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# DNA methylation aberrancies delineate clinically distinct subsets of colorectal cancer and provide novel targets for epigenetic therapies

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## Abstract

Colorectal cancer (CRC) is a worldwide health concern with respect to both incidence and mortality, and as a result, CRC tumorigenesis, progression and metastasis have been heavily studied, especially with respect to identifying genetic, epigenetic, transcriptomic and proteomic profiles of disease. DNA methylation alterations are hallmarks of CRC, and epigenetic driver genes have been identified that are thought to be involved in early stages of tumorigenesis. Moreover, distinct CRC patient subgroups are organized based on DNA methylation profiles. CRC tumors displaying CpG island methylator phenotypes (CIMPs), defined as DNA hypermethylation at specific CpG islands in subsets of tumors, show high concordance with specific genetic alterations, disease risk factors and patient outcome. This review details the DNA methylation alterations in CRC, the significance of CIMP status, the development of treatments based on specific molecular profiles and the application of epigenetic therapies for CRC patient treatment.

## INTRODUCTION TO CRC

Colorectal cancer (CRC) is a significant global health burden, with an incidence of 1.4 million cases and ~700 000 deaths worldwide in 2012.[1] CRC is the third leading cause of cancer mortality in the United States and the second leading cause of cancer mortality in Europe. Owing to more widely implemented screening modalities, such as colonoscopy and image-based detection, as well as effective therapies, CRC mortality has decreased in many countries,[1] and the median survival of patients with metastatic disease now approaches 30 months.[2,3] In contrast to survival rates of 65% for patients with localized disease, < 10% of

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CONFLICT OF INTEREST

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metastatic CRC patients survive 5 years after diagnosis. Owing to its prevalence in the population and occurrence as both sporadic and familial diseases, CRC has been well studied at the molecular level in order to characterize the genetic, epigenetic, transcriptomic and proteomic changes for the purposes of disease detection, surveillance and ultimately to develop novel therapeutic approaches to improve patient outcome and survival.

## GENETIC ALTERATIONS GUIDING CRC TUMOR DEVELOPMENT AND PROGRESSION

The majority of CRCs (70%) develop sporadically, whereas the remaining cases develop through genetic predisposition or familial influence. Genetic predisposition, or genetic susceptibility, describes the increased risk of developing disease owing to inherited genetic alterations. Only a small percentage (5%) of all CRCs are hereditary, in which family members develop cancer via germline transmission of genetic alterations. Approximately 2–4% of CRCs are characterized as Lynch Syndrome (formerly described as hereditary non-polyposis colorectal cancer), an autosomal dominant disease that arises due to mutations in the DNA mismatch repair genes *MLH1* (42%), *MSH2* (33%), *MSH6* (18%) and *PMS2* (7–8%) (reviewed in reference 4).

Fearon and Vogelstein first proposed a multistep model of colon cancer tumorigenesis in 1990,[5] in which chromosomal instability, namely mutations and deletions of key oncogenes and tumor suppressors, correlate with disease progression. *APC* mutations or deletions are thought to be among the earliest events in CRC tumorigenesis in which normal colonic mucosa transitions to hyperproliferative epithelium. Subsequent development of adenomas occurs mainly in the distal colon and involves activating *KRAS* mutations (*KRAS*-mut), losses of *DCC* on chromosome 18q, and inactivation of TGF-β response by *SMAD2/SMAD4* changes.[6] Finally, *TP53* mutations and/or losses correlate with the development of adenocarcinomas, whereas additional alterations are associated with tumor metastases.

Sottoriva *et al.*[7] recently provided evidence for a Big Bang model of CRC tumorigenesis, challenging the Vogelstein model, after sequencing multiple regions of individual tumor glands. Instead of stepwise accumulation of genomic alterations described in the Vogelstein model, the Big Bang model suggests that CRC tumors grow as a single expansion after the initial cellular transformation. The expanded cells contain the genomic alterations present in the initial transformed cell, and accumulate more alterations as a result of cellular growth and expansion. Although these additional changes provide growth advantages, the earliest changes are prevalent, and latter changes are present only in small tumor subpopulations.

There does seem to be room for both models in cancer biology. The stepwise Vogelstein model is supported by epidemiological data of CRC incidence, and is generally thought to describe the accumulation of somatic driver mutations with selection and clonal expansion, but does not support intratumor heterogeneity, a frequent confounder in cancer genomics. The Big Bang model, although may not apply to all tumors, does support intratumor heterogeneity and its occurrence early and continuously during tumorigenesis.[7] Moreover, the Big Bang model corroborates with observations that clonal selection is infrequent after the tumor becomes advanced.[7]

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Irrespective of the tumorigenic model, *APC* mutations are hallmarks of CRC, are present in up to 70% of all CRCs, and result in the failure to block the G1–S phase of the cell cycle. In addition, wild-type APC functions to negatively regulate WNT signaling by degrading beta catenin. A report from The Cancer Genome Atlas Research Network showed that over 90% of all CRCs involve WNT signaling pathway alterations, especially with respect to the presence of *APC* alterations in CRCs.[8] The Cancer Genome Atlas Research Network has identified other high-frequency driver alterations for CRC, including *TP53, KRAS, PIK3CA, ACVR2A, TGFBR2, BRAF, MSH3* and *MSH6*. Integrated analyses of mutation, copy number and gene expression data show that in addition to WNT signaling alterations, CRCs show activation of TGF-β and p53 signaling, as well as inactivation of RAS and PI3K pathways.

Although most (70%) CRC tumors are thought to develop from the traditional adenoma pathway, ~ 30% of CRCs develop from the serrated pathway[9] and include hyperplastic polyps, traditional serrated adenomas and sessile serrated adenomas. Hyperplastic polyps are mainly located in the distal (left) side of the colon, can be stratified into microvesicular, goblet cell and mucin poor subgroups, but do not progress past the adenoma state. Traditional serrated adenomas are the least frequent serrated polyps, present in the distal colon with a saw-tooth like appearance and are enriched for *KRAS* mutations. Sessile serrated adenomas are mainly located in the proximal (right) colon, and are highlighted by large size, pronounced serration, flattened appearance as well as dilated and horizontal colon crypts. Sessile serrated adenomas are mainly enriched for *BRAF* mutations and unique DNA methylation alterations.

## EPIGENETIC ALTERATIONS INVOLVED IN CRC DEVELOPMENT AND PROGRESSION

### Introduction to epigenetics

Models of CRC tumorigenesis and progression are mainly based on genetic alterations, however, epigenetic changes are highly prevalent in CRCs. Epigenetics is defined as changes in gene expression that are not due to changes in gene sequence, and include DNA methylation, histone modifications, microRNAs (miRNAs) and nucleosome positioning. Unlike genetic alterations, epigenetic changes are reversible due to enzymatic activity and via pharmacological treatment with small molecule inhibitors, namely those that target DNA methylation and chromatin modifications.

### DNA methylation

DNA methylation in mammalian organisms mostly occurs by the addition of a methyl group to the C-5 position of cytosine in a 5′-CG-3′ or CpG sequence context. Non-CpG DNA methylation occurs at low levels in somatic cells and is generally found in embryonic stem cells.[10] CpG methylation is evolutionary unstable as methylated CpGs spontaneously deaminate to thymine faster than the rate at which unmethylated CpGs deaminate to uracil. As a result, CpG content in the human genome is only 20% of what is expected by sequence context alone (reviewed in reference 11). The human genome is CpG depleted, and ~70% of all CpGs are methylated,[11,12] mostly in transposable elements and intergenic regions of the

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human genome. However, there are regions of the genome, CpG islands, that contain their expected CpG content, are unmethylated in normal somatic tissues and are more often (>50%) located in gene promoter regions.

Cytosine DNA methylation marks are placed by the enzymatic activities of DNA methyltransferases (DNMTs) using *S*-adenosylmethionine as a co-factor. DNMT1, DNMT3A and DNMT3B are largely responsible for catalyzing DNA methylation in human tissues. DNMT1 is mainly involved in maintenance DNA methylation to copy DNA methylation patterns from parental DNA onto daughter DNA strands in conjunction with cellular DNA replication.[13] DNMT3A and DNMT3B are classified as *de novo* DNMTs and place new DNA methylation marks at CpG sites that were previously unmethylated. DNMT3A and DNMT3B are predominantly expressed in embryonic stem cells and are responsible for placing *de novo* DNA methylation marks during development. Although DNMT3A and DNMT3B are expressed at low levels in somatic tissues, both are overexpressed in human cancers, including CRC, and are thought to be involved in generating cancer-specific DNA methylation profiles.

