

Molecular Complexity of Colorectal Cancer: Pathways, Biomarkers, and Therapeutic Strategies

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Abstract: Colorectal cancer (CRC) is a diverse disease entity and a leading cause of cancer-related mortality worldwide. CRC results from the accumulation of multiple genetic and epigenetic alterations. This heterogeneity of CRC underscores the significance of understanding its molecular landscape, as variations in tumor genetics can greatly influence both patient prognosis and therapeutic response. The molecular complexity of CRC is defined by three major carcinogenesis pathways: chromosomal instability (CIN), microsatellite instability (MSI), and the CpG island methylator phenotype (CIMP). These pathways contribute to the onset and progression of CRC through mutations, epigenetic modifications, and dysregulated cellular signalling networks. The heterogeneous nature of CRC continues to pose challenges in identifying universally effective treatments, highlighting the need for personalized approaches. Hence, the present review aims at unravelling the molecular complexity of CRC that is essential for improving diagnosis, prognostication, and treatment. We detail on the current understanding of the molecular framework of CRC, central signalling pathways of CRC associated with its initiation to a malignant phenotype, further invasion, progression, metastases, and response to therapy. Continued research into CRC's pathways and biomarkers will pave the way for the development of more precise and effective therapeutic strategies, ultimately improving patient outcomes.

Keywords: colorectal cancer, molecular mechanisms, pathways, chromosomal instability, adenomatous polyposis coli, biomarkers

Introduction

Colorectal cancer (CRC) refers to the uncontrolled growth of cells formed at the lower end of the digestive tract. Cancerous growth in the colon and rectum is known as colorectal cancer (CRC). Worldwide, CRC is the third leading cause of cancer-related mortality worldwide. In 2020, an estimated 1,880,725 individuals were diagnosed with CRC. It is the third most commonly diagnosed cancer and the second leading cause of cancer-related deaths globally, following lung cancer.¹ Furthermore, in the US, CRC has been identified as the second most common cause of cancer-related deaths due to cancer when the numbers of men and women are both considered with an estimated 153,020 new cases of CRC and were expected to be diagnosed, and around 52,550 deaths were projected.²

CRC manifests itself in the beginning as noncancerous polyps. It develops in the inner mucosal layers of the colon and rectum. Although polyps are commonly detected in individuals 50 years of age or older during colonoscopy, fewer than 10% of these polyps are estimated to progress towards developing cancer.³ Adenomatous (ie adenoma) or serrated polyps are common cancer precursors. Accumulating evidence suggests that CRCs are a group of molecularly heterogeneous diseases with many genetic and epigenetic alterations that participate in the development of the disease.^{4,5} This highly heterogeneous nature of the disease tends to significantly influence tumor initiation, its progression, resistance, response to therapy and overall clinical outcome. Despite significant progress, critical gaps remain in understanding how this molecular complexity can be fully harnessed to improve patient outcomes. A significant challenge lies in tumor heterogeneity, both between patients (inter-tumoral) and within a single tumor (intra-tumoral), which complicates the discovery of universal biomarkers and treatment approaches. Although certain biomarkers, such as microsatellite instability (MSI) and mutations in genes like KRAS, BRAF, and TP53, have clinical significance, numerous others remain unexplored. Furthermore, resistance to existing therapies, especially in advanced and metastatic CRC, underscores the need for a better understanding of how molecular changes contribute to treatment failure. The development of

novel biomarkers for early detection, prognosis, and therapy response is critical, as is the identification of new therapeutic strategies that can address the inherent heterogeneity and treatment resistance in CRC.

Here, we present a simplified insight into the current knowledge and recent developments pertaining to the molecular network and central signalling pathways of CRC associated with its initiation to a malignant phenotype, further invasion, progression, metastases, and response to therapy. This understanding is essential for the discovery and testing of future treatment therapies targeting the respective molecular pathways. Three major molecular pathways leading to CRC are discussed in detail: a) Chromosomal Instability (CIN) pathway, b) CpG Island Methylator Phenotype (CIMP) pathway, and c) Microsatellite Instability (MSI) pathway.

