations *TP53* is allowed function (Levine 1997; Vogelstein et al. 2000).

2.2.4.6 Aneuploidy: 18q Loss

Allelic loss at chromosome 18q has been identified in as many as 70 % of CRCs, particularly at advanced stages. Candidate tumor suppressors located on 18q include *deleted in colorectal carcinoma (DCC)*, *SMAD2*, *SMAD4*, and *Cables*. *DCC* gene expression is absent or markedly reduced in a majority of advanced colorectal cancers (Fearon et al. 1990; Takagi et al. 1996; Mehlen and Fearon 2004). *DCC* encodes a receptor for netrin-1 and induces apoptosis unless bound to its ligand (Mehlen et al. 1998). However, a *DCC* mutant mouse model did not develop cancer, so doubts were raised about the role of *DCC* in carcinogenesis (Fazeli et al. 1997). A group led by Patric Mehlen recently reported that mice in which the proapoptotic activity of *DCC* is genetically silenced develop spontaneous intestinal neoplasia and, in an *Apc* mutant background, more invasive adenocarcinoma. Thus, *DCC* suppresses colorectal tumor formation via induction of tumor cell apoptosis (Castets et al. 2011). *SMAD2* and *SMAD4* mutations have been found in 10 % and 15 % of CRCs, respectively (Takagi et al. 1998). Mutations in *SMAD4* are found in a subset of patients with juvenile polyposis syndrome (JPS), which is characterized by

childhood onset of multiple hamartomatous polyps throughout the GI tract and an increased incidence of stomach, small intestinal, colon, and pancreatic cancers (Merg and Howe 2004). Cables protein increases tyrosine phosphorylation of cyclin-dependent kinases (cdk2, cdk3, and cdk5) by nonreceptor tyrosine kinases (Src, Abl, and Wee1). Loss of Cables expressions is found in 60–70 % of sporadic CRC, and loss of Cables in mice potentiates carcinogen-induced colonic tumorigenesis (Park et al. 2007; Kirley et al. 2005).

2.2.4.7 TGF- β Type II Receptor

Inactivating mutations in the TGF- β type II receptor (*TGF β IIIR* or *TGFBR2*) are found in approximately 25 % of CRCs, principally in those with MSI (see Sect. 3.5). In addition to MSI-associated tumors, somatic *TGF β IIIR* mutations are found in 15 % of MSS tumors. TGF- β -mediated receptor phosphorylation regulates the function of the SMAD2 and SMAD3 proteins (Grady et al. 1999).

2.2.4.8 Aneuploidy: Inactivation of CDC4 and Chromosome 1p Deletion

Chromosome 1p deletions occur at an early stage of colorectal carcinogenesis (Lothe et al. 1995; Bomme et al. 1994; Di Vinci et al. 1996) and are linked to karyotypic evolution during CRC development (Höglund et al. 2002). Introduction of chromosomal band 1p36 into CRC cell lines suppressed tumorigenicity (Tanaka et al. 1993). Interestingly, 76 % of patients with deletions in chromosome 1p in colorectal cancers were reported to harbor similar 1p deletions in distant normal-appearing mucosa (Cianciulli et al. 2004). Chromosome 1p deletions may influence carcinogenesis via loss of genes associated with DNA repair, spindle checkpoint function, apoptosis, miRNAs, the Wnt signaling pathway, tumor suppression, antioxidant functions, and defense against environmental toxins (Roschke et al. 2008; Negrini et al. 2010).

2.2.4.9 CMYC, CCNE1, and FBW7

The role of the *CMYC* gene in human cancer was first identified in the early 1980s, in the setting of chromosomal translocation in lymphoma and gene amplifications in small-cell lung cancer (Eilers and Eisenman 2008). The c-Myc protein is a transcription factor that regulates genes involved in cell-cycle progression and cellular survival. High and moderate copy amplification of the *CMYC* gene is seen in 10 % and 30 % of CRCs, respectively (Camps et al. 2009; Leary et al. 2008). Expression of *CMYC* is repressed by wild-type APC and activated by β -catenin, and this effect is mediated by TCF-4 binding sites in the *CMYC* promoter. *APC* inactivation may thus in part explain amplifications in *CMYC* expression (He et al. 1998).

High copy amplification of the cyclin E gene (*CCNE1*) is found in 5 % of CRCs, although modest increases are found in 15–20 % of CRCs (Leary et al. 2008; Bondi et al. 2005). More commonly, elevated cyclin E protein expression is due to inactivating mutations in the *FBXW7* gene, the human homologue of yeast gene *Cdc4*. Fbxw7/hCdc4 is a member of the F-box family of proteins, which acts as a substrate recognition component for the SCG ubiquitin ligase complex. Inactivation of Fbxw7/hCdc4 in CRC cells results in a CIN phenotype due to a defect in execution of metaphase (Rajagopalan et al. 2004). Fbxw7/hCdc4 mediates the ubiquitin-dependent proteolysis of several oncoproteins including cyclin E, c-Myc, c-Jun, and Notch (Tan et al. 2008). Somatic mutations that inactivate *FBXW7* are found in 9 % of CRCs (Akhoondi et al. 2007). Low tumor *FBXW7* mRNA expression corresponds to significantly poorer prognosis in CRC patients (Iwatsuki et al. 2010). Together, these data implicate *FBXW7* as a tumor suppressor in CRC.

2.2.4.10 CDK8

The *CDK8* oncogene, located at 13q12, is amplified in approximately 10–15 % of CRCs. CDK8 is a cyclin-dependent kinase that complexes with cyclin C to phosphorylate substrates such as RNA polymerase II and DNA-binding transcription factors. CDK8 kinase activity is necessary for β -catenin activity and for expression of several β -catenin transcriptional targets (Firestein et al. 2008). Overexpression of the *CDK8* gene is associated with increased CRC-related mortality (Firestein and Hahn 2009; Firestein et al. 2010).

2.2.4.11 COX2

Overexpression of cyclooxygenase-2 (COX2) is believed to play a role in CRC tumorigenesis. The *COX2* gene is overexpressed in 43 % of adenomas and 86 % of carcinomas (Eberhart et al. 1994), which is consistent with epidemiologic data for a protective role of aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) in CRC (Hahn et al. 2010; Garcia-Albeniz and Chan 2011; Ruder et al. 2011). Direct evidence for a role for COX2 in CRC carcinogenesis came from a study in which the number of small intestinal polyps in *APC Δ^{716}* knockout mice was reduced by 34 % when one copy of *COX2* was knocked out and by 86 % when both alleles were deleted (Oshima et al. 2001). A recent meta-analysis found that aspirin users in four randomized, placebo-controlled trials had a pooled risk ratio of 0.83 (95 % CI, 0.72–0.96) for any adenoma and 0.72 (95 % CI, 0.57–0.90) for advanced adenomas (Cole et al. 2009). Three randomized trials showed that the COX-2 selective inhibitors celecoxib and rofecoxib prevent adenoma recurrence among patients with a history of adenoma (Arber et al. 2006; Bertagnolli et al. 2006; Baron et al. 2006), but enthusiasm for chemoprevention of CRC with COX2 inhibitors was dampened after reports of increased cardiovascular mortality in the COX2 arms of these trials (Bresalier et al. 2005; Curfman et al. 2005).