HCT116 colon cancer cells have been instrumental in determining the mechanisms of DNA methylation in human cancers. HCT116 colon cancer cells harboring hypomorphic knockdown of DNMT1 ($DNMT1^{\ 2\text{-}5}$) and/or knockout of DNMT3B ($DNMT3B^{-/-}$) showed that down regulating DNMT1 or DNMT3B alone did not substantially alter global DNA methylation levels. However, DNMT1/DNMT3B double knockout (DKO, $DNMT1^{-/-}$ $DNMT3B^{-/-}$) cells display near complete (95%) DNA demethylation, suggesting that DNMT1 and DNMT3B work in concert to maintain DNA methylation marks.[14-16] DNMT3B is expressed as ~ 30 alternatively spliced variants that play important roles by: (1) serving as an accessory protein to recruit DNMT3A to sites requiring DNA methylation and (2) maintaining or restoring DNA methylation of CpGs located in gene bodies or transcribed regions by recognition of histone H3 lysine 36 trimethylation (H3K36me3) marks,[17,18] which are positively correlated with actively expressed genes.

## DNA methylation alterations in human cancers

DNA methylation changes are hallmarks of CRC and virtually all tumor types, highlighted by gene-specific DNA hypermethylation occurring together with DNA hypomethylation of repetitive elements and CpG-poor regions.[19-21] DNA methylation alterations may result in gene expression changes, including gene silencing via CpG island promoter DNA hypermethylation and gene activation owing to DNA hypomethylation of CpG-poor gene promoters. Gene body DNA hypermethylation is associated with oncogene overexpression,[18] suggesting that genes regulated by DNA methylation are driving events in tumorigenesis. In addition, DNA methylation alterations can also be exploited for use as diagnostic, predictive and prognostic biomarkers for CRC tumorigenesis and metastasis.

## DNA methylation-based driver genes in CRC

The search for genetic-based changes in human cancers is ultimately aimed at identifying a select set of alterations that are essential for tumorigenesis. These elements are linked to the concept of oncogenic addiction, defined as the dependence on a single oncogenic pathway

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for cancer cell survival.[22] Oncogenic addiction supports the idea that targeting these pathways will lead to effective therapeutic treatments, as these pathways are generally not constitutively active in normal cells. Examples of addicted oncogenes are *BRAF, EGFR, HER2, MYC* and *RAS*, as well as others, across CRC and several other forms of human cancer.[23] Addiction can also be applied to cancer epigenetics, specifically retained DNA hypermethylation of selected genes is essential for cancer cell growth and survival after evaluation of DNA methylation in human colon cancer cells deficient for one or more DNMTs (*DNMT1*$^{-/-}$, *DNMT3B*$^{-/-}$).[24] Indeed, DNA hypermethylation of *ADAM2, ARMCX1, BCHE, CDO1, ESX1, IRAK3, P2RY14* and *SYCP3* are required for cancer cell growth and survival, and DNA demethylation of these genes resulted in cell death and apoptosis.

Attesting to their importance in CRC tumorigenesis, promoter DNA hypermethylation results in the silencing of genes essential for DNA repair, cell cycle progression, signaling pathway checkpoints, among others and include: (1) *MLH1* (mut-L homolog 1); (2) *CDKN2A*$^{(INK4A)}$ (*p16*, cyclin-dependent kinase inhibitor 2A), (3) *MGMT* (*O*-6-methylguanine methyltransferase), (4) *RUNX3* (runt-related transcription factor 3), (5) *TPEF* (transmembrane protein with EGF-like and two follistatin-like domains 2), (6) *VIM* (vimentin), (7) *SFRP1/2/4/5* (secreted frizzled-related protein) family, and others. Of note, SFRPs inhibit WNT signaling, and their silencing is one mechanism of WNT signaling alterations that are plentiful in colorectal tumors.[25]

## miRNA epigenetic silencing

miRNAs are short RNA sequences of 20–22 nucleotides in length that are transcribed from their own promoters or from intronic gene regions (reviewed in reference 26,27). MiRNAs form double-stranded complexes with target mRNAs, which signals either the degradation of the mRNA-miRNA complex or translational inhibition. As a result, a single miRNA can regulate multiple mRNAs, implicating miRNAs in substantially altering translation and enzymatic signaling. miRNAs are frequently altered in human cancers,[26,27] and miRNA expression is also altered in human cancers via enhancer function, binding of hormones and growth factors at individual miRNA promoters, and miRNA promoter DNA hypermethylation.

Indeed, miRNA promoter DNA hypermethylation is prevalent in CRCs and virtually every cancer type, implicating these miRNAs as tumor suppressors. Saito *et al.*[28] provided the first evidence of miRNA epigenetic silencing in cancer cells (*miR-127*), and additional miRNAs silenced by DNA hypermethylation have been described in CRC, the first of which was *miR-124a*,[29] which allows for cyclin D kinase 6 activation and subsequent RB phosphorylation. Activated cyclin D kinase 6 acts in an oncogenic capacity in phosphorylating RB, thereby inactivating the enzyme, thus leading to loss of cell cycle control and tumor progression.[30] Additional examples of miRNA silencing by DNA hypermethylation include: (1) *miR34b* and *miR34c,* which share a CpG island with the tumor suppressor gene B-cell translocation gene 4;[31] (2) *miR-137,* which regulates the lysine specific demethylase *KDM1A/LSD1,*[32] *miR-342,* which targets DNMT1, thereby leading to activation of *RASSF1, ADAM23*, *RECK* and *HINT1* as a result of promoter DNA

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hypomethylation,[33] as well as several others (reviewed in references 34-36). miRNA silencing via promoter DNA hypermethylation has downstream effects owing to the inability of the specific miRNA to regulate gene expression and cellular programming.

### DNA methylation biomarkers of CRC

Cancer-specific DNA methylation can be identified not only in tumors, but also in adenomas, circulating tumor DNA, cfDNA (cell-free DNA) in patient blood (plasma/serum), urine and fecal matter as sensitive (early) detection protocols (reviewed in reference 37). DNA hypermethylation of key CRC epigenetic driver genes has been identified in human stool/fecal matter (*MLH1, CDKN2A, MGMT, VIM, SFRP2*),[38-44] urine ( *VIM, WIF1, ALX4, NDRG4* (reviewed in reference 45) and blood (*MLH1, APC, MGMT, RASSF2A, TMEFF2* (reviewed in reference 37). DNA methylation markers of CRC tumor recurrence and patient survival have also been identified[46-49] and include *CDKN2A, HLTF* and *TPEF*. Finally, CRC patients with promoter DNA hypermethylation of *p14*$^{ARF}$, *RASSF1* or *APC1A* showed poor prognosis, while patients with *MGMT* promoter DNA methylation show improved prognosis.[50]

DNA methylation-based biomarkers with unknown biological relevance to CRC have also been recently identified through genome-wide and genome-scale approaches.[51,52] Recent examples include *THBS1, C9ORF50* and *SEPT9*. *THBS* and *C9ORF50* were identified by Lange *et al*.[52] from publicly-available TCGA DNA methylation data, and displayed CRC-specific DNA hypermethylation after comparison with 14 other tumor types. Both markers validated in cfDNA in pre-therapeutic plasma and serum samples from CRC patients, and outperformed the carcino-embryonic antigen blood test used in the clinic with respect to tumor detection sensitivity and specificity.

*SEPT9* was first identified from a genome-wide screen of CRC-specific DNA methylation profiles.[53] *SEPT9* DNA methylation of the v2 variant promoter region occurs in nearly all colorectal tumors and adenomas, but not in normal colonic mucosa. The first release of the cfDNA SEPT9 DNA methylation assay showed high sensitivity (72%) and specificity (86%) of CRC detection in plasma,[53] and evaluations of an updated version of the assay (Epi proColon 2.0) showed similar sensitivities (68–95%) and specificities (80–99%) in detecting CRCs in blood plasma. Interestingly, the Epi proColon 2.0 assay identified stage I disease in 60–84% of cases and 80–100% of stage II disease (reviewed in reference 54). Importantly, DNA methylation of *SEPT9* and *TAC1* in post-operative serum blood samples serves as independent predictors of CRC recurrence and patient survival.[55] The *SEPT9* assays outperform fecal occult blood and carcino-embryonic antigen tests with respect to detection sensitivity and specificity. The *SEPT9* DNA methylation assays, unlike colonoscopy, are noninvasive, cost effective and do not require outpatient medical procedures. *SEPT9* is one example of the power of DNA methylation biomarkers as clinically important and effective means of CRC detection.