CIN Pathway and CRC

The frequency of the baseline mutation rates under normal conditions is too low and insufficient for cancer development. Thus, a chromosomal instability is observed in as high as 65–70% cases of colorectal cancers.^{5,6} CIN refers to a type of genomic instability with accelerated rates of unstable chromosomes, wherein either the whole chromosome or a portion of the chromosome is duplicated or deleted.⁷ Numerical CIN, also known as aneuploidy, is a common form seen. Under normal conditions, errors in chromosomal segregation leading to changes in the number of chromosomes occur in less than 1% of cell divisions but cells with CIN exhibit enhanced error rate of ~20%.⁸ Structural CIN is different, and portions of whole chromosomes may be duplicated or deleted, or there may be a rearrangement of parts of the chromosomes.⁹ Multiple causes lead to a higher normal rate of abnormalities in the whole or part of the chromosome. Not diving into the detail, briefly, the major causes include a) breakdown in the cell's repair mechanism may lead to chromosome rearrangements that result in the loss, amplification, and/or exchange of chromosome segments. For example, ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) protein kinases are DNA repair proteins, whose inactivation mutations have been implicated in the development of human cancer.¹⁰ With inactivating mutations in TP53 that result in the loss of its function, uncontrolled entry into the cell cycle occurs, and this functional loss of TP53 has been directly implicated in human CRC.^{11,12} Telomere dysfunction is a major cause of CIN. Telomeres are specific DNA-protein structures present at both ends of each chromosome and play a crucial role in protecting against nucleolytic degradation, unnecessary recombination, inter-chromosomal fusion, and breakage during segregation. During every round of cell division, a portion of telomeric DNA is lost.¹³ Once a significant number of divisions occurs (25–50), telomeres can be completely lost with the induction of p53 expression, which permanently arrests the cell or induces apoptosis. The shortening of telomeres and p53 expression form a protective barrier against uncontrolled replication and tumour development. However, inactivating mutations leading to nonfunctional p53 cause shortening of telomeres with eroded portions prone to chromosomal rearrangements through recombination and repeated breakage-fusion-bridge (B/F/B) cycles.¹⁴ Continuation of B/F/B cycles for multiple cell divisions may lead to undesirable changes in genome organisation. Studies have indicated that shorter telomeres were observed in 77% to 90% of colon cancer samples, compared to adjacent normal tissues.^{15,16} Defects in chromosome segregation during the mitotic cycle are another cause of CIN. Spindle assembly checkpoint (SAC) and mitotic checkpoint controls are key regulators of chromosome segregation during mitosis and meiosis. It delays the start of the anaphase until all pairs of duplicated chromatids are correctly positioned on the metaphase plate.¹⁷ Checkpoint signalling defects cause chromosome mis-segregation and subsequent aneuploidy, in which daughter cells receive abnormally large numbers of chromosomes.¹⁸ The CIN pathway has been associated with several genetic events. Whether CIN enables the creation of an appropriate environment for the accumulation of these mutations or whether these mutations lead to CIN remains unresolved. The major genes involved in this process are discussed below.

APC (Adenomatous Polyposis Coli) Gene Mutation

The most important of all is the APC gene which is classified as a tumor suppressor gene. This gene is located on chromosome 5q21-q22, which consists of 8535 nucleotides with 21 exons. *APC* encodes a 310 kDa protein, *Adenomatous polyposis coli* (APC). This protein has multiple domains that primarily consist of an oligomerization domain, an armadillo repeat domain, 15- or 20-residue repeat domain, a SAMP repeat domain, a basic domain, and a C-terminal domains.^{19,20} Through its multiple binding partners, APC is involved in cellular processes, such as cell

migration, adhesion, differentiation, and chromosomal segregation. APC functions as a critical negative regulator of the canonical WNT/β-catenin pathway. It acts as a scaffold structure for the destruction complex that promotes phosphorylation and subsequent ubiquitin-dependent degradation of CTNNB1(Catenin beta-1), a key WNT pathway activator.^{21,22} APC enhances the effectiveness of this destruction machinery by encouraging axin multimerization and stabilising the axin complex.²³ If the APC is rendered inactive by a mutation, an excessive build-up of CTNNB1 in the cytoplasm results, which then translocates to the nucleus. This controls transcriptional changes that promote *MYC* expression and activation of other oncogenes. Interruption of the WNT pathway dysregulates colonic epithelial cell proliferation and proper differentiation, leading to progression from low-grade to high-grade adenomas.^{5,24} This is because other tumour suppressor genes are rendered inactive. Additionally, functional APC loss could affect the regulation of mitotic processes, which adds to the structural or numerical CIN. Approximately, 60% of colon tumours and 82% of rectal cancers have APC mutations.²⁵