2.2.4.12 LKB1

LKB1 is a tumor suppressor gene that encodes a ubiquitously expressed and evolutionarily conserved serine–threonine kinase, which in turn regulates a number of downstream kinases. *LKB1* inactivation leads to stimulation of the mammalian target of rapamycin (mTOR) pathway, which promotes cell division and growth. Deletion mutations in *LKB1* are found in a majority of cases of Peutz–Jeghers syndrome (PJS), a rare autosomal dominant syndrome. Loss of function *LKB1* mutations have also been identified in 5–15 % of sporadic nonsmall lung cancers and 5 % of pancreatic cancers and melanomas (Hezel and Bardeesy 2008).

2.2.5 Timing of CIN

Is CIN the cause or a consequence of colorectal carcinogenesis? A number of studies have found allelic imbalances in early stages of tumorigenesis; Shih et al. found allelic imbalances of at least one chromosomal arm in over 90 % of adenomas 2 mm in size (Bardi et al. 1997; Stoler et al. 1999; Shih et al. 2001; Cardoso et al. 2006). However, these studies did not ask whether CIN occurred before or after APC inactivation. Nowak et al. used a stochastic mathematical model to conclude that under a variety of conditions, CIN mutation is likely the initiating event or the second event following mutation of one allele of *APC* (Nowak et al. 2002). APC inactivation has been proposed as a potential initiator of CIN. Mouse embryonic stem cells with *APC* mutations, but not wild-type cells, became aneuploid and accumulated chromosomal abnormalities (Fodde et al. 2001; Kaplan et al. 2001), while other studies have found that Wnt signaling might contribute to CIN (Aoki et al. 2007; Hadjihannas et al. 2006). Chromosomal instability has not been conclusively linked to acquisition of key mutations required for colorectal carcinogenesis but is common in the early stages of malignancy and likely increases mutation rate and facilitates CRC progression.

2.2.6 Clinical Implications of CIN

Our insights into the genetic basis for CRC have allowed the identification of prognostic molecular markers. Patients with activating *KRAS* and *BRAF* mutations may experience worse overall survival outcomes compared to wild-type patients (Van Cutsem et al. 2011; Ogino et al. 2009a, 2011). Patients with tumor harboring *KRAS* and *PIK3CA* mutations are more likely to develop liver metastases compared to wild-type patients (Li et al. 2011). *TP53* mutation may be associated with greater mortality, but this risk may be limited to patients with metastatic disease (Munro et al. 2005; Russo et al. 2005). There are contradictory reports on whether deletion of chromosome 18q is associated with poor outcomes; individual chromosomal

deletions are currently used as molecular markers for CRC prognosis (Zhou et al. 2002; Diep et al. 2003; Ogino et al. 2009b).

Years of research on the molecular mechanisms of CRC are slowly translating into the clinic. Patients with *KRAS* mutant tumors do not appreciably respond to inhibition of the EGFR; use of agents such as Cetuximab is thus limited to patients with *KRAS* wild-type cancer (Karapetis et al. 2008). A recent phase I clinical trial examined treatment of *BRAF*^{V600E} CRC with Vemurafenib, a specific inhibitor of the *BRAF*^{V600E} protein and demonstrated mixed results, which suggest the presence of primary resistance mechanisms (Tol et al. 2009). Inhibition of the PI3K and downstream mTOR pathways has shown efficacy in a mouse model for *PIK3CA* wild-type CRC, and phase I clinical trials are planned (Roper et al. 2011). Small molecule inhibitors of Aurora kinase, Plks, and the spindle motor protein Eg5 have shown promise in preclinical studies and have demonstrated safety and antitumor efficacy in phase I human trials (Jani et al. 2010; Schöffski et al. 2012; Infante et al. 2012).

2.3 Microsatellite Instability in Colorectal Cancer

In 1993, Manuel Perucho and colleagues performed PCR amplification of thousands of sequences in colon cancer and matched normal tissue samples using randomly chosen primers. His group found that 12 % of the tumors had bands that were shorter in length. The sequences from these bands contained simple repetitive elements (i.e., microsatellites), primarily in polyadenine (A_n) tracts associated with *Alu* sequences. Further work revealed that tumors with these somatic mutations were associated with distinct clinical characteristics. The tumors were significantly more likely to arise in the proximal colon, less likely to be invasive, less likely to harbor mutations in *KRAS* or *TP53*, more likely to be poorly differentiated, and were found in younger patients (Ionov et al. 1993). Concurrently, the laboratory of Stephen Thibodeau identified deletion mutations in $[CA]_n$ sequences in chromosomes 5q, 15q, 17p, and 18q in colorectal tumors and coined the term *microsatellite instability*. Similar to Perucho's findings, Thibodeau's group reported MSI in 28 % of colorectal tumors and found that 89 % of tumors with MSI were located in the proximal colon and were associated with a better prognosis than MSS tumors (Blake et al. 2001; Thibodeau et al. 1993). Allotyping studies of CRC found that 15 % of CRCs had no apparent LOH; these tumors were later found to harbor MSI (Thibodeau et al. 1993; Vogelstein et al. 1989). Both the Perucho and Thibodeau groups recognized that microsatellite instability represents a unique pathway to CRC development.

2.3.1 DNA MMR System

Further investigations revealed that MSI arises from defects in the DNA mismatch repair (MMR) system, which is one of a number of DNA repair systems. In

prokaryotes, the MMR system consists of a family of enzymes encoded by the *mutS* and *mutL* genes that detect DNA replication errors in which the newly synthesized strand has incorporated the wrong nucleotide. These single base-pair mismatches usually result in point mutations. DNA polymerase is more likely to make such errors during replication of long repetitive DNA sequences such as microsatellites. Slippage during replication of a repetitive sequence results in formation of an insertion–deletion loop that can be identified and corrected by the MMR system. If this loop is not repaired a frameshift mutation results, which can produce a truncated, nonfunctional protein. This results in MSI (Boland and Goel 2010). In yeast, MMR is encoded by the genes *Mut S* homologue (*MSH*), *Mut L* homologue (*MLH*), and *postmeiotic segregation-1* (*PMS1*). Homologous copies of these genes are designated *MSH1* to *MSH6*, and *MLH1* through *MLH3*.