## CPG ISLAND METHYLATOR PHENOTYPES (CIMPS) IN CRC

DNA methylation aberrancies substantially outnumber somatic mutations in human cancers,[56] and individual tumor types can be stratified into subgroups based on DNA methylation

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profiles. In 1999, Toyota and colleagues first identified a unique subset of colorectal tumors positive for a CIMP (now classified as CIMP-high (CIMP-H)) that display extensive DNA hypermethylation at a unique set of CpG islands that remained unmethylated in other colorectal tumors and normal tissues[57] (Figure 1). Follow-up experiments showed that CIMP-H tumors are preferentially located in the proximal (right) colon, are enriched in women of older age, patients with a family history of CRC, and harbor the *BRAF* V600E (*BRAF*-mut) point mutation, as well as *MLH1* epigenetic silencing due to promoter DNA hypermethylation, microsatellite instability (MSI), diploid copy number and the absence of *TP53* mutations[51,58] (Figure 2). Moreover, tumors with CIMP-H, MSI and *BRAF*-mut are positively associated with smoking[51,59,60] and body mass index in women.[51]

A second CIMP subgroup, CIMP-low (CIMP-L) was identified as another distinct subgroup of CRCs.[61] CIMP-L tumors display an attenuated and partial DNA methylation status at CIMP-defining regions, both with respect to the number of methylated CIMP loci and their DNA methylation levels (Figure 1). In support of this, a genome-scale DNA methylation analysis of primary CRCs showed that 20% of CIMP-H sites are also methylated in CIMP-L tumors.[62] CIMP-L tumors are enriched in *KRAS* and *TP53* mutations and male gender (Figure 2). Although generally located on the right side of the colon, CIMP-L tumors are not like CIMP-H tumors as they are non-hypermutated, chromosomal instability positive and do not harbor *MLH1* DNA hypermethylation.

Shen *et al.*[63] reported the CIMP2 subgroup along with CIMP1 (CIMP-H) and non-CIMP subgroups. Like non-CIMP tumors, CIMP2 tumors also harbor *KRAS* and *TP53* mutations, but are generally located in the proximal colon. Yagi *et al.*[64] categorized three CRC subgroups, with the high-methylation epigenotype enriched for *BRAF* mutations and the intermediate-methylation epigenotype harboring *KRAS* mutations. Finally, Hinoue *et al*[62] and TCGA[8] categorized CRCs into four groups based on unsupervised clustering: CIMP-H, CIMP-L and two non-CIMP groups.

An analysis of the consequences of CIMP DNA methylation on gene expression showed that only a small percentage (7%) of genes with DNA hypermethylation in CIMP-H tumors were downregulated in expression.[62] However, these appear to be enriched for genes essential for CRC tumorigenesis. Indeed, a search for epigenetic driver genes in colorectal tumors identified a handful of genes displaying both DNA hypermethylation and gene expression reduction in CIMP-H and non-CIMP tumors. Interestingly, these include *SFRP1, SFRP2, FOXD2* and *TMEFF2/HPP1,* and are key regulators of the WNT pathway (SFRP1, SFRP2), have roles in transcription factor associated gene regulation (*FOXD2*) and coordinate cellular proliferation, differentiation and apoptosis (*TMEFF2/HPP1*). These epigenetic driver genes are attractive therapeutic targets, such that their activation by treatment with DNA methylation inhibitors may result in resetting of cellular programs.

## CONSENSUS MOLECULAR SUBGROUP (CMS) CLASSIFICATION OF CRC BASED ON GENETIC AND EPIGENETIC ALTERATIONS

The CRC Subtyping Consortium performed comprehensive cross-comparative analyses of tumor subtype assignments based on publicly-available molecular data sets and six existing

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algorithms for determining CRC subgroups to assess whether the subtype assignments correlated with patient outcome, and ultimately, to institute a translational approach for the use of molecular subtypes in the clinic.[65] The classifier identified four CMS of CRC: CMS1 (MSI immune), CMS2 (Canonical), CMS3 (Metabolic) and CMS4 (Mesenchymal) (Figure 2). CMS1 tumors display CIMP-H, MSI, *BRAF*-mut, DNA hypermutation, as well as immune infiltration and activation, and CMS1 patients show poor survival after relapse. CMS2 tumors are non-CIMP, with SCNAs (somatic copy number alterations), as well as activation of WNT and MYC signaling. CMS3 tumors are characterized by CIMP-L, MSI/microsatellite stable (MSS) status, low SCNAs, *KRAS*-mut and metabolic dysregulation. Finally, CMS4 patients are also non-CIMP and display SCNAs, infiltration of stromal cells, as well as activated TGF-β and angiogenic signaling. In addition, CMS4 patients show worse relapse-free survival and overall survival (OS).

## CRC THERAPEUTICS

### The intersection of CIMP with EGFR and VEGFR signaling pathway status

The EGFR and VEGFR pathways are instrumental for determining appropriate and effective CRC treatments. EGFR, upon activation by binding EGF ligand, activates KRAS, which activates BRAF. Activated BRAF then stimulates MEK1 and MEK2, and subsequently ERK signaling. ERK signaling activates oncogenic transcription factors (MYC, ELK1, FOS, JUN) that in turn activate genes that drive cell proliferation, cell cycle progression and differentiation (Figure 3). The VEGFR pathway also overlaps with the EGFR pathway via RAS and BRAF signaling, but also activates transcription factors through PI3K/AKT/mTOR signaling to promote cell growth, differentiation and angiogenesis (Figure 3).

Even though large numbers of CRC genomes have been sequenced to identify potential novel drug targets, this has not yet resulted in the identification of new and highly penetrant mutations or novel therapeutics. Only *RAS*-mut status exists to guide CRC therapeutic decisions, however, 40–60% of RAS-wt tumors are resistant to EGFR-based treatments.[66] The CRYSTAL and OPUS phase III clinical trials[67,68] showed that adding cetuximab to FOLFOX (5-florouracil (5-FU), folinic acid and oxaliplatin) or FOLFIRI (5-FU, folinic acid and irinotecan) increased patient OS, progression-free survival (PFS) and objective response in first-line treatment of *RAS*-wt (*KRAS* or *NRAS*) metastatic CRCs, whereas patients with *RAS* mutations did not benefit from these treatment schemes. *KRAS* mutations predict resistance to EGFR-based antibodies (panitumumab and cetuximab), as KRAS is involved in signal transduction from ligand-bound EGFR from the cell membrane to the nucleus (Figure 3). DNA methylation alterations in *KRAS*-mut CRCs, especially those related to the CIMP-L CRC subset, may play important roles in EGFR resistance; however, CIMP-L-specific DNA methylation signatures have not yet been identified.

*EGFR* silencing may also be involved in drug resistance, especially in CIMP-H tumors that display extensive DNA hypermethylation and correlated gene repression. In support of this, Scartozzi *et al.*[69] demonstrated *EGFR* promoter DNA hypermethylation in ~ 60% of primary colon tumors, and showed that patients with *EGFR* promoter DNA methylation have 5 month shorter PFS and ~ 12 month shorter OS than patients without *EGFR* promoter DNA methylation. This was supported by a recent report by Demurtas and colleagues in which 88

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CRC patients were evaluated.[70] In contrast, Geißler *et al.*[71] showed that *EGFR* promoter DNA methylation, CIMP status and MSI status are not correlated with patient response after treatment with cetuximab and/or panitumumab. However, it should be noted that only a small number ($n = 25$) of CRC patients were studied in the Geißler report, and only two were CIMP-positive, and highlights the importance of CIMP status in selecting patients for treatment.

Interestingly, the Geißler report also showed that treatment responses were linked to *PIK3CA* mutations, whereas non-responders were associated with *ATM* mutations and low CDH1 expression.[71] ATM forms a protein complex with EGFR and causes AKT phosphorylation. E-cadherin is involved in recruiting and activating EGFR. In contrast, cells with low or no E-cadherin expression can bypass EGFR signaling and become resistant to EGFR-based antibodies. Therefore, restoring E-cadherin expression is an important facet of tumor sensitivity to EGFR-targeted antibodies.