K-RAS Gene Mutation

KRAS (12p12) is another important gene in the CIN pathway; however, it is also associated with the CIMP pathway. The Kirsten rat sarcoma virus oncogene homolog (Ki-ras2) gene is an oncogene that produces the 21 kDa GTPase transductor protein KRAS. KRAS controls cell division because of its ability to relay external signals to the cell nucleus.²⁶ Most human cells express KRAS, a membrane-bound GTP/GDP-binding protein with an intrinsic GTPase activity. Under normal conditions, binding of growth factors to their respective cell surface receptors activates guanine exchange factors (GEF), such as SOS (son of sevenless), which are attached by the adaptor protein GRB2 (growth-factor-receptor bound protein 2). Bound GDP is released from RAS and exchanged for GTP in response to SOS stimulation, creating an active RAS-GTP conformation. Multiple effector pathways, including the Raf-MEK-ERK pathway, PI3K, RALGDS, RALGDS-like gene (RLG), and RGL2, are regulated by the activated RAS. These downstream effectors have a wide range of effects, such as prevention of apoptosis, stimulation of cell growth, cell transformation, angiogenesis, migration, and differentiation.^{26,27} GTPase-activating proteins (GAP) can bind to RAS-GTP and accelerate its conversion to guanosine diphosphate (RAS-GDP), causing signal termination.²⁸ KRAS mutations decrease the intrinsic GTPase activity of the protein, causing a build-up of active GTP-bound KRAS proteins. Codons 12 and 13 of exon 2 and, to a lesser extent, codon 61 of exon 3 are the most common single nucleotide point mutations.²⁹ Mutant KRAS stops responding to GAPs and quickly converts GDP to GTP, thereby locking it in its active state.²⁶ This results in the constitutive activation of downstream effector activities, thus promoting uncontrolled cell growth and transformation, cancer spread, and enhanced resistance to chemotherapy in many cancer types, including CRC.^{30,31} KRAS mutations have been linked to poor prognosis for CRC and lung and liver metastases according to a number of studies. Approximately 30–40% of CRCs harbour *KRAS* mutations.^{32,33} KRAS mutations in colon cancer have been associated with poor survival, increased tumour aggressiveness, and resistance to select treatment strategies.^{31,34} Table 1 highlights

Table 1 Major Molecules Targeting the KRAS Pathway, Developed for Therapeutic Testing Against CRC Development Along with Category, Their Mode of Action and Current Clinical Status

Category	Candidate Molecule	Mode of Action	Current Status	Related Reference
Direct KRAS Inhibitors	AMG 510 (Sotorasib)	A specific, irreversible inhibitor of KRAS G12C that traps the KRAS in its inactive GDP-bound state	Approved in NSCLC, ongoing clinical trials in CRC	[35]
Direct KRAS Inhibitors	MRTX849 (Adagrasib)	Small-molecule specific inhibitor of mutant KRAS G12C protein	Phase II/III clinical trials	[36]
Polo-like kinase 1 (PLK1) inhibitors	Onvansertib	Selective and oral inhibitor of PLK1, a key regulator of cell division and mitosis.	Phase I/II clinical trials	[37]

(Continued)

Table 1 (Continued).

Category	Candidate Molecule	Mode of Action	Current Status	Related Reference
Antimicrobial peptides (AMPs)	KR12	Recognizes and Alkylates adenine residues on the template strand at codon 12 (GTT and GAT), exon 2 of mutated KRAS, producing strand cleavage and decreasing the proliferation rate of the CRC cell with G12D/G12 V mutation.	Tested only in preclinical studies	[38]
KRAS-PDEδ interaction inhibitor.	Deltarasin	Inhibition of Prenyl-binding protein PDEδ (a protein essential for maintaining the spatial organization of RAS for effective signalling) blocking the oncogenic RAS signalling	Phase I/II clinical trials	[39]
Immuno-oncolytic virus therapy	Pelareorep	Oncolytic reoviruses that selectively infects the KRAS mutated CRC cells inducing lysis and promoting autophagy	Currently advancing towards Phase III clinical trials	[40,41]
Nucleic acid antisense oligonucleotide	AZD4785	Genetically engineered molecule functions as an antisense oligonucleotide complementary to mRNA sequences of KRAS causing exhaustion of intracellular KRAS mRNA and protein.	Studied in Phase I clinical trials	[42]
Small molecule kinase inhibitors	AZD6244 (Selumetinib)	Selective oral mitogen-activated protein kinase (MAPKK, or MEK) pathway inhibitor. The drug interacts with MEK1/2 by turning MEK1/2 into their inactive conformational states.	Studied in Phase I and II settings	[43]

the major biomolecules based on the KRAS pathway that have been developed for therapeutic purposes, with their mechanisms of action and current clinical status.

Impairment of TP53

One of the key genes responsible for the transformation of colorectal cancer (CRC), and its aggressive and metastatic characteristics, is TP53. As one of the most crucial components of the body's anticancer defense system, this gene is a critical tumour suppressor. The gene product, which is a 393 amino acid transcription factor located on the short arm of chromosome 17, serves as a tumour suppressor and a key coordinator of cellular responses to oxidative stress, DNA damage, and aberrant proliferative signals.^{44,45} The transcription of hundreds of genes involved in DNA metabolism, apoptosis, cell cycle regulation, senescence, angiogenesis, immunological response, cell differentiation, motility, and migration is regulated by the master regulator p53.^{46,47} Under normal conditions, once activated, MDM2 (murine/human double minute 2), a negative regulator, is upregulated. MDM2 acts as an E3 ubiquitin ligase and attaches to the p53 transactivation domain, causing ubiquitination and p53's destruction.^{48,49} This creates a negative feedback loop that maintains low p53 levels in the normal cells.