2.3.2 Lynch Syndrome

Lynch syndrome (also known as hereditary nonpolyposis CRC or HNPCC), one of the first inherited disease syndromes to be identified, was first described in 1913 by Warthin (1913). Many years later, Henry Lynch and colleagues further characterized kindreds with autosomal dominant patterns of CRC that lacked extensive polyposis. Patients with Lynch syndrome develop CRC at early ages, at a mean age of 40, and also present with extracolonic tumors of the endometrium, stomach, ovary, urinary tract, small intestine, and other sites (Vasen 2005). Without a putative genetic etiology to define the syndrome, the Amsterdam Criteria were developed to facilitate clinical diagnosis and research on families with clustering of CRC. According to these criteria, Lynch syndrome is defined as three CRC cases in a family in which one individual is a first-degree relative of the other two, CRC in at least two generations (in which FAP is excluded), and one affected family member younger than age 50 (Vasen et al. 1991). The Amsterdam II Criteria were developed in 1999 to include the presence of noncolonic tumors (i.e., cancer of the endometrium or small bowel, and transitional cell carcinoma of the ureter or renal pelvis) in the diagnosis (Vasen et al. 1999).

2.3.3 Sporadic MSI

Two of the three initial descriptions of MSI were made in samples from sporadic colon cancers, rather than tumors from patients with familial CRC (Ionov et al. 1993; Blake et al. 2001). Approximately 12–17 % of all colorectal tumors have MSI, whereas only 3 % of CRCs are identified in Lynch syndrome families; thus, most CRCs with MSI are sporadic (Ward et al. 2001; Hampel et al. 2005). Characteristically, sporadic CRC with MSI is associated with (1) absence of

significant clustering in families, (2) biallelic methylation of the *MLH1* promoter (Veigl et al. 1998), (3) absence of MLH1 and PMS2 proteins (not MSH2), (4) diploidy (74 %), (5) frequent mutation in *BRAF* (usually V600E) (Carragher et al. 2010), and (6) better prognosis than MSS tumors (Sinicrope et al. 2006). Nevertheless, MSI is associated with poorer survival in metastatic CRC in the context of *BRAF* mutation (Tran et al. 2011). Patients with sporadic CRC with MSI tend to be older than those with microsatellite stable sporadic CRC, and loss of *MLH1* expression increases with age (Kakar et al. 2003).

2.3.4 Epigenetic Changes in CRC and CpG Island Methylation Phenotype

Unlike colorectal tumors from Lynch syndrome, sporadic CRC with MSI arises via a mechanism involving the CIMP (Toyota et al. 1999). The combination of a cytosine nucleotide followed by a guanine nucleotide (CpG dinucleotide) is relatively uncommon in the human genome. However, pockets of CpG dinucleotides, termed CpG islands, are found in the promoter regions of approximately 50 % of all genes (Bird 1986). The addition of a methyl group to cytosine bases in these CpG regions (i.e., DNA methylation) has been associated with silencing of genes that encode tumor suppressors (e.g., *p16*, *insulin-like growth factor 2*, and *HIC1*), DNA repair genes such as *methylguanine methyltransferase (MGMT)* and *MLH1*, and Wnt signaling antagonists known as SFRPs (secreted Frizzled-related proteins), leading to cancer (Jones and Laird 1999; Kim et al. 2010a). Hypermethylation of *MLH1* is the major cause of MSI in sporadic CRC (Kane et al. 1997). Other tumor suppressor genes are also more commonly silenced by methylation in MSI associated, compared to MSS-associated CRC; this lead to the observation that 20–30 % of colorectal cancers are associated with hypermethylation of CpG islands—a phenomenon that was termed CIMP (Benatti et al. 2005; Des Guetz et al. 2010). A subsequent study used a more sensitive method for detecting methylation to develop a more specific classification of CIMP and found the phenotype in 18 % of colorectal tumors (Weisenberger et al. 2006). Although most sporadic MSI-associated tumors have CIMP, half of all tumors with CIMP do not have methylation of *MLH1* or MSI (Samowitz et al. 2005a; Hawkins et al. 2002). Many of these tumors carry *BRAF* mutations and arise from the serrated pathway (discussed later in this chapter) (Leggett and Whitehall 2010).

In contrast to the specific hypermethylation found in CpG islands, in benign and malignant colorectal tumors there is an overall decrease in total DNA methylation (i.e., hypomethylation) compared to adjacent normal tissue, perhaps leading to activation of oncogenes, though the functional significance of this finding is still unclear (Goelz et al. 1985; Feinberg et al. 1988). DNA hypomethylation of pericentrosomic sequences may impair chromosomal segregation, a theory that would link hypomethylation to the CIN pathway (Ji et al. 1997).

2.3.5 Pathophysiology of Colorectal Carcinogenesis with MSI

In 1995, Markowitz et al. examined the role of transforming growth factor- β (TGF- β) in MSI; TGF- β signaling inhibits proliferation of colonic epithelial cells. They found that *transforming growth factor B (TGF- β) type II receptor (TGF β R2)* was not expressed in CRC cell lines with MSI but was expressed in MSS cell lines. Those cell lines without TGF β R2 expression did not slow proliferation in response to TGF- β . The group further demonstrated that a single base-pair deletion in a repetitive A₁₀ sequence in TGF β R2 was found in 90 % of 111 MSI-positive colorectal tumor samples, which suggested a model in which repetitive DNA sequences are sensitive to loss of DNA MMR activity, leading to frameshift mutations, premature stop codons, and gene inactivation (Markowitz et al. 1995). An additional tumor suppressor gene in MSI-H CRC is *ACVR2A*, which encodes the activin type II receptor. Both alleles of the *ACVR2A* gene are somatically mutated in a polyadenine repeat tract at exon 10 in approximately 85 % of MSI-H CRCs. The resulting frameshift mutation is associated with loss of the activin type II receptor and poorer prognosis. MSI-H CRC cells in which *ACVR2* or *TGF β R2* function has been restored exhibit slower growth (Jung et al. 2006, 2007). Approximately one-third of MSI-H CRCs harbor mutations in a repeat tract of the *TCF7L2* gene, which encodes the TCF4 protein. TCF4 suppresses DNA transcription of Wnt pathway target genes in the setting of stabilized β -catenin, which may provide an additional pathway for Wnt activation in MSI-H cancer (Cuilliere-Dartigues et al. 2006).