## Tumor location and CIMP status as predictive and prognostic tool

As CIMP-H tumors are mainly found in the right side of the colon, tumor location may be an important determinant of CRC patient outcome. Left-sided tissues derive from the hindgut, whereas right-sided tissues arise from the midgut. Indeed, major differences in embryonic patterning genes and crypt stem cell populations begin in normal colonic tissue in both mouse and human systems.[72-74] Left- and right-sided colon cancers differ extensively in terms of gene expression, DNA mutation and DNA methylation profiles,[72] however, how these relate to embryological origin or other site-associated factors, is still unknown. Clinically, left- and right-sided colon tumors have different epidemiologic trends and outcomes. Driver germline genetic alterations in hereditary syndromes show non-random propensity to develop left- or right-sided polyps and tumors. Lynch syndrome patients, for example, predominantly develop right-sided tumors. Most sporadic colon cancers are left-sided, whereas a minority of sporadic tumors are right-sided and have unique molecular profiles.

In the Cancer and Leukemia Group B and Southwest Oncology Group 80 405 trial, 1104 metastatic colon cancer patients with a *KRAS*-wt genotype were treated with either FOLFIRI or FOLFOX prior to adjuvant therapy with cetuximab or bevacizumab. Although overall patient survival rates were not significantly different between treatment arms, significant differences in OS and PFS were evident after stratification by tumor location (left vs right),[75,76] suggesting that tumor location is an important predictor of patient outcome. Tejpar *et al.*[77] supported this observation by showing that *RAS*-wt patients with left-sided tumors had improved PFS and OS compared with patients with right-sided tumors. Moreover, patients with right-sided *RAS*-wt tumors showed comparable to nominally improved treatment efficacy by adding cetuximab to FOLFIRI, but a marked improvement of the same treatment regimen in CRC patients with left-sided tumors and *RAS*-wt status.

Loupakis *et al.*[78] showed that patients with right-sided (generally CIMP-H) tumors exhibit shorter OS and PFS, as well as higher mortality rates than patients with left-sided tumors (generally non-CIMP). An analysis of CIMP data from over 10 000 patients[79] showed that CIMP-H CRC patients display shorter disease-free survival (DFS) and OS than non-CIMP

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patients, both with MSI or MSS disease. These results were supported by a recent study from the HE6C/05 trial of 441 patients with stage II/III disease treated with either XELOX (capecitabine, oxaliplatin) or FOLFOX.[80] No differences in survival were found between CIMP-H and non-CIMP patients, however, stage II patients showed lower risk of relapse, and patients with lower stage and left-sided tumors displayed a lower risk of death.

The prognostic value of CIMP is not well understood. In agreement with other clinical trials, CIMP-H, stage III CRC patients had shorter OS after surgical resection than CIMP-negative patients,[81] and Ahn *et al.*[82] showed that CIMP-H, *BRAF*-mut and proximal location correlated with a significantly worse DFS. However, the prognostic utility of CIMP status in mixtures of stage II and III patients shows either a decreased DFS in CIMP-positive patients or no difference in DFS between CIMP and non-CIMP patients (summarized in reference[83]).

CIMP-positive patients show improved survival after 5-FU treatment. DNA hypermethylation-associated silencing of *DPYD* (dihydropyrimidine dehydrogenase), a gene specific for 5-FU degradation, is prevalent in CIMP-positive CRC patients, and may explain the correlation between CIMP status and 5-FU sensitivity[84] (reviewed in[83]). Moreover, DNA hypermethylation-based silencing of *GGH* (gamma-glutamyl hydrolase), a regulator of folate levels for methyl-transfer and nucleotide biosynthesis, may also help explain this association. However, this may also be confounded by MSI, which although enriched in CIMP-positive tumors, is also present in non-CIMP tumors.[83] Nonetheless, the CIMP-specific DNA methylation and silencing of *DPYD* may impede the tumor cells ability to degrade and deactivate 5-FU, thereby resulting in drug sensitivity, whereas epigenetic silencing of *GGH* may result in increased cellular folate concentrations and dysregulated nucleotide synthesis.[85]

The association of CIMP with positive response to therapeutic agents also appears to be limited to 5-FU adjuvant therapy. In one study,[86] CIMP patients showed improved outcome after 5-FU treatment and improved OS after FOLFIRI treatment as compared with non-CIMP patients. A report from Van Rijnsoever showed that CIMP-positive patients show shorter OS as compared with non-CIMP patients, yet CIMP patients showed improved OS after 5-FU adjuvant treatment.[81] A survival benefit was also shown for stage II CRC patients after 5-FU treatment.[87] Finally, Min *et al.*[88] showed that CIMP-positive patients had improved DFS after 5-FU treatment.

DNA methylation of candidate genes and repetitive elements can be used for prognostic and predictive purposes. DNA hypomethylation of *LINE-1*-repetitive elements, a surrogate marker for global DNA methylation, is associated with poor patient outcome, and worse PFS and OS following FOLFOX-based chemotherapy. *LINE-1* DNA hypomethylation is independently associated with poor prognosis as well as resistance to FOLFOX treatment.[89] In addition, DNA methylation of *HYLA2* (hyaluronoglucosaminidase 2) was associated with positive response of 5-FU in stage II and III CRC patients.[90]

## Mechanisms of CIMP-specific DNA methylation in BRAF-mutant CRCs

*BRAF*-mut results in a constitutively active BRAF protein and resistance to MEK and EGFR inhibitors. In addition, the relationship of *BRAF*-mut and CIMP, whereas highly associated,

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has not been understood until recently, nor are the reasons as to why CIMP-H tumors are almost entirely *KRAS*-wt and CIMP-L tumors are mainly KRAS-mut. Fang *et al.*[91] identified the transcriptional repressor MAFG as essential for CIMP-H DNA hypermethylation and silencing in *BRAF*-mut tumors. MAFG binds to CIMP-H genes, and recruits co-repressor proteins including BACH1, the chromatin remodeler CHD8 and DNMT3B to methylate and silence CIMP-H target genes (Figure 4). MAFG is overexpressed in CRCs, and MAFG-binding sites are located in the majority of CIMP-H-defining genes.

MAFG is a substrate for the BRAF/MEK/ERK pathway. The BRAF-mut protein is constitutively active in CIMP-H tumors, leading to increased BRAF/MEK/ERK signaling. ERK then phosphorylates MAFG, resulting in decreased MAFG ubiquitination and subsequent MAFG protein stability (Figure 4). Ultimately, this leads to high expression of MAFG protein, resulting in MAFG binding to CIMP-H defining loci. This model suggests a direct connection between BRAF-mut activity, MAFG levels and CIMP-H-specific DNA methylation. This signaling system appears to be specific for BRAF-mut CRCs, as MAFG knockdown in *KRAS*-mut tumors did not show an effect. However, *KRAS*-mut CRCs utilize a unique set of co-repressors and include ZNF304, which recruits KAP1, SETDB1 and DNMT1 to CIMP-L target regions, resulting in DNA hypermethylation and gene silencing[92] (Figure 4). ZNF304 does not have a role in *BRAF*-mut CRCs, thus indicating two separate pathways for DNA methylation in CIMP-H and CIMP-L CRCs (Figure 4).

## DNA METHYLATION INHIBITION AS A THERAPEUTIC APPROACH FOR CANCER TREATMENT

### 5-Azacytidine-based DNA methylation inhibitors

The extensive DNA methylation alterations in CRC patients suggest that a substantial number of patients may benefit from epigenetic therapies, especially with DNA methylation inhibitors. Small molecule DNA methylation inhibitors, such as nucleoside and non-nucleoside based molecules, have played important roles in understanding human methylomes in normal and tumor cells. The first DNA methylation inhibitors, 5-azacytidine (5-Aza-CR) and 5-aza-2'-deoxycytidine (5-Aza-CdR), were designed and synthesized in the 1960s by Sorm and colleagues (reviewed in reference 93) as cytotoxic anticancer drugs, akin to 5-FU. Aza-substituted analogs are converted to Aza-triphosphates after entering the nucleus, and are then incorporated into newly synthesized DNA during DNA replication. Aza-incorporated DNA traps DNMTs to genomic DNA, leading to their depletion and passive DNA demethylation.[94,95]

A modification of 5-Aza-CdR, Guadecitabine (SGI-110), was more recently developed, and consists of 5-Aza-CdR followed by deoxyguanosine.[96,97] SGI-110 shows promising clinical utility, displays improved stability and lower toxicity over 5-Aza-CdR alone, is better tolerated upon delivery, and is effective in mice and patient-derived xenograft models of cancer.[96] Currently, SGI-110 is under evaluation in clinical trials for CRC patients, as well as patients with other malignancies.

