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Epigenetic alterations in the gastrointestinal tract: Current and emerging use for biomarkers of cancer

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Abstract

Colorectal cancer, liver cancer, stomach cancer, pancreatic cancer, and esophageal cancer are leading causes of cancer related deaths worldwide. A fundamental trait of virtually all gastrointestinal cancers is genomic and epigenomic DNA alterations. Cancer cells acquire genetic and epigenetic alterations that drive the initiation and progression of the cancers by altering the molecular and cell biological processes of the cells. These alterations, as well as other host and microenvironment factors, ultimately mediate the clinical behavior of the pre-cancers and cancers and can be used as biomarkers for cancer risk determination, early detection of cancer and pre-cancer, determination of the prognosis of cancer and prediction of the response to therapy. Epigenetic alterations have emerged as one of most robust classes of biomarkers and are the basis for a growing number of clinical tests for cancer screening and surveillance.

Keywords

DNA methylation; noncoding RNA; chromatin; histone; colorectal cancer; esophageal cancer; gastric cancer; pancreatic cancer; Barretts esophagus; biomarkers; diagnosis; prognosis; predictive; treatment

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INTRODUCTION

DNA Alterations In Cancer

Cancer develops as a consequence of disruption of the mechanisms that regulate fundamental processes, such as cellular proliferation, cell metabolism, angiogenesis, cell death, invasion, metastasis, as well as other hallmark behaviors of cancer[1, 2]. (Figure 1) These disruptions arise through an evolutionary process that stably encodes acquired oncogenic alterations in the genome and epigenome, which can then accumulate in clonal lineages. Genetic alterations, including gene sequence mutations, gene copy number alterations, insertions, deletions, and recombination events, were the first and most clearly demonstrated of these oncogenic alterations of DNA, leading to the prominence of the model of cancer genetics in cancer biology. More recently epigenetic alterations have been established as another fundamental oncogenic mechanism and appear to play a prominent role in the pathogenesis of cancer by inducing hallmark cancer cell behaviors. Genome-wide genomic and epigenomic analyses have revealed the widespread occurrence of mutations in epigenetic regulators as well as the breadth of alterations to the epigenome in cancer cells[3, 4]. It is clear that genetic and epigenetic mechanisms influence and cooperate with each other to enable the acquisition of the hallmarks of cancer[2].

The prevailing consensus suggests that epigenetic alterations in cancer occur early and are more common than genetic alterations. In addition, advances in genomic and epigenomic analysis technologies have led to the identification of a variety of specific epigenetic alterations that can be used as potential clinical biomarkers for gastrointestinal (GI) cancer patients. The use of these recently developed technologies has led to insights into cancer epidemiology and hereditary cancer syndromes as well as cancer biology. Because of the potential for environmental factors to modify epigenetic states, the association of cancer risk factors, such as tobacco exposure, on cancer epigenetics has also been assessed. There is modestly robust evidence to date suggesting such factors have significant effects in humans[5, 6]. It is also notable that GI cancer syndromes, such as Lynch syndrome, can arise from germline epigenetic alterations[7, 8]. However, familial epigenetic cancer syndromes are rare and appear to be rarely transmitted to offspring[9]. This review briefly outlines the fundamental basis of epigenetic alterations in cancer, and details the current state of the field regarding the promise and clinical usefulness of various epigenetic alterations as biomarkers for the early detection, diagnosis, prognosis and management of GI pre-cancer and cancer, with a primary focus of colorectal and esophageal cancer. The classes of cancer epigenetic alterations that will be the focus of this review will be DNA methylation and chromatin and histone structure and function. Noncoding RNAs and microRNAs are beyond the scope of this review and the interested reader is directed to recent reviews of this subject for more information[10, 11].