Several other genes affected by MSI have since been identified that encode regulators of cellular proliferation (*GTB1*, *TCG-4*, *WISP3*, *insulin-like growth factor-2 receptor*, *axin-2*, and *CDX2*), cell cycle (*BAX*, *caspase-5*, *RIZ*, *BCL-10*, *PTEN*, *hG4-1*, and *FAS*), and DNA repair (*MBD-4*, *BLM*, *CHK1*, *MLH3*, *RAD50*, *MSH3*, and *MSH6*) (O'Brien et al. 2006). However, it is unclear which of these mutations are of functional significance (as has been determined for TGF β R2) and which are simply markers of MSI, because biallelic inactivation of these genes has not been documented in all of the tumors. For instance, a recent retrospective study found no association between *BAX* mutations in MSI-H tumors and patient survival (Shima et al. 2011). Genes associated with MSI in CRC are summarized in Table 2.2. The key steps in the MSI pathway to CRC are outlined in Fig. 2.4.

The discovery of multiple genetic targets of MMR deficiency that differ from the classic Fearon and Vogelstein model indicates that MSI-associated CRC occurs via a different biological pathway than conventional MSS tumors. Tumors in the CIN pathway arise from a combination of genetic mutations and LOH, resulting in biallelic inactivation of APC. Colorectal tumors with MSI, on the other hand, harbor an increased number of point mutations compared to MSS cancers, are more likely to be diploid, and do not exhibit widespread LOH. A vast majority of MSI-associated tumors have normal expression of APC but have mutations in β -catenin that prevent binding to the APC protein and degradation, which is functionally equivalent to loss of the APC protein (Miyaki et al. 1999; Mirabelli-Primdahl et al. 1999). Other MSI-associated tumors have neither inactivated APC nor mutated β -catenin but instead have frameshift mutations in other Wnt pathway factors such as *TCF-4* (Boland and Goel 2010).

Table 2.2 Genes that regulate chromosomal instability

Microsatellite length	Gene
A10	<i>AIM2</i>
	<i>CASPASE-5</i>
	<i>MBD-4</i>
	<i>OGT</i>
	<i>SEC63 (also, A9)</i>
	<i>TGFBR2</i>
A9	<i>BLM</i>
	<i>CHK1</i>
	<i>GRB-14</i>
	<i>MLH3</i>
	<i>RAD50</i>
	<i>RHAMM</i>
	<i>RIZ (also, A8)</i>
	<i>TCF-4</i>
A8	<i>WISP3</i>
	<i>ACVR2</i>
	<i>APAF</i>
	<i>BCL-10</i>
	<i>hG4-1</i>
	<i>MSH3</i>
A6	<i>PTEN</i>
T10	<i>OGT</i>
T9	<i>KIAA0971</i>
G8	<i>NIADH-UOB</i>
	<i>BAX</i>
	<i>IGF2R</i>
C9	<i>SLC23A1</i>
C8	<i>MSH6</i>
G7	<i>AXIN-2</i>
T7	<i>CDX2</i>
	<i>FAS</i>

Modified from Duval A, Hamelin R. Mutations at coding repeat sequences in mismatch repair-deficient human cancers: toward a new concept of target genes for instability. *Cancer Res* 2002;62(9):2447–54

2.3.6 MSI and Inflammatory Bowel Disease

CRC risk is increased in inflammatory bowel disease, but the mechanisms are not well established. Inflammation may increase mutagenesis via generation of oxidative stress and free radicals that may promote proliferation of colorectal cells. Although seemingly paradoxical, oxidative stress can inactivate the DNA MMR system and is associated with an increased mutation rate (Lee et al. 2003; Chang et al. 2002; Gasche et al. 2001). MSI has been identified in colorectal cancers of patients with ulcerative colitis; 21 % of 63 colitis-associated tumors and areas of dysplasia had at least 1 of 5 dinucleotide repeat markers mutated (Suzuki et al. 1994). Interestingly, MSI has been found in at least 1 of 7 dinucleotide repeat

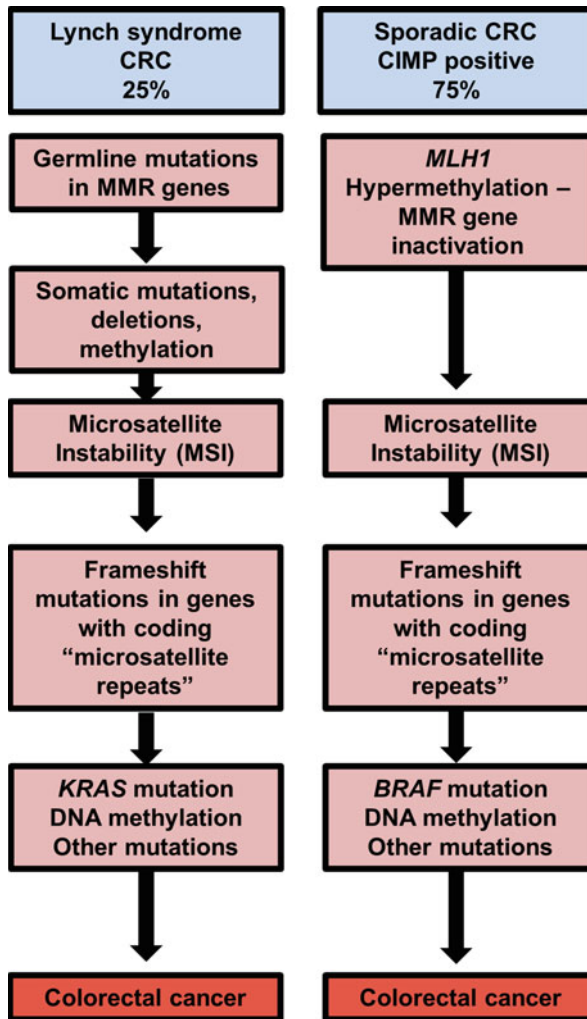


Fig. 2.4 Molecular pathways to MSI-associated colorectal cancer (CRC). Approximately 25 % of MSI-associated CRC arises from the Lynch syndrome, in which inactivating germline mutations in MMR genes are inherited in an autosomal dominant pattern. Additional “second hits” to the wild-type copy of the gene (inherited from the unaffected parent) in the form of somatic mutations, deletions, and methylation lead to MSI. 75 % of MSI-associated CRC is sporadic. These cancers are associated with CIMP and undergo MMR gene inactivation via hypermethylation of the *MLH1* promoter. Both Lynch syndrome and sporadic CIMP positive-associated defects in MMR lead to MSI and rapid accumulation of somatic mutations in genes with coding “microsatellite repeats.” Many of these microsatellite repeats may not contribute to carcinogenesis but provide a signature that can be used for identification of MSI. Lynch-associated CRCs often harbor *KRAS* mutations, whereas sporadic CIMP-associated tumors are often *BRAF* mutant. Modified from Boland CR, Goel A (2010) Microsatellite instability in colorectal cancer. *Gastroenterology* 138(6):2073–2087.e3

markers in 50 % of nonneoplastic tissue in patients with chronic ulcerative colitis, but not in controls with acute infectious colitis (Brentnall et al. 1996).