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## DNA methylation as a therapeutic target

The DNA demethylation and gene activation aspects of 5-Aza-CdR have been well characterized with respect to tumor suppressor and DNA repair systems. However, 5-Aza-CdR treatment also reduces the overexpression of genes through DNA demethylation of gene bodies and transcribed regions, which are normally methylated in actively expressed genes.[18] Genes whose expression is downregulated after 5-Aza-CdR treatment include oncogenes and those involved in c-MYC regulated processes (Figure 3), suggesting that combining 5-Aza-CdR with EGFR and VEGFR therapies may have synergistic anti-tumor effects.

In addition, ERVs (endogenous retroviruses) and other repetitive elements located in transcribed (gene body) regions, normally silenced by DNA methylation in cancer cells, are reactivated after treatment with DNA methylation inhibitors.[98-101] Demethylated and activated ERVs trigger activation of an interferon response and essentially mislead the cancer cell to operate in a viral-infected state, and therefore susceptible to immuno-modulating drugs that have shown success in the clinic (Figure 5). Li *et al.*[102] showed that treatment of human colon cancer cell lines with 5-Aza-CR resulted in activation of immunomodulatory pathways, namely interferon, inflammation, cytokine/chemokine and cancer testis antigen-signaling pathways. The activation of an immune response, coupled with activation of tumor suppressors (*p14*, *p15*, *p16*), DNA repair genes (*MLH1, MGMT*) and the reduction in oncogene (MYC) expression, provide substantial evidence of the efficacy of DNA methylation inhibition as a treatment option for CRC patients (Figure 5).

## TET enzyme-based DNA demethylation

Although DNMTs and DNA methylation inhibitors are well described, DNA demethylases have only been recently characterized. The Ten Eleven Translocase (TET) family of enzymes (TET1, TET2, TET3) were shown to convert 5-methylcytosine to 5-hydroxymethylcytosine using ascorbic acid (vitamin C) as a co-factor.[103,104] TETs can further oxidize 5hmC to 5-formylcytosine (5fC) and 5-carboxylcytosine,[105,106] with both 5-formylcytosine and 5caC marks removed and replaced with an unmethylated cytosine residue via DNA glycosylase-involved base excision repair.

The correlation between TET activity and cancer-specific DNA methylation was first shown in human glioblastoma. A specific heterozygous point mutation in isocitrate dehydrogenase 1 results in the catalysis of an oncometabolite that inhibits DNA demethylation. Although isocitrate dehydrogenase 1 functions as a dimer in the citric acid cycle by converting isocitrate to alpha-ketoglutarate,[107] mutant isocitrate dehydrogenase 1 further converts alpha-ketoglutarate to D-2-hydroxyglutarate,[108] which is an inhibitor of TET activity[109] and DNA demethylation, resulting in DNA hypermethylation.

Interestingly, only low frequency *IDH* and *TET* mutations were identified in CRCs,[8] suggesting that CRC DNA methylation profiles are also generated independent of *TET* and *IDH* mutations alone. In addition to alpha-ketoglutarate, TET enzymes also require oxygen as a substrate for activity, as well as Fe(II) and vitamin C as cofactors.[110] Many tumor types, including CRC, display hypoxia, described as decreased cellular oxygen levels, which

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inhibit TET function, thereby retaining DNA methylation profiles. In addition, cancer patients commonly present with vitamin C deficiency,[111] also resulting in TET enzyme inhibition and retained DNA methylation.

Vitamin C is an effector of 5-Aza-CdR based DNA demethylation. Combining vitamin C with 5-Aza-CdR treatment of cancer cells results in a synergistic boost in DNA demethylation, as both active (TET) and passive (Aza) mechanisms of DNA demethylation are activated.[102] Vitamin C enhances the activation of ERVs, tumor suppressors, DNA repair genes and other genes silenced by DNA promoter hypermethylation. Vitamin C is orally available, cost-effective and only physiological concentrations (57 μM) are required for synergistic DNA demethylation.

## DNA METHYLATION INHIBITION AS A TREATMENT OPTION FOR CRC PATIENTS

Treatment of human colon cancer cell lines with DNA methylation inhibitors has provided evidence for potential treatment efficacy. In one report, human colon cancer cell lines were treated with combinations of conventional chemotherapies (5-FU, irinotecan, oxaliplatin), DNA methylation inhibitors (5-Aza-CR, 5-Aza-CdR, zebularine) and histone deacetylase inhibitors (Trichostatin A, SAHA, valproic acid) to determine whether epigenetic therapies improve tumor toxicity.[112] The addition of DNA methylation inhibitors resulted in synergistic effects incurred by chemotherapy, and in particular, 5-Aza-CdR showed the most potent synergistic effect and enhanced oxaliplatin cytotoxicity. Moreover, 5-Aza-CdR added to 5-FU or oxaliplatin treatments of CRC cell lines showed synergism based on cell viability and cell counts.[113]

Early clinical trials evaluating efficacy of 5-Aza based DNA methylation inhibitors in CRC patients have shown inconsistent findings. A total of 11 clinical trials involving 5-Aza-CR (Vidaza), 5-Aza-CdR (Decitabine) or SGI-110 are active or have been completed (Table 1; clinicaltrials.gov). A recent phase II study of 5-Aza-CR and the histone deacetylase inhibitor entinostat,[114] although tolerated, did not result in clinical activity. However, DNA demethylation occurred in a subset of patients and was correlated with improved PFS. A separate phase I/II trial[115] was performed by treating CIMP-H CRC patients who are resistant to 5-FU and oxaliplatin with 5-Aza-CR and CAPOX (capecitabine and oxaliplatin). DNA demethylation was detected, but did not correlate with occurrence of stable disease. Moreover, CIMP status did not correlate with stable disease or PFS, suggesting that evaluating additional drug combinations, both in the clinic and in the laboratory, are required to determine treatment efficacy for CRC patients. Finally, a phase I/II trial[116] to assess the performance of 5-Aza-CdR and panitumumab in metastatic CRC patients with *KRAS*-wt tumors showed tolerance and activity to this drug combination. Partial responses were observed in 2/20 (10%) of patients and stable disease was observed in 10/20 (50%) of patients, suggesting that this drug combination may improve survival and quality of life in patients with metastatic colon cancer.

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## CONCLUSIONS AND FUTURE DIRECTIONS

DNA methylation alterations are not only abundant in CRCs, but also have clinical importance. The correlation of CIMP-H with *BRAF*-mut involves cell-signaling aberrancies that dictate the types of effective treatments for CRC patients. However, DNA methylation inhibition is only at the clinical trial phase for treating CRC patients. There is substantial evidence that DNA methylation inhibition by 5-Aza-CdR/SGI-110 and vitamin C sensitizes the tumor cells to traditional chemotherapies, immune-based therapies and DNA repair inhibitors. These attributes, as well as the activation of tumor suppressors and miRNAs and the down regulation of oncogenes by DNA demethylating agents, make DNA methylation inhibition an attractive therapeutic strategy. Combining DNA methylation inhibitors with EGFR antibodies may also show clinical promise, as the *EGFR* promoter is hypermethylated in a substantial proportion of CRCs, and DNA methylation inhibition may boost efficacy of EGFR inhibitors by blocking BRAF-mut signaling and reducing MYC signaling to inhibit cellular proliferation.

Combining DNA methylation inhibition with targeted agents, cytotoxic agents and immuno-modulating drugs in CIMP-H/CIMP-L patients will help determine the prognostic utility of CIMP status on response to treatment and patient outcome. Determining the DNA methylation status of gene regions and their correlation with clinical outcome is also important to determine whether CIMP or candidate gene regions are better predictive and prognostic biomarkers. These DNA methylation-based signatures, such as DNA methylation of *MMP9* and *RASSF1*, can be used to determine specific patients who may benefit from epigenetic therapies.