Documented data suggest that 4%–26% of adenomas, 50% of adenomas with invasive foci, and 50%–75% of CRCs have been associated with functional loss of p53. Thirty-four percent of proximal colon tumours and 45% of distal colorectal tumours in CRC have been shown to have p53 mutations. These statistics highlight the key role of these molecules in the transformation of adenomas to carcinomas.^{50,51} The majority of TP53 mutations are missense changes that affect exons 5 to 8 (the DNA-binding domain), primarily at hotspot codons, such as 175, 245, 248, 273, and 282, which result in the synthesis of an inactive protein with an abnormally extended half-life.^{51,52} p53 mutations are associated with lymphatic invasion in proximal colon cancer and are strongly correlated with both lymphatic and vascular invasion in distal colon cancer. Additionally, individuals with CRC who have mutant p53 show higher chemoresistance and worse prognosis than those with wild-type p53.⁵³ These observations suggest that the reactivation and restoration of p53 function have a great potential as novel therapeutic strategies for CRC treatment. **Table 2** briefly details the various p53-based treatment options explored for the treatment of CRC. Most of the candidate molecules have shown promising results as tested in cell lines or in animal models, and clinical data on these are the need of the hour so as to translate these p53-based therapies for clinical use for CRC patients.

Other Genes

In addition to the above genes, later events in the pathogenesis of CRC include deletions on chromosome 18q, with a high proportion observed in cases of colorectal cancer.⁶³ One gene, *Cables*, a novel regulatory protein mapped to

Table 2 Molecules Based on p53-Based Treatment Approaches Being Explored for CRC, Along with Their Mode of Action and Clinical Progress Status

Candidate Molecule	Mechanism of Action	Therapeutic Potential in CRC	Ongoing Status	Related Reference
PRIMA-1 (p53 Reactivation and Induction of Massive Apoptosis)	Restores wild-type function of mutant p53, inducing apoptosis.	Induces apoptosis in CRC cells harbouring mutant p53, sensitizes to other treatments.	Preclinical and early clinical trials	[54]
MI-219	MDM2-p53 inhibitor	Induces apoptosis in HCT-116 colon cancer	Promise in preclinical studies, not yet advanced further	[55]
Nutlin-3 (R1772)	Occupy the binding pocket of MDM2, thus disrupting MDM2-p53 interaction	Reactivates p53-dependent apoptosis in CRC cells, enhancing chemotherapy efficacy	Preclinical and early clinical trials	[56]
RITA	Directly binds to p53 and induces a conformational change in p53, causing interfered p53-MDM2 interaction causing p53 accumulation and cellular apoptosis	Induces apoptosis in CRC cells, both p53 wild-type and mutant.	Preclinical trials	[57]
Tenovins	Inhibit SIRT1/2 deacetylases, stabilizing p53 and enhancing its tumor-suppressing activity.	Increases p53-mediated apoptosis and enhances response to chemotherapy in CRC.	Preclinical trials	[58]
Machinic acid (MA)	Natural triterpene from <i>Olea europaea</i> , that works by inducing the expression of JNK (c-Jun NH ₂ -terminal kinase) and p53 resulting in cell cycle arrest and apoptosis	Shows anticancer activity by reactivating p53 in CRC cells.	Preclinical trials	[59]
Epicatechin gallate	Natural polyphenol found in green tea, causing stimulated expression of p53, p21, and MAPKs (mitogen-activated protein kinases) in CRC cell lines	Inhibits CRC cell growth via p53 activation, enhances antioxidant defense.	Preclinical trials	[60]
Lipoic acid (α -LA)	α -LA inhibits proliferation and induces apoptosis in colon cancer cells by preventing p53 degradation	Enhances p53-mediated apoptosis in CRC, reduces cancer cell viability.	Preclinical trials	[61,62]

chromosome 18q11.2–12.1, is lost at a high frequency in colon cancer. This protein connects to, or acts as a cable for, other crucial proteins involved in carcinogenesis and cell proliferation. Cables interact with serine/threonine protein kinase cyclin-dependent kinase (cdk) and increase tyrosine phosphorylation, which reduces kinase activity and decreases cell growth. Cables interact with the p53 tumour suppressor gene to increase p53-induced apoptosis.⁶⁴ Loss of *Cables1* expression, evaluated by immunohistochemistry, has been found to be decreased or absent in 65% of primary CRCs,⁶⁵ correlating with the loss of heterozygosity at 18q. Kirley et al⁶³ studied the susceptibility of *Cables*-/- mice to colon tumour development. This Twenty weeks of subcutaneous 1,2-dimethylhydrazine (DMH) injections were administered to *Cables*-/- mice and their *Cables*+/+ littermates. Compared with their *Cables*+/+ littermates, *Cables*-/- mice had considerably lower median survival times following DMH injections. The number of colorectal tumours that developed in *Cables*-/- mice was 46 tumours versus 21 tumours. In another study by Arnason et al,⁶⁵ the role of *Cables1* employing

an *in vivo* mouse model of intestinal adenocarcinoma. *ApcMin/+* mice were crossed with mice harbouring the targeted inactivation of *Cables1* (*Cables1-/-*). Results showed that the mean number of small intestinal tumors per mouse was 3.1 ± 0.6 in *Cables1+/+ ApcMin/+* mice, and in *Cables1-/-ApcMin/+* mice, this value was 32.4 ± 3.5 . In addition, tumors from *Cables1-/-ApcMin/+* mice demonstrated an increased nuclear expression of β -catenin, showing that loss of *Cables1* enhanced tumor progression in the *ApcMin/+* mouse model and activated the Wnt/ β -catenin signalling pathway.