2.3.7 *Clinical Diagnosis of MSI*

The definition of MSI was standardized at an international consensus meeting in 1997. The term “MSI” refers to MSI-high, in which >30 % of a defined microsatellite marker panel is mutated. Those CRCs in which at least 1, but <30 %, of the markers are mutated are called MSI-low and have clinical features of MSS tumors (Boland et al. 1998). Another type of MSI has been recognized, called “elevated microsatellite alterations at selected tetranucleotide repeats” (EMAST). EMAST is largely found in noncolonic tumors such as lung, is associated with *TP53* mutations, and is not caused by inactivation of the MMR system (Ahrendt et al. 2000).

MSI testing is used clinically to identify patients with Lynch syndrome, which comprises 2–3 % of all CRCs. MSI identifies MMR-deficient colorectal tumors with 93 % sensitivity, whereas the sensitivity and specificity of immunohistochemical analysis of MLH1 and MSH2 is 92.3 % and 100 %, respectively (Shia 2008). The sensitivity of IHC improves with expression of MSH6 and PMS2 are included in the analysis. Staining of tumors for MMR proteins can be heterogeneous, which may limit sensitivity (Shia 2008; Zhang 2008). MSI-H tumors can also be distinguished from MSS tumors by the presence of tumor-infiltrating cytotoxic lymphocytes on histologic examination, the degree of which independently confers improved survival (Ogino et al. 2009c; Phillips et al. 2004).

2.3.8 *MSI and Response to Chemotherapy*

The MMR phenotype is associated with resistance to cytotoxic agents in human CRC cell lines such as HCT-116 (Bhattacharyya et al. 1994). Stable restoration of MMR activity in cell lines increases sensitivity to alkylating agents, 6-thioguanine, 5-fluorouracil, and platinum compounds (Mäkinen et al. 2001; Samowitz et al. 2005b; Chan et al. 2002; Wynter et al. 2004; O’Brien et al. 2004; Minoo et al. 2006). With the exception of one study with potential methodological flaws (Elsaleh et al. 2000), multiple studies, including two meta-analyses, have shown no benefit for chemotherapy among patients with MSI-associated colorectal tumors (de Vos tot Nederveen Cappel et al. 2004; Ribic et al. 2003; Storojeva et al. 2005; Benatti et al. 2005; Popat et al. 2005; Lanza et al. 2006; Jover et al. 2006; Kim et al. 2007; Des Guetz et al. 2010). The largest such study, a prospective, multicenter, randomized controlled trial, a threefold increased mortality was found in Stage II CRC patients with MSI-associated tumors compared to without (Ribic et al. 2003). However, MSI is associated with improved response to regimens containing a topoisomerase I inhibitor, irinotecan (Bertagnolli et al. 2009; Fallik et al. 2003).

2.4 The Serrated Pathway in Colorectal Cancer Pathogenesis

Colorectal polyps have traditionally been classified as either hyperplastic or adenomatous, with only the latter progressing into carcinoma. However, beginning in the late 1980s, an increasing number of reports suggested that CRC can arise from hyperplastic polyps in the setting of what is now known as the hyperplastic polyposis syndrome (HPS), in which a large number of hyperplastic polyps are found throughout the colon (these polyps are distinguished from typical hyperplastic polyps, which are small, left sided, and benign) (Samowitz et al. 2006; Ji et al. 2006; Pérez et al. 2010; Shrubsole et al. 2008; Chirieac et al. 2005; Ogino et al. 2006b; Ward et al. 2003; Glazer et al. 2008). These studies identified a 35 % risk of CRC in patients with HPS, as well as increased risk of synchronous cancers (Boparai et al. 2010). Polyps in patients with HPS are characterized by gland serrations, which led pathologists to reexamine the malignant potential of other polyps with histologic serrations. Data from screening colonoscopy cohorts have demonstrated that serrated polyps are strongly associated with the development of synchronous and metachronous advanced adenoma and CRC (Li et al. 2009; Schreiner et al. 2010).

2.4.1 *Classification of Serrated Polyps*

Serrated polyps are characterized by a “sawtooth” pattern, or serrations, in the colonic crypts. In 1990, Longacre and Fenoglio-Preiser proposed the term “serrated adenoma” for polyps exhibiting features of both adenomatous and hyperplastic polyps (Longacre and Fenoglio-Preiser 1990). In 1996, Torlakovic and Snover first showed that polyps in HPS have serrated features similar to serrated adenomas, though with less atypia, and were more likely to be sessile than standard hyperplastic polyps (Torlakovic and Snover 1996). Further detailed work identified a subset of serrated polyps with abnormal proliferation, crypt distortion, and dilation that were typically sessile and found on the right side of the colon. These polyps were distinguished from traditional serrated adenomas (TSAs), which more closely resembled conventional adenomas (Torlakovic et al. 2003). These findings eventually led to a proposal for a new nomenclature for serrated polyps in 2005 (Snover et al. 2005).

The use of the term “adenoma” to describe sessile lesions has been controversial because conventional adenomas are dysplastic, whereas SSAs lack cytological dysplasia, though they manifest disordered proliferation and crypt architecture. Robert Odze and colleagues have thus opted for the term “sessile serrated polyp” in a recent pathology textbook (Hornick and Odze 2009), whereas a recent European publication chose the term “sessile serrated lesion.” (Lambert et al. 2009) As the term SSA has grown in research and clinical practice, we will use it in this chapter.

2.4.1.1 Hyperplastic Polyp

Hyperplastic polyps (HPs) have a narrow crypt base lined with proliferative cells and serrations in the upper third of the gland. HPs have been subdivided into goblet cell-rich type, microvesicular type (which are precursors to SSAs), and the rare mucin-poor variant. Overall, HPs are highly prevalent sessile lesions that are commonly located in the distal colon and rectum (Tedesco et al. 1982; Imperiale et al. 2002). Endoscopically, HPs are identified by their smooth, symmetrical, and pale appearance. Microvesicular type HPs are precursor lesions to SSAs and, like SSAs, harbor *BRAF*V^{600E} mutations. Goblet cell HPs, on the other hand, often contain *KRAS* mutations (43 % in one study), which are mutually exclusive of *BRAF* mutations (O'Brien et al. 2006). Large goblet cell HPs may progress into *KRAS* mutant dysplastic serrated polyps (Boparai et al. 2008).