One aspect of epigenetics that has been overlooked is the role of tumor heterogeneity in epigenetic targeting. Although this has been a focus of genetic and mutation-based analyses, the extent of DNA methylation heterogeneity is not fully understood in primary colorectal tumors. Only cellular-based tumor cell contamination, especially white blood cells, has been documented in DNA methylation-based analyses.[117]

Additional future directions involve the development of next-generation DNA methylation inhibitors, especially those that result in sustained DNA methylation inhibition concurrent with low cellular toxicity, as well as addressing drug activity and patient response using both quantitative and qualitative methods. There is a need for targeted delivery of DNA methylation inhibitors to specific tissues and/or tumor cells so as to achieve an optimal response and to avoid off-target effects. This is exceptionally challenging and requires engineering to not only target tumor cells but also ensure their delivery into the cell and nucleus. Combining highly focused epigenetic therapies with immune-modulating therapies, for instance, may be an effective strategy for targeted cancer treatment. Exploiting expressed cell surface markers for targeted therapy of specific tissue types may also provide an efficacious drug delivery system.

Determining treatment efficacy and patient response are also challenges that require the development of highly sensitive and specific biomarkers, and/or the ability to obtain quantitative data on circulating tumor DNA, cfDNA and small amounts of primary or

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metastatic tissues. These methods need to be time-effective so that treatments can be fine-tuned and tailored for each patient in order to achieve a durable and prolonged effect.

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## ABBREVIATIONS

| | |
|---|---|
| **2-HG** | 2-hydroxyglutarate |
| **5-Aza-CR** | 5-aza-cytidine |
| **5-Aza-CdR** | 5- aza-2’-deoxycytidine |
| **5-FU** | 5-fluorouracil |
| **5caC** | 5-carboxylcytosine |
| **5fC** | 5-formylcytosine |
| **5hmC** | 5-hydroxymethylcytosine |
| **5mC** | 5-methylcytosine |
| **α-KG** | alpha-ketoglutarate |
| **ADAM2** | ADAM metallopeptidase domain 2 |
| **ADAM23** | ADAM metallopeptidase domain 23 |
| **APC** | adenomatous polyposis coli |
| **ARMCX1** | armadillo repeat containing, X-linked 1 |
| **ATM** | ataxia telangiectasia mutated |
| **BACH1** | BTB domain and CNC homolog 1 |
| **BCHE** | butyrylcholinesterase |
| **BRAF** | B-Raf proto-oncogene, serine/threonine kinase |
| **BRAF-mut** | mutant BRAF |
| **BTG4** | BTG anti-proliferation factor 4 |
| **C9ORF50** | chromosome 9 open reading frame 50 |
| **CALGB** | aancer and leukemia group B |
| **CAPOX** | capecitabine and oxaliplatin |
| **CDKN2A** | cyclin-dependent kinase inhibitor 2A |

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|---|---|
| **CDH1** | E-cadherin |
| **CDO1** | cysteine dioxygenase type 1 |
| **CEA** | carcino-embryonic antigen |
| **cfDNA** | cell-free DNA |
| **CHD8** | chromodomain-helicase-DNA-binding protein 8 |
| **CIN** | chromosomal instability |
| **CRC** | colorectal cancer |
| **CIMP** | CpG island methylator phenotype |
| **CIMP-H** | CIMP-high |
| **CIMP-L** | CIMP-low |
| **CMS** | Consensus molecular subgroup |
| **CRCSC** | CRC subtyping condortium |
| **ctDNA** | circulating tumor DNA |
| **DCC** | deleted in colorectal cancer |
| **DKO** | DNMT1/DNMT3B double knockout |
| **DNMT** | DNA Methyltransferase |
| **DNMT1** | DNA methyltransferase 1 |
| **DNMT3A** | DNA methyltransferase 3A |
| **DNMT3B** | DNA methyltransferase 3B |
| **DPYD** | dihydropyrimidine dehydrogenase |
| **EGFR** | epidermal growth factor receptor |
| **ES** | embryonic stem |
| **ESX1** | ESX homeobox 1 |
| **EPCAM** | epithelial cell adhesion molecule |
| **FOLFIRI** | 5-FU, folinic acid and irinotecan |
| **FOLFOX** | folinic acid, 5-FU and oxaliplatin |
| **FOXD2** | forkhead box D2 |
| **GGH** | gamma-glutamyl hydrolase |
| **H3K36me3** | histone H3 lysine 36 trimethylation |

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|---|---|
| **HER2** | ERBB2 (erb-b2 receptor tyrosine kinase 2) |
| **HINT1** | histidine triad nucleotide-binding protein 1 |
| **HNPCC** | hereditary non-polyposis colorectal cancer |
| **HPP** | hyperplastic polyp |
| **IDH1** | isocitrate dehydrogenase 1 |
| **IME** | intermediate methylation epigenotype |
| **IRAK3** | interleukin receptor associated kinase 3 |
| **KAP1** | KRAB-associated protein 1 |
| **KRAS** | V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog |
| **KRAS-mut** | mutant KRAS |
| **MAFG** | MAF BZIP transcription factor G |
| **MEK1** | mitogen-activated protein kinase 1 |
| **MEK2** | mitogen-activated protein kinase 2 |
| **MGMT** | *O*-6-methylguanine-DNA methyltransferase |
| **miRNA** | microRNA |
| **MMP9** | matrix metallopeptidase 9 |
| **MSH2** | MutS homolog 2 |
| **MSI** | microsatellite instability |
| **MSI-H** | MSI-high |
| **MSI-L** | MSI-low |
| **MLH1** | Mut-L homolog 1 |
| **MSH6** | MutS homolog 6 |
| **MSS** | microsatellite stable |
| **MYC** | MYC proto-oncogene, bHLH transcription factor |
| **OS** | overall survival |
| **P2RY14** | purigenic receptor P2Y14 |
| **PFS** | progression-free survival |
| **PIK3CA** | phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha |

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|---|---|
| **PMS1** | post-meiotic separation increased 1 |
| **RASSF1** | RAS association domain family member 1 |
| **RB** | retino-blastoma |
| **RECK** | reversion inducing cysteine-rich protein with kazal motifs |
| **RFS** | relapse-free survival |
| **RUNX3** | Runt-related transcription factor 3 |
| **SAHA** | suberoylanilide hydroxamic acid |
| **SAM** | *S*-adenosylmethionine |
| **SCNA** | somatic copy number alteration |
| **SEPT9** | septin 9 |
| **SETDB1** | SET domain bifurcated 1 |
| **SFRP** | secreted frizzled-related protein 1 |
| **SGI-110** | Guadecitabine |
| **SMAD2** | SMAD family member 2 |
| **SMAD4** | SMAD family member 4 |
| **SSA** | sessile serrated sdenoma |
| **SWOG** | southwest oncology group |
| **SYCP3** | synaptonemal complex protein 3 |
| **TAC1** | techykinin precursor 1 |
| **TCGA** | The cancer genome atlas |
| **TET** | Ten eleven translocase |
| **TGF-β** | transforming growth factor beta |
| **THBS1** | thrombospondin 1 |
| **TMEFF2** | transmembrane protein with EGF-like and two follistatin-like domains 2 |
| **TP53** | Tumor protein 53 |
| **TPEF** | transmembrane protein with EGF-like and two follistatin-like domains 2 |
| **TSA** | traditional serrated adenoma |
| **VEGFR** | vascular endothelial growth factor receptors |

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|---|---|
| **VIM** | vimentin |
| **WNT** | wingless-related integration site |
| **ZNF304** | zinc finger protein 304 |