Additional genes reported in 18q were deleted in Colorectal Carcinoma (*DCC*), *SMAD4*, and *SMAD2*.^{66,67} However, the product of *DCC* is a cell surface receptor for the neuronal protein netrin-1, and *DCC* mutations have rarely been detected in colorectal tumors (6%).^{7,68} Similarly, *SMAD4* and *SMAD2* mutations have been found in less than 10–20% cases of colon cancers,^{69,70} and thus more data need to be examined to understand the role of these genes, if any, in CRCs.

Overexpression of COX-2 in CRC

In addition, evidence suggests that Cyclooxygenase-2 (COX-2) plays a significant role in the development of CRC.^{71,72} There are three isoforms of COXs: crucial regulators of angiogenesis, inflammation, and carcinogenesis (COX 1–3). COX-2 is an inducible isoform in normal tissues, such as the colon, kidney, reproductive organs, and stomach organs.⁷³ High expression with constant upregulation of COX-2 is observed in various premalignant and malignant lesions of epithelial origin as well as in different regions of the gastrointestinal tract.^{71,74,75} Tumors with high levels of COX-2 are relatively more aggressive,⁷¹ and patients with these tumors have significantly reduced survival rates.⁷⁶ In a study by Oshima et al,⁷⁷ which was the first to directly demonstrate the role of COX-2 overexpression in the early stages of polyp development, the *Apcdelta716* mouse's intestinal and colonic polyps decreased in number and size in a dose-dependent manner after treatment with a specific COX-2 inhibitor, rofecoxib.

Arachidonic acid (AA) serves as a substrate for COX-2, which mediates the biosynthesis and release of prostaglandins. PGE2 is the primary prostaglandin, contributing to colorectal progression. PGE2 operates on receptors (EP1, EP2, EP3, and EP4) to induce a signal cascade with changes in intracellular calcium and cAMP, and a prolonged PGE2 increase initiates pathological events, cancer genesis, and spread.⁷⁸ Recent investigations have shown that PGE2 may enhance the progression of colorectal cancer^{79,80} and that EP4 is a therapeutic target for cancer therapy.^{81,82} In addition, COX-2 is involved in regulating angiogenesis, and overexpression of COX-2 increases the production of pro-angiogenic factors that contribute to tumor vascularity and growth.⁸³ Negi et al⁷² studied mRNA expression levels of COX-2 in thirty CRC patients. They found that COX-2 overexpression in patients with colorectal cancer was related to clinicopathological factors and that COX-2 mRNA expression may serve as a biomarker for the diagnosis of CRC.

MSI Pathway and CRC

Microsatellites, also referred to as simple sequence repeats (SSRs), are repeat DNA sequences that range in length from one to six base pairs and are found next to one another in the genome. Microsatellite instability (MSI) is a genetic hypermutability condition caused by a faulty DNA mismatch repair (MMR).^{84,85} More specifically, mutations inserted into microsatellite regions are not corrected when there are errors in mismatch repair (MMR) genes (*MLH1*, *PMS2*, *MSH2*, *MSH6*), leading to MSI.⁸⁶ It has been observed that 10–15% of CRC are associated with MSI and are MMR deficient (dMMR).⁸⁷ Under normal conditions, the cell's MMR system effectively starts to function to take care of any introduced microsatellite instability through a cascade of events involving the interaction of MMR proteins as heterodimers, such as *MSH2–MSH6* (*MutS α*), *MLH1–PMS2* (*MutL α*), and *SH2–MSH3* (*MutS β*), and further excision with the help of proteins, that is, exonuclease 1 and proliferating-cell-nuclear antigen, followed by re-synthesis and re-ligation of the DNA strand.^{86,88} MSI tumors carry mutations in the coding regions of several genes including *BRAFV600E*, *TGF β RII*, *BAX*, and *IGF1R*. Two molecular pathways lead to the development of CRCs with MSI. The first are germline mutations in MMR genes, which lead to hereditary nonpolyposis colorectal cancer (HNPCC), that is Lynch syndrome, an autosomal dominant disorder. The second most common mechanism is epigenetic inactivation of *MLH1* due to somatic hypermethylation of CpG islands surrounding the promoter region of *MLH1* and other genes. Approximately 15% of all colorectal malignancies have MSI; 3% of them are linked to Lynch syndrome, while the remaining 12% are brought on by spontaneously acquired hypermethylation.^{89,90} However, many CRCs have an intact