2.4.1.2 Sessile Serrated Polyp

Sessile serrated adenomas (SSAs) are characterized by crypt architectural alterations that reflect disordered growth. These include serration of the epithelium, often at the base of the crypts; dilation of the base of the crypts; and T- or L-shaped crypts. SSAs may contain areas of cytologic dysplasia and adenocarcinoma; tumors with neoplastic progression tend to lose serrated features (Fujita et al. 2011). SSAs likely evolve from preexisting microvesicular type HPs (Spring et al. 2006). Endoscopically, SSAs are usually larger than 5 mm, flat or sessile (height one half or less than width), and often mucous covered (Jaramillo et al. 2005). They are generally larger than HPs and located in the proximal colon. The surface is often smooth, and the edges are poorly defined and irregular. These features make SSAs difficult to detect endoscopically (Higuchi et al. 2005).

2.4.1.3 Dysplastic Serrated Polyps

Dysplastic serrated polyps contain gland serrations and cytologic dysplasia. There are two categories of dysplastic serrated polyp (1) SSA with dysplasia, which exhibits SSA morphologic characteristics contiguous to an area of conventional dysplasia and (2) TSA, which has not only serrations but also dysplastic epithelial cells and ectopic crypts with bases not adjacent to the muscularis mucosa, in contrast to SSAs in which new crypts are generally anchored to the muscularis mucosa. TSAs differ from SSAs in that they are typically distally located, polypoid, contain tubulovillous architecture, and marked cytoplasmic eosinophilia (O'Brien 2007; Torlakovic et al. 2008). TSAs not only are frequently *KRAS* mutant (which heralds an aggressive phenotype) but may also be *KRAS/BRAF* wild-type or *BRAF* mutant (Kim et al. 2010b).

2.4.2 Epidemiology

It is estimated that up to 20 % of CRCs arise from the serrated pathway, or nearly 30,000 cases annually (Jass 2007). One study reported a prevalence of 29 % HPs, 9 % SSAs, 1.7 % mixed polyps, and 0.7 % TSAs from a cohort of colonoscopy-resected specimens (Spring et al. 2006). Dysplastic serrated polyps are much less common than conventional polyps or HPs, representing 1–2 % of all polyps (Higuchi et al. 2005; Jass et al. 2006).

The molecular basis of the serration of the crypt epithelium has not been determined, though it has been proposed that serrations occur due to cell crowding or because of failure of apoptosis or anoikis (Tateyama et al. 2002). Crypt serration is strongly associated with the presence of BRAF mutation; hyperplastic polyps with KRAS rather than BRAF mutation have less, or absence of, gland serration.

2.4.3 Serrated Polyps, MSI, CIMP, and BRAF

Tumors associated with HPS have a higher than expected incidence of MSI (Leggett and Whitehall 2010; Jeevaratnam et al. 1996; Rashid et al. 2000; Jass et al. 2000). Serrated polyps from colectomy specimens were more likely to have MSI than MSS, and another study found that MSI was more common in serrated adenomas than in control tumors (37.5 % vs. 11 %, respectively) (Hawkins and Ward 2001; Mäkinen et al. 2001). O'Brien et al. found MSI only in the areas of advanced SSAs with carcinoma, which suggests that MSI develops late in the serrated pathway. Epigenetic silencing of *MLH1* is the underlying cause of MSI in serrated lesions and is an important driver of the progression to invasive cancer (O'Brien et al. 2006). A large proportion serrated cancers are MSS and frequently have *TP53* mutation, which may explain their more aggressive phenotype and poorer prognosis than MSI-associated tumors [hazard rate ratio (HRR), 2.97; 95 % CI, 2.05–4.32] (Samowitz et al. 2005b).

CIMP is commonly observed in both HPs and in proximal SSA (Chan et al. 2002; Wynter et al. 2004; O'Brien et al. 2004). Yang et al. detected CIMP in microvesicular HP (47 %), SSA (75 %), and TSA (80 %). Using a narrower definition of methylation ($\geq 4/5$ markers), CIMP was detected in 11 % of MVHP, compared to 40 % of SSA²⁸⁴. CIMP has even been detected in histologically normal colonic mucosa of HPS patients (Minoo et al. 2006). Higher CIMP levels (four or more markers positive) were more frequently found in SSAs (with or without carcinoma) than in conventional adenomas or carcinomas (O'Brien et al. 2006). Together, these data indicate that methylation of specific CIMP loci may facilitate the transition from microvesicular HP to SSA.

In a systematic genome-wide screen for genes affecting cell proliferation and death, activating mutations in *BRAF* were identified in a high proportion of melanomas and in a small fraction of other cancers including colon. BRAF is a serine/

threonine kinase that is part of the mitogen-activated protein kinase (MAPK) cell signaling pathway; mutations in *BRAF* result in constitutive activation of the MAPK pathway and transcription of genes promoting cell growth and proliferation (Davies et al. 2002). Rajagopalan et al. sequenced *BRAF* and *KRAS* mutations in colorectal tumors and found that (1) 10 % of tumors harbored somatic mutations in *BRAF* and (2) no tumors exhibited mutations in both *BRAF* and *KRAS* (Rajagopalan et al. 2002). Another group confirmed these findings (Yuen et al. 2002). Chan et al. examined *BRAF* and *KRAS* mutations in a series of serrated polyps and found *BRAF* mutations in 36 % of HPs and 100 % of SSAs. The *BRAF*^{V600E} substitution is the most common *BRAF* mutation in human cancers including serrated CRCs (Davies et al. 2002). Using current histologic definitions, 70–76 % of MVHPs and 75–83 % of SSAs have *BRAF*^{V600E} mutations. *BRAF* and *KRAS* mutations are mutually exclusive (O'Brien et al. 2006). The *BRAF*^{V600E} mutation was found in 5 % of a cohort of MSS tumors and 52 % of MSI-associated tumors (Samowitz et al. 2005b). However, histological reviews have confirmed that *BRAF* is almost never mutated in conventional adenomas or in Lynch syndrome, highlighting the association of *BRAF* mutation with the serrated pathway rather than MSI (O'Brien et al. 2006; Kambara et al. 2004; Wang et al. 2003). Mutation of *BRAF* strongly correlates with CIMP (Weisenberger et al. 2006). These findings support the role of CIMP and the MAPK pathway via activating mutation in *BRAF* or *KRAS* in the serrated adenoma pathway.

2.4.4 Initiation and Progression of the Serrated Pathway

Activation of *BRAF* in normal melanocyte epithelium and in mouse gastrointestinal epithelium results in an initial burst of proliferation followed by cell senescence (Carragher et al. 2010; Campisi 2005). Methylation-induced silencing of *p16INK4a* is an early event in the serrated pathway and may be sufficient to allow colorectal cells (and possible microvesicular HPs) to escape *BRAF*-induced senescence (Chen et al. 2005). In melanocytes, activated *BRAF* is sufficient for synthesis and secretion of insulin-like growth factor binding protein 7 (IGFBP7) which in turn inhibits MAPK signaling and induces senescence and apoptosis (Wajapeyee et al. 2008). The large columnar vacuolated cells of the upper crypts of the microvesicular HP and SSA are a manifestation of cell senescence (Minoo and Jass 2006). Silencing via methylation of *IGFBP7* in *BRAF*-mutant, CIMP-positive CRC cells permits unrestrained cell proliferation and progression to SSA by enabling escape from p53-induced senescence (Suzuki et al. 2010). Therefore, the additive tumorigenic effects of mutated *BRAF* and *CIMP* may result from silencing of tumor suppressor genes such as *p16INK4a* and *IGFBP7* via hypermethylation.