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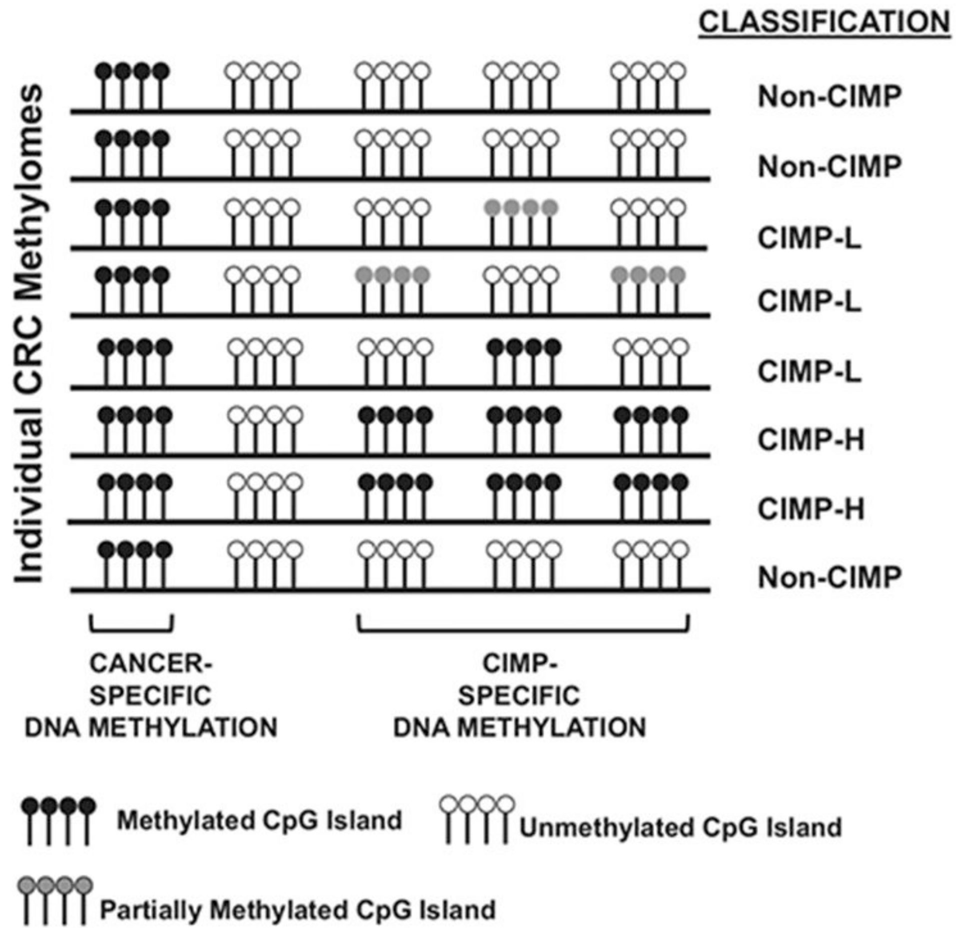


**Figure 1.**
Description of CIMP-H, CIMP-L and non-CIMP CpG island DNA methylation in CRCs. Each row represents a CRC methylome, and lollipop clusters indicate CpG islands. Black lollipops indicate methylated CpG islands, white lollipops indicate unmethylated CpG islands and gray lollipops indicate partially methylated CpG islands. Classification of each methylome as CIMP-H, CIMP-L or non-CIMP are indicated to the right of each methylome. Tumor and CIMP-specific DNA methylation profiles are indicated in the figure.

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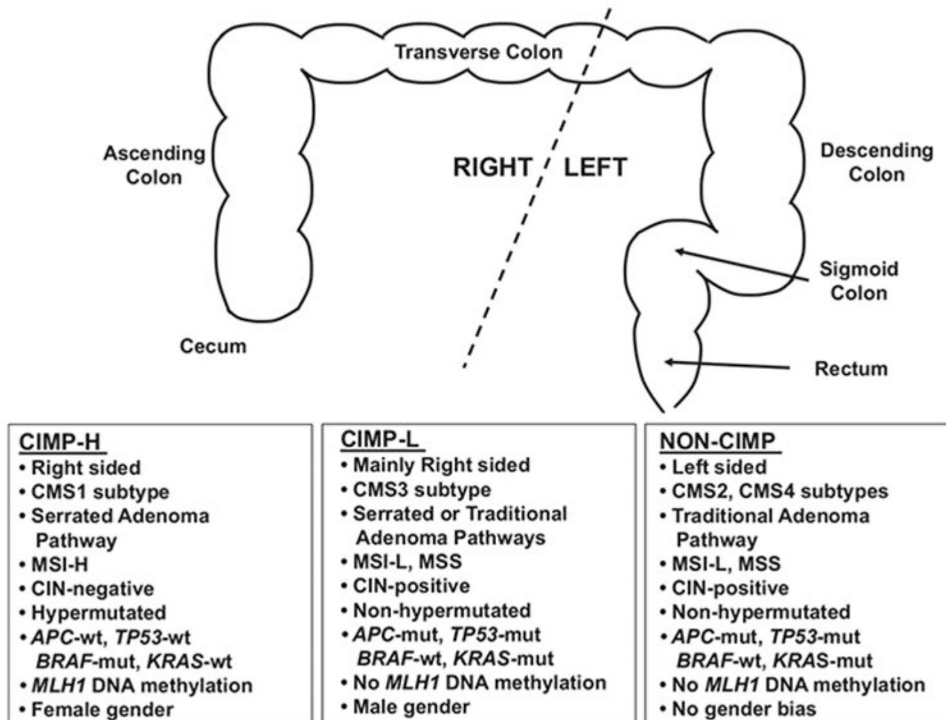


**Figure 2.**

Description of CIMP-H, CIMP-L and non-CIMP tumors. Top section, graphic representation of the colorectum, stratified by location as left or right sides. Bottom section, correlations of each CIMP subgroup with location, CMS subtype, adenoma pathway, mutation status, *MLH1* DNA methylation status and gender bias.

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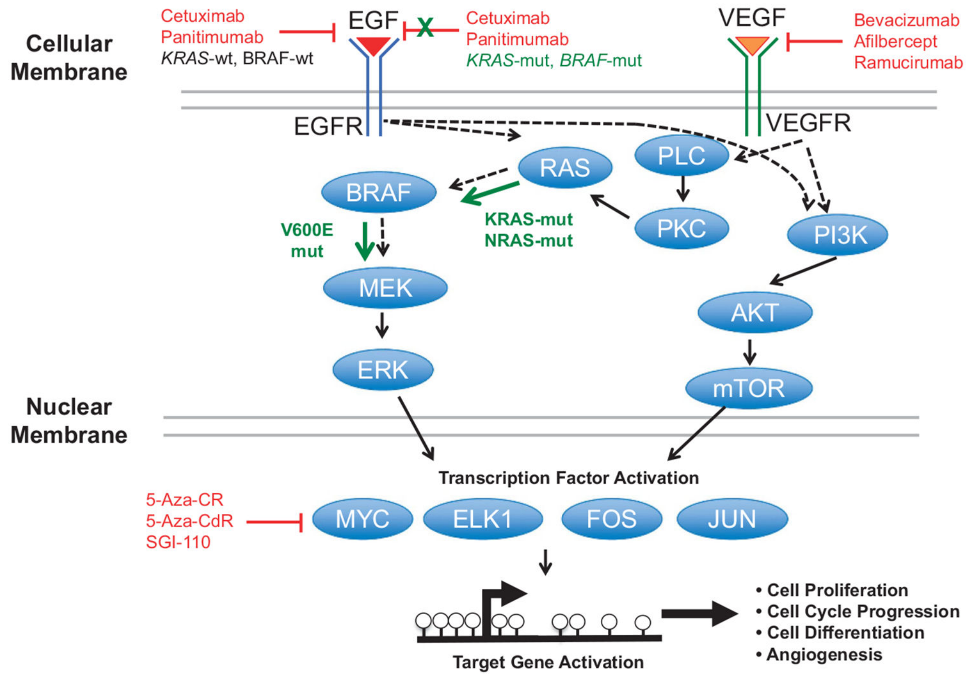


**Figure 3.**

EGFR and VEGFR signaling in CRCs. Protein signaling from EGF and VEGF binding to their respective receptors. Black arrows indicate traditional signaling, whereas green arrows indicate constitutive signaling. Red indicates inhibition of specific aspects of the pathways.