MMR system but frameshift mutations in a few microsatellites.⁹¹ CRCs with MSI have distinctive pathological features, including a tendency to arise in the proximal colon, poorly differentiated tissue, high mucinogens, and tumour-infiltrating lymphocytes.^{92,93} CRC with MSI does not respond to 5-fluorouracil (5-FU) treatment; thus, MSI is a negative predictive marker of the response to 5-FU. HCT116 colon cancer cells with a homozygous mutation in hMLH1 on human chromosome 3, which display microsatellite instability and resistance to 5-FU, were used in a study by Koi et al.⁹⁴ However, with the transfer of chromosome 3, which restored the copy number of the functional MLH1 gene, the sensitivity to 5-FU was restored (39) was regained back. Similar observations were noted when studying the response to 5-FU in human colon cancers (hCC) with MSI orthotopically xenografted into nude mice.⁹⁵ In a systematic study by Popat and team,⁹⁰ it was found that the survival rate of CRC patients with MSI was higher than that of CRC patients with microsatellite stable (MSS) tumours, and colorectal tumours with MSI have a slightly better prognosis than colorectal tumours without MSI.⁹⁶ However, more conclusive data from larger prospective trials are required to establish the clinical significance of MSI in CRC treatment.

The CpG Island Methylator Phenotype (CIMP) Pathway

The second most frequent pathway in sporadic CRCs is CIMP. Fifteen% of sporadic cases are caused by the CIMP pathway.^{97,98} The CIMP pathway is the basis of epigenetic instability. Epigenetic changes refer to changes in gene expression without corresponding changes in DNA sequence. Studies have stressed the key role of epigenetic alterations such as DNA methylation, histone modification, nucleosome positioning, and the role of small non-coding RNAs in tumourigenic events, disease progression, metastases, and chemoresistance in CRC.^{99,100} These epigenetic changes, DNA methylation in selected gene promoters, are the most common and widely investigated molecular alterations associated with human tumours, including CRC.

During methylation, methyl groups are added to cytosine at the 5-position, producing 5-methylcytosine by DNA methyltransferases (DNMT). These cytosine residues are often clustered in CpG islands (CpG islands are short stretches of palindromic DNA with repeated cytosine and guanine nucleotides), which are mostly associated with the promoter regions of most genes.^{101,102} Toyota et al¹⁰³ reported that methylation of cancer-specific clones was only observed in a subset of colorectal cancers that had a CpG island methylator phenotype (CIMP), while comprehending the global patterns of CpG island methylation in colorectal cancer.

The CpG island methylator phenotype (CIMP) promotes hypermethylation in promoter CpG regions of tumour suppressor genes, causing their inactivation or transcriptional silencing, leading to the development and progression of CRC.¹⁰³ Other DNA methylation biomarkers are associated with CRC development of CRC. These epigenetic biomarkers form a useful panel of genes, whose methylation levels form the basis of a useful screening strategy for patients with CRC.

SFRPs

Secreted Frizzled-related proteins (SFRPs) are a family of secreted proteins that are extracellular regulators of the Wnt signalling pathway. Since SFRPs have a cysteine-rich domain (CRD) identical to frizzled receptors, they can bind to these receptors to create inactive complexes that block Wnt signalling.¹⁰⁴ The Wnt antagonists SFRP1 and SFRP2 act as tumour suppressors by interacting with Wnt-1 and Wnt-5 ligands to control the growth and apoptosis of cancer cells. However, abnormal SFRP1 and SFRP2 DNA hypermethylation results in reduced or lost gene expression, inhibition of gene function, and upregulation of the Wnt signalling pathway, all of which contribute to the emergence and development of CRC.^{105,106} Cadwell et al¹⁰⁷ showed that SFRP1 mRNA expression was downregulated in 28 (76%) cases out of 51 cases of locally advanced colorectal cancers compared with normal mucosa. Additionally, only 11 of 36 matched normal mucosal samples and 40 of 49 (82%) tumours showed hypermethylation. Similarly, Qi et al¹⁰⁸ observed that SFRP1 mRNA expression was abnormally hypermethylated in adenomas and CRC, unlike in normal mucosal cells. According to Huang et al,¹⁰⁹ SFRP1 and SFRP5 mRNA levels were considerably downregulated in 85 and 80% of CRC, indicating that SFRPs play key roles in tumour progression by inhibiting Wnt signalling. Identification of SFRP2 and SFRP1 methylation in stool samples can detect CRC with good sensitivity and specificity. While SFRP1 stool DNA methylation assay had a sensitivity of 89% and specificity of 86% for diagnosing colorectal neoplasia tumours, SFRP2 stool methylation assay demonstrated sensitivity and specificity of 90% and 77%, respectively.¹¹⁰