The Wnt signaling pathway is another major regulator of cellular proliferation in CRC. In the absence of APC protein, β -catenin accumulates in the cell nucleus instead of undergoing degradation. Three studies found positive nuclear β -catenin immunostaining in 0–50 % of HPs and 38–67 % of SSAs, 36 % of TSAs, and 100 % of tubular adenomas (TA). 29 % of SSAs without dysplasia and all SSAs with

dysplasia displayed aberrant β -catenin staining. Nuclear β -catenin was identified only in the setting of *BRAF*^{V600E} mutation (Wu et al. 2008; Yachida et al. 2009; Sandmeier et al. 2009). A recent histological study found that nuclear β -catenin staining in SSA was limited to dysplastic areas of the polyps, and histologically these dysplastic areas lost serrated features and become more tubulovillous (Fujita et al. 2011). Unlike conventional adenomas, however, *APC* mutation is found in only a minority (19 %) of serrated polyps, and β -catenin gain-of-function mutation in *CTNNB1* has not been identified in serrated polyps (Yachida et al. 2009; de Vogel et al. 2009). A mouse model for *BRAF*^{V600E} CRC demonstrated that expression of *BRAF*^{V600E} in intestinal crypts was sufficient for β -catenin nuclear localization via MAPK-dependent, Akt-independent phosphorylation of Gsk3 β (Carragher et al. 2010). However, this mechanism of Wnt activation has not been confirmed in the human serrated pathway. These findings suggest that activation of the Wnt signaling pathway follows *BRAF* mutation and plays an important role in the progression (but not initiation) of the serrated pathway. The molecular steps in the initiation and progression of sessile serrated adenomas are summarized in Fig. 2.5.

2.4.5 An Alternate Serrated Pathway

Recognition of the heterogeneity of serrated polyps has led to the hypothesis that there are two parallel serrated pathways to colorectal carcinogenesis: one driven by *BRAF* mutation and the other driven by *KRAS* mutation (O'Brien et al. 2006; O'Brien 2007; Yang et al. 2004). The *BRAF* pathway has been discussed in detail above. *KRAS* mutant serrated carcinomas have relatively low levels of CIMP, but it is possible that rather than being a true CIMP-low group, these cancers are methylated at different loci (Weisenberger et al. 2006). Silencing of the DNA repair gene *MGMT* by promoter hypermethylation has been associated with *KRAS* mutation and CIMP-low status (Ogino et al. 2006a, 2007; Whitehall et al. 2001). However, no specific panel of markers has been validated to study this pathway. No precursor lesion to *KRAS* mutant serrated carcinomas has been identified, though it has been proposed that large goblet cell HPs, tubulovillous adenomas, and/or serrated polyps with dysplasia may be relevant to the “alternate pathway” (Boparai et al. 2008; Jass et al. 2006).

2.4.6 Risk Factors for Serrated CRC

Susceptibility to serrated neoplasia may be associated with a genetic predisposition to hypermethylation of gene promoters. Rare families with multiple members affected by HPS have been described (Jeevaratnam et al. 1996; Rashid et al. 2000; Chow et al. 2006). Most cases of CIMP-high, *BRAF* mutant serrated polyps appear to be sporadic, although a few families with high incidences of CRC and serrated polyps have been identified (Des Guetz et al. 2010)^{306,307}. However, residents of

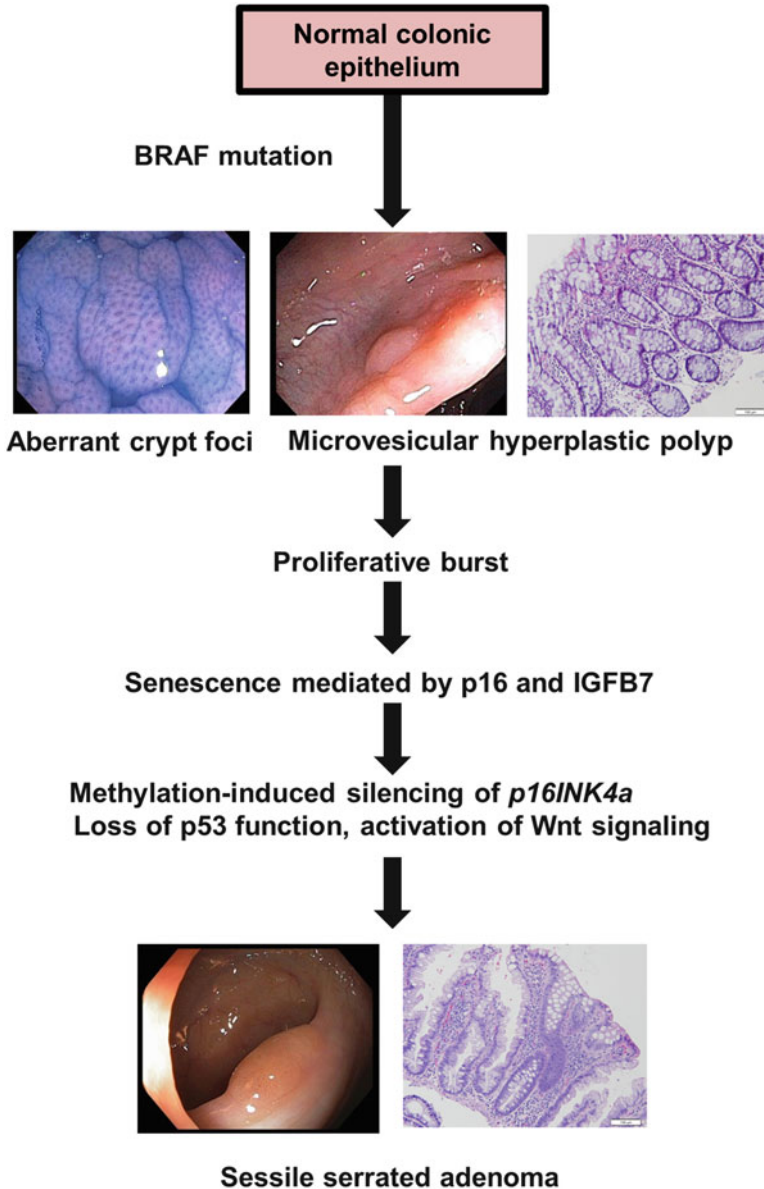


Fig. 2.5 The sessile serrated pathway. Activation of BRAF induces the formation of ACF with serrated features and microvesicular hyperplastic polyp (MVHP). Further cell proliferation is controlled by cell senescence, which is mediated by *p16INK4a* expression and IGFBP7 secretion. Methylation-induced silencing of *p16INK4a* or loss p53 function allows early polyps to escape the senescent state and develop into sessile serrated adenomas. Endoscopic images courtesy of Moises Guelrud, Tufts Medical Center; pathology images courtesy of Barbara Weinstein, Tufts Medical Center. Modified from Leggett B, Whitehall V (2010) Role of the serrated pathway in CRC pathogenesis. *Gastroenterology* 138(6):2088–2100