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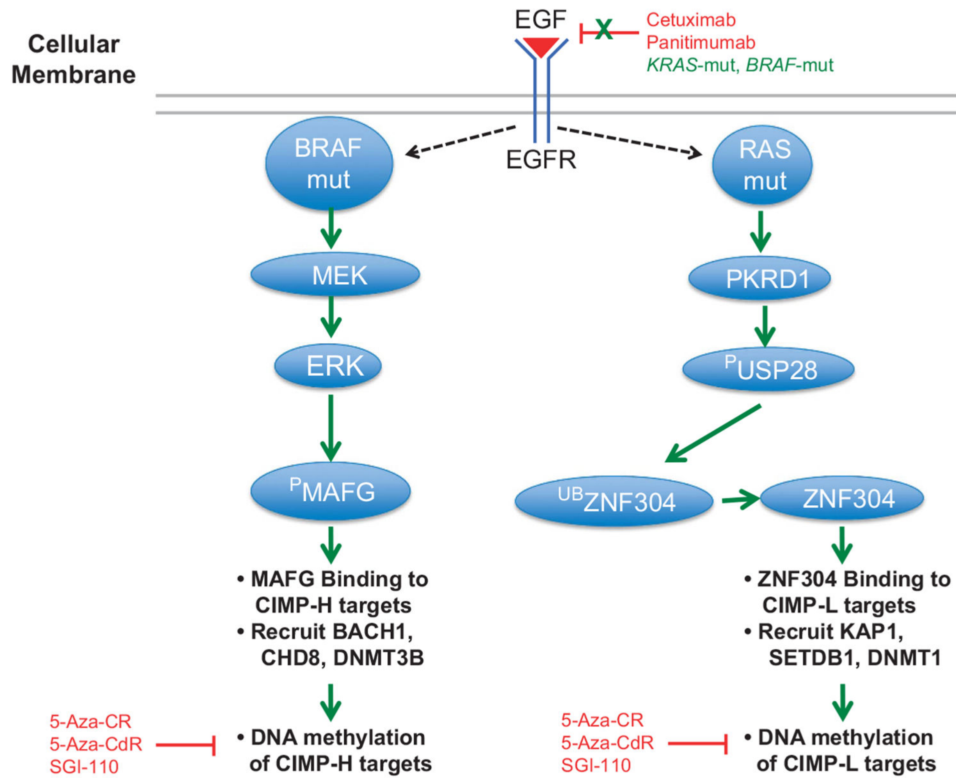


**Figure 4.**

Effects of KRAS-mut and BRAF-mut on EGFR signaling. Models are based on previously described reports.[91,92] Left panel: BRAF-mut constitutively activates MEK and ERK signaling. The ERK enzyme phosphorylates MAFG, stabilizing the protein and allowing MAFG to bind to CIMP-H target regions. MAFG recruits BACH1 and CHD8 co-repressors, as well as DNMT3B to place DNA methylation marks at CIMP-H loci. Right panel: KRAS-mut activates PKRD1, which phosphorylates USP28 ($^{P}$USP28), thereby activating the protein. $^{P}$USP28 removes ubiquitin moieties from ZNF304, thus allowing ZNF304 to bind to CIMP-L-defining loci. ZNF304 binding recruits KAP1, SETDB1 co-repressors, as well as DNMT1, which is thought to methylate CIMP-L loci. Black arrows indicate traditional signaling, whereas green arrows indicate constitutive signaling. Red indicates inhibition of specific aspects of the pathways.

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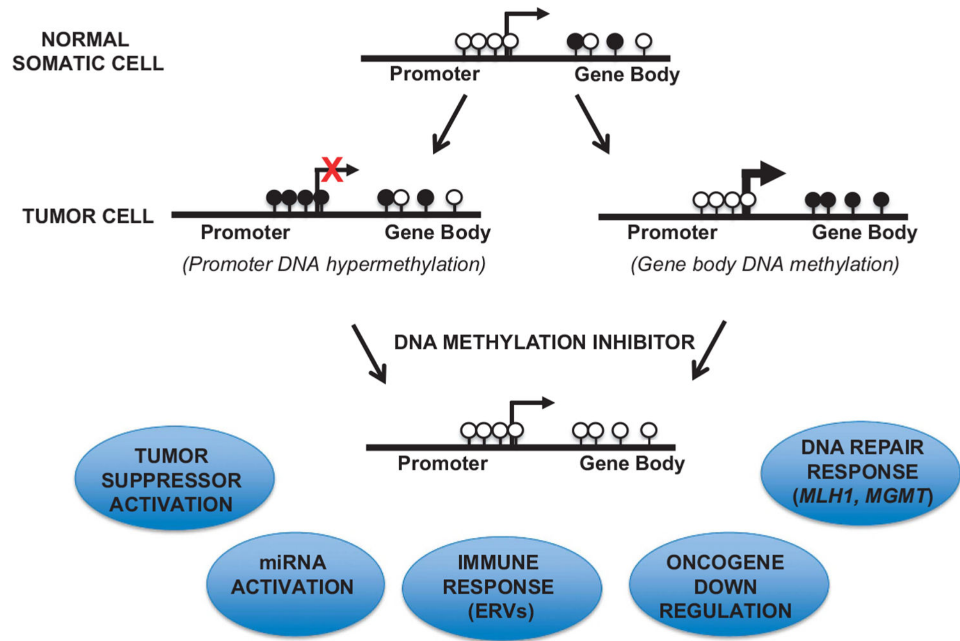


**Figure 5.**

Potential efficacy of DNA methylation inhibition for CRC therapy. Top, promoter and gene body DNA methylation in normal somatic cells. Black lollipops indicate methylated CpG islands, white lollipops indicate unmethylated CpG islands. Middle, promoter (left) or gene body (right) DNA hypermethylation in human cancers. Bottom, promoter DNA hypermethylation may correlate with gene silencing, whereas gene body DNA methylation is associated with actively expressed genes. Treatment with DNA methylation inhibitors results in demethylation of gene promoters and gene body regions, resulting in activation of tumor suppressors, DNA repair response, miRNAs and ERVs, with suppression of oncogenes.

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**Table 1.**

Clinical trials involving DNA methylation inhibitors listed on www.clinicaltrials.gov

| Number | Trial ID | Drug | Phase | Clinical test |
|---|---|---|---|---|
| 1 | NCT01193517 | 5-Azacitidine (Vidaza), capecitabine and oxaliplatin | Phase I/II | Phase I: Find the highest tolerable dose of 5-Azacitidine with capecitabine and oxaliplatin (CAPOX). Phase II: Determine whether 5-Azacitidine with CAPOX is effective in metastatic colon cancer patients |
| 2 | NCT02260440 | 5-Azacitidine and Pembrolizumab | Phase II | To determine the anti-tumor, safety and tolerability of 5-Azacitidine and Pembrolizumab in patients with chemo-resistant metastatic CRC |
| 3 | NCT01105377 | 5-azacitidine and entinostat | Phase II | Efficacy of 5-azacitidine and entinostat in metastatic CRC patients |
| 4 | NCT02316028 | Decitabine | Phase I | Efficacy of Decitabine delivered by hepatic arterial infusion in patients with non-resectable liver metastases |
| 5 | NCT02959437 | 5-Azacitidine with Pembrolizumab and Epacadostat | Phase I/II | Phase I: Dose escalation of 5-Azacitidine with Pembrolizumab and Epacadostat. Phase II: Expansion cohort of MSS CRC patients with recommended dose from Phase I. |
| 6 | NCT02811497 | 5-Azacitidine and Durvalumab | Phase II | To determine the anti-tumor activity of 5-Azacitidine and durvalumab in MSS CRC patients |
| 7 | NCT02512172 | Romidepsin and/or 5-Azacitidine with MK-3475 | Phase I | To evaluate the anti-tumor activity of Romidepsin and/or 5-Azacitidine with MK-3475 in MSS CRC patients |
| 8 | NCT00879385 | Ddcitabine and panitumumab | Phase I | To determine the clinical utility of Decitabine and panitumumab in KRAS-wt in second or third line treatment of metastatic CRC patients |
| 9 | NCT01966289 | SGI-110 with Allogeneic Colon Cancer Cell Vaccine (GVAX) and Cyclophosphamide (CY) | Phase I | SGI-110 with Allogeneic Colon Cancer Cell Vaccine (GVAX) and Cyclophosphamide (CY) in metastatic CRC patients |
| 10 | NCT01896856 | SGI-110 with irinotecan or regorafenib | Phase I/II | Phase I: SGI-110 and/or irinotecan. Phase II: SGI-110 and/or irinotecan vs regorafenib |
| 11 | NCT01882660 | Decitabine | | To determine whether: (1) pre-operative decitabine treatment increases Wnt-target gene expression; (2) DNA demethylation indices more favorable tumor characteristics, and (3) to measure Wnt signaling, DNA methylation and tumor characteristics comparing Wnt methylated vs Wnt unmethylated tumors |