TFPI2

Another marker that has been shown to undergo aberrant methylation of the promoter CpG islands leading to loss of its expression is tissue factor pathway inhibitor-2 (TFPI-2). A structural homologue of tissue factor pathway inhibitor (TFPI), TFPI-2, is a member of the Kunitz-type serine proteinase inhibitor family. Plasmin, plasma kallikrein, factor XIa, trypsin, and chymotrypsin are serine proteinases that are inhibited by TFPI-2 and the tissue factor/factor VIIa (TF/VIIa) complex.^{111,112} It has been reported that abnormal methylation of the TFPI-2 promoter within the CpG island is linked to reduced expression and production of TFPI-2 protein in human malignancies and cancer cell lines.¹¹³ In one such study, TFPI2 methylation was found in stool DNA from patients with stage I to stage III CRC, with a sensitivity of 76% to 89% and a specificity of 79% to 93%. TFPI2 methylation is a frequent and early occurrence in CRCs.¹¹⁴ Stool DNA testing to identify TFPI2 methylation represents a useful adjunct method for use in conjunction with other non-invasive biomarkers for early CRC screening.¹¹⁵

MGMT

O⁶-methylguanine DNA methyltransferase (MGMT) is a DNA-repair enzyme. This enzyme also referred as DNA “suicide” repair enzyme plays a key role in repairing damaged guanine nucleotides by removing the alkyl groups from the O⁶-position of the guanine, thus acting as an acceptor. This enzyme is essential to avoid gene mutations, cell death, and tumourigenesis caused by alkylating agents.^{116,117} When CpG dinucleotides in the MGMT promoter region are methylated, MGMT production is lost, and the enzyme is unable to remove alkyl groups from the methylated guanine.¹¹⁸ MGMT promoter methylation has been implicated as a frequent and relevant event in CRC cases, and a low expression of MGMT was seen in 27 to 40% of metastatic cases of CRC showing chemoresistance.¹¹⁹ In a metastatic report by Li et al¹² that incorporated the results of 14 relevant studies, the team concluded that MGMT methylation frequency was much higher in CRC tissues than in normal tissues and that the MGMT promoter was more commonly methylated in CRC patients than in adenoma patients. DNA methylation levels of MGMT serve as a promising screening approach.¹²⁰ Stool testing of faecal DNA for methylated genes, including MGMT, represents a simple non-invasive method for screening the early stages of CRC and precancerous lesions.¹²¹ In this study, methylated MGMT was found in 48.1% of patients with CRC and 28.6% of patients with adenoma, respectively, with a 93.7% sensitivity and 77.1% specificity for diagnosing CRC and precancerous lesions.

Vimentin

Vimentin is a type III intermediate filament (IF) protein encoded by VIM. This structural protein is the major cytoskeletal component of mesenchymal cells.¹²² Vimentin is also employed as a biomarker of cells generated from mesenchyme or cells undergoing epithelial-to-mesenchymal transition (EMT) during both metastatic progression and normal development.^{123,124} Aberrant promoter methylation of the vimentin gene linked to the pathogenesis of CRC was reported in a meta-analysis based on seven clinical cohort studies, including a total of 467 CRC subjects.¹²⁵ A significantly higher frequency of vimentin promoter methylation in CRC tissues (odds ratio, ie, OR) = 32.41, 95% CI = 21.04 ~ 49.93) than in normal and benign tissues (OR = OR = 1.60, 95% CI 1.05 ~ 2.42) was observed. A similar observation was reported wherein faecal DNA from patients with CRC showed aberrant methylation of exon-1 sequences within the non-transcribed vimentin gene.¹²⁶ In this study, vimentin exon-1 sequences were methylated in 83% (38 of 46) and 53% (57 of 107) of tumours from colon cancer patients, thus suggesting that *VMT* gene methylation may also serve as a novel molecular biomarker for colon cancer screening.

In addition to the above genes showing hypermethylation events, studies have reported that hypomethylation of genes is also observed in CRC cases.¹²⁷ Hypomethylation of long interspersed nuclear element 1 (LINE-1) is associated with colorectal carcinogenesis.^{128,129} Long interspersed nuclear elements (LINEs) refer to a class of non-LTR (long terminal repeat) retrotransposons that are widely distributed in the human genome. They are also referred to as “long interspersed nucleotide elements” or “long interspersed elements”. The LINEs account for up to 21.1% of the human genome.¹³⁰ In normal somatic cells, LINE-1s are heavily methylated, which restricts the activity of retrotransposal elements and prevents genomic instability.¹³¹ However, global DNA hypomethylation levels are been found to be higher in neoplastic lesions (including hyperplastic polyps and adenomas) than in the normal mucosa.¹²⁷ Matsuzaki et al¹²⁸ analysed global

LINE-1 methylation levels in 80 sporadic colorectal cancers, 51 adjacent normal tissues, and 20 normal tissues. Results showed that colorectal cancers had significantly lower global methylation levels than did normal tissues ($41.0 \pm 9.7\%$ versus $54.3 \pm 6.5\%$; $P < 0.001$). Also, tumours with global hypomethylation levels $\leq 40\%$ had higher number of LOH (+) chromosomal loci unlike seen in tumours without global hypomethylation. This suggests that global hypomethylation plays a role in inducing genomic instability in sporadic CRC. The tumour LINE-1 methylation level may be a useful prognostic biomarker for identifying aggressive carcinomas among MSI CRCs, and the detection of LINE-1 hypomethylation levels in circulating cell-free DNA in plasma represents a potential biomarker for early-stage CRC.^{132,133}