Melbourne, Australia of Anglo-Celtic origin were found to have a higher risk of CIMP and *BRAF* mutant CRC compared to those of southern European origin, and serrated polyps were more frequent in Caucasians compared to Hispanics and African Americans (English et al. 2008; Wallace et al. 2009). Cigarette smoking has been strongly associated with CIMP and *BRAF* mutation and is a stronger risk factor for HPs than adenomatous polyps in multiple studies, although one report found no association between smoking and HPs (Samowitz et al. 2006; Ji et al. 2006; Pérez et al. 2010; Shrubsole et al. 2008). Aspirin is protective against serrated polyps, as with conventional polyps (Wallace et al. 2009). A study on risk factors for CRC found that obesity, smoking, dietary fat, caloric intake, and red meat intake were associated with increased risk for distal, but not proximal, serrated polyps (Wallace et al. 2009).

2.4.7 Clinical Characteristics of Serrated CRC

The presence or absence of *BRAF* mutation does not affect the excellent prognosis of MSI-associated CRC (Samowitz et al. 2005b). Cancers that arise via the serrated pathway, whether with or without MSI, tend to be proximal, mucinous, occur in older individuals, and present at more advanced stage (Samowitz et al. 2005a; Hawkins et al. 2002; Chirieac et al. 2005; Ogino et al. 2006b). In the context of MSS, increased DNA methylation and *BRAF* mutation is associated with worse prognosis (Weisenberger et al. 2006; Ward et al. 2003). Serrated polyps are strongly associated with synchronous advanced neoplasia (defined as invasive carcinoma, tubular adenoma 1 cm, or adenoma with any villous histology or high-grade dysplasia), particularly proximal CRCs, in large colonoscopy cohort studies (Glazer et al. 2008; Hiraoka et al. 2010; Li et al. 2009; Schreiner et al. 2010).

HPS is an uncommon condition characterized by multiple and/or large HPs. Several reports of CRC in patients with HPS led to the hypothesis that serrated polyps may develop into CRC (Jeevaratnam et al. 1996). The incidence of CRC in HPS is estimated to be 40–50 % (Buchanan et al. 2010; Leggett et al. 2001; Rubio et al. 2006). Type I HPS is defined multiple (five or more), large, proximally located SSAs. There is a high frequency of CIMP and mutated *BRAF*. Type II HPS, a more heterogeneous condition, describes the finding of numerous (≥ 30) small HPS distributed throughout the colon, and is believed to have a lower risk of CRC than type I HPS (Ferrández et al. 2004). Although the syndrome has no proven genetic basis, there are reports of familial HPS and ethnic associations in population studies (Young and Jass 2006; Young et al. 2007).

2.4.8 Detection and Surveillance of Serrated Polyps

Detection of serrated polyps via currently available screening modalities may be difficult. Serrated polyps are less likely to bleed, and hence may not be detected by

fecal occult blood testing (East et al. 2008). CT Colonography may be less likely to detect flat or sessile lesions, though this has not been studied. Colonoscopy performs relatively poorly in the detection of serrated polyps, which may partly explain findings that mortality rates from left-sided CRC, but not right-sided CRC, have decreased in recent years (Baxter et al. 2009; Brenner et al. 2010). This may be due to poor colonic prep on the right side of the colon and/or poor visualization of flat, mucous-covered lesions. Randomized trials have demonstrated that chromoendoscopy improves detection of serrated polyps by twofold. The importance of detection and removal of serrated polyps is highlighted by the findings that interval cancers (found despite appropriate screening or surveillance colonoscopy) were four times as likely to be associated with MSI (Sawhney et al. 2006) and CIMP, and more likely to be proximal and mucinous, which are all features suggestive of *BRAF* mutation (Leggett et al. 1997; Farrar et al. 2006; Bressler et al. 2004; Arain et al. 2010).

2.4.9 Models of the Serrated Pathway

Isogenic *BRAF*^{V600E} human CRC cell lines (VACO432 and RKO) have been developed in which either the endogenous wild-type or mutant allele has been inactivated through targeted homologous recombination (Yun et al. 2009). Carragher et al. published a Cre-lox-regulated knockin mouse in which *Braf*^{V600E} is expressed from the endogenous *Braf* gene in the proliferative cells of the intestinal crypts. They showed that intestinal *Braf*^{V600E} is not only sufficient for formation of hyperplastic crypts via activation of the MAPK and Wnt pathways but also induces cell senescence, and that inactivation of *p16INK4a* through DNA methylation is necessary for tumor progression. However, polyps in this model are adenomas, not carcinomas, and are confined to the small bowel (Carragher et al. 2010). Kenneth Hung and colleagues developed a novel genetically engineered mouse model in which mice with a conditional *Apc* allele were crossed with those with a latent *Braf*^{V600E} allele. They showed that combination treatment with BRAF and PI3K/mTOR inhibitors was required to induce apoptosis and tumor regression. This model offers several advantages for preclinical drug testing (1) solitary tumors develop rapidly along a reproducible time line in the colon; (2) tumors can be continuously monitored throughout drug treatment via colonoscopy; and (3) tumors recapitulate the serrated pathway seen in humans, including HPs, SSAs, SSAs with dysplasia, and SSAs with congruent invasive adenocarcinoma (Coffee et al., manuscript under review).

2.5 Conclusions

CRC continues to be a significant public health burden. Whereas there have been significant advances in the development of targeted therapies, the 5-year prognosis for metastatic CRC still continues to be less than 10 %. However, our increased understanding of the molecular events underlying CRC carcinogenesis will enable

the development of new targeted therapies and the identification of clinical biomarkers that will inform their effective usage. This is an exciting time for cancer medicine and we believe that the field is poised to make significant therapeutic breakthroughs.

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Molecular Pathogenesis of Colorectal Cancer

Haigis, K.M. (Ed.)

2013, VIII, 316 p. 30 illus., 29 illus. in color.,

ISBN: 978-1-4614-8412-7