Clinical Implications and Advances

In this section, we highlight on the notable advances in the treatment of CRC based on our better understanding of the molecular pathways and identified biomarkers related to the disease progression. One of the major implications of identifying key molecular biomarkers in colorectal cancer (CRC) is “Precision medicine” which is advancing rapidly. This involves tailoring medical treatment to the individual characteristics of each patient, particularly the specific genetic mutations and molecular alterations in their cancer cells.¹³⁴ For this, the researchers are exploring the possibility of multi-gene panel testing to match patients with specific targeted therapies based on their tumor’s molecular profile. This approach is being used to develop personalized treatment plans for individual CRC patients, incorporating biomarkers like KRAS, BRAF, MSI, and HER2 status into clinical decision-making.¹³⁵

Other major advancement is the liquid biopsies that are non-invasive tests that detect circulating tumor DNA (ctDNA), circulating tumor cells (CTCs), and other molecular components (such as RNA, proteins, and exosomes) from a patient’s blood sample.¹³⁶ The key biomarkers being detected in ctDNA for CRC include KRAS/NRAS Mutations, BRAF V600E, MSI-H/dMMR mutation, HER2 amplification, etc.¹³⁷ The ability of ctDNA will offer advantage in very early detection of adenomas (precancerous polyps) and early carcinomas even before symptoms develop,¹³⁸ predicting recurrence rates in patients post-surgery allowing earlier intervention and personalized treatment plans¹³⁹ as well as ctDNA levels during treatment (real-time monitoring) may help the physician to monitor the success of ongoing treatment and the need to change to alternative plan.

In addition to the above, the new era of use of artificial intelligence (AI) and machine learning-assisted models represents a major breakthrough in the fight against colorectal cancer.^{140,141} Deep learning techniques can integrate multi-omic datasets to identify complex biomarker signatures that may not be detectable through traditional methods. AI algorithms can also predict how CRC patients will respond to certain therapies based on their molecular biomarker profile. In a study by Zhang et al,¹⁴² a machine learning survival predictive system for CRC patients was developed based on their immune prognostic marker profile. Briefly, twenty immune genes were recognized and a prognostic nomogram developed. The prognostic model was able to discriminate between the high-risk patients with poor prognosis from low-risk patients with favourable prognosis, thus predicting the overall survival curves. Thus, machine learning models can forecast responses to immune checkpoint inhibitors by detecting molecular patterns, such as microsatellite instability-high (MSI-H) or tumor mutational burden (TMB). This strategy enhances treatment planning by ensuring that therapies are given primarily to patients who are more likely to benefit from them.¹⁴³ Further, the presence of specific mutations in APC, TP53, or PIK3CA can be integrated into predictive algorithms to predict the likelihood of CRC development, recurrence and metastasis.^{144,145} Ahmadieh-Yazdi and team¹⁴⁶ investigated CRC metastasis-related biomarkers by employing a machine learning (ML) approach. In this, the team first obtained the gene expression profile of CRC patients with liver metastasis and studied the differentially expressed genes between primary and metastatic samples. Out of the eleven genes selected by machine learning algorithms [Penalized Support Vector Machine (P-SVM) and Least Absolute Shrinkage and Selection Operator (LASSO) algorithms], seven had significant prognostic value in colorectal cancer. Thus, ML algorithms were able to select out and identify a set of potential biomarkers for CRC metastasis essential for timely intervention.

Conclusion

In conclusion, CRC represents a complex and heterogeneous group of disorders at the molecular level, involving multiple signalling pathways. Various trends in genetic mutations and epigenetic changes influence the development and

progression of CRC as well as the way in which it responds to various therapies. Multiple signalling pathways are activated in CRC, making it difficult to address the disease with a single therapy and a single approach. Understanding molecular complexity provides crucial insights that can propel future research and treatment advancements. Detailed molecular profiling will help identify novel biomarkers and actionable mutations in CRC subtypes, leading to personalized treatment regimens that improve efficacy and reduce side effects. This complexity is a valuable source for discovering predictive and prognostic biomarkers, aiding in early diagnosis, treatment monitoring, and response prediction. Investigating resistance-related molecular pathways (eg, Wnt, MAPK) can help develop strategies to overcome treatment resistance. Further, the intra-tumor molecular variability may help identify more effective treatment regimens for metastatic CRC by addressing different clones within the tumor. The study of molecular crosstalk between different pathways (eg, immune evasion, apoptosis) will enable to reveal novel combinations of therapies, optimizing treatment efficacy while minimizing toxicity.

By leveraging the molecular complexity of CRC, future research has the potential to transform treatment approaches, advancing toward more personalized and effective therapies.

Data Sharing Statement

All data are available within the manuscript, and there are no associated data.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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