Cancer Epigenetics

Epigenetic mechanisms stably regulate cell behavior by controlling the transcriptional availability of various parts of the genome through differential DNA methylation, chromatin marking and DNA packaging via histone modifications. The best studied of these mechanisms involve direct DNA modifications (primarily CpG cytosine-5’

methylation[12, 13], as well as hydroxylation, formylation, and carboxylation[14, 15]); nucleosome occupancy and positioning[16–18]; nucleosome alterations (e.g. histone variants, different histone modifications)[19]; and noncoding RNAs[20–22]. Cancer related epigenetic alterations cooperate through a network of mutually reinforcing or counteracting signals. Genome-scale projects charting the human epigenome are rapidly extending our understanding of epigenetic marks and how they interact[23]. (The interested reader can visit the NIH Roadmap Epigenomics Project for more details at http://www.roadmapepigenomics.org/) Importantly, epigenetic mechanisms induce stable cell phenotypes but can adapt to changing developmental or environmental needs through the activities of proteins and noncoding RNAs (ncRNA), that regulate the epigenetic state of the genome and its effects on transcription. Recent studies of the aberrant regulation of gene enhancers and super-enhancers in cancer have further extended our understanding of the effects of epigenetic alterations on tumor formation and have shown how epigenetic alterations in noncoding DNA elements can promote cancer formation. The interested reader is directed to recent reviews of this topic[24, 25] The epigenome regulators can be classified as: 1) initiators (e.g. long noncoding RNAs, transcription factors); 2) histone writers, which establish the epigenetic marks; 3) readers, which interpret the epigenetic marks; 4) erasers, which remove the epigenetic marks; 5) remodelers, which can alter chromatin states; and 6) insulators, which form boundaries between epigenetic domains. The establishment, maintenance, and modification of epigenetic marks are intricately regulated, with crosstalk among the marks and writers to shape and alter the epigenetic landscape[23].

DNA Methylation

DNA methylation is an epigenetic DNA modification that can mediate a variety of processes in cells, such as maintenance of genome integrity, genomic imprinting, transcriptional regulation, and developmental processes[26]. DNA methylation in eukaryotic cells primarily occurs at the 5-prime position of the cytosine ring within CpG dinucleotides and regulates gene transcription via effects on promoters and noncoding DNA elements, such as enhancers. The modification at 5-methyl cytosine is catalyzed by a family of DNA methyltransferases (DNMTs) that include DNMT1, a DNA methylation maintenance enzyme, and DNMT3A and DNMT3B, which are de novo enzymes that target unmethylated CpGs to initiate methylation. DNMT3A and DNMT3B are highly expressed during embryogenesis and only minimally in adult tissues; whereas DNMT1 is constitutively active in all adult replicating tissues[26]. Another family member is DNMT-3L that lacks intrinsic methyltransferase activity and interacts with DNMT3A to facilitate DNA methylation[27]. In normal cells, DNA methylation occurs predominantly in repetitive genomic regions, including satellite DNA and parasitic elements such as long interspersed transposable elements (LINEs) and short interspersed transposable elements (SINEs), maintaining genomic integrity[28, 29]. (Figure 2A) Methylated cytosines account for approximately 1% of total nucleotides and are found in about 75% of all CpG dinucleotides in the human genome (approximately 28 million CpGs/genome). The CpG dinucleotides are unevenly distributed across the human genome and are concentrated in areas called CpG islands (CGIs). Roughly 50–60% of gene promoters lie within CpG islands, and it is estimated that the human genome contains approximately 29,000 CGI sequences[30]. Unlike the rest of the genome, CpG islands particularly those associated with promoters are generally unmethylated in

normal cells, providing access to transcription factors and chromatin-associated proteins for the expression of most housekeeping genes and several other regulated genes, although some of them (~6%) become methylated in a tissue-specific manner during early development or in differentiated tissues[26, 31]. DNA methylation can inhibit gene expression directly, by inhibiting the binding of specific transcription factors, and indirectly, by recruiting methyl-CpG-binding domain (MBD) proteins. The associated MBD family members in turn recruit histone-modifying and chromatin-remodeling complexes to methylated sites[32]. To date, six methyl-CpG-binding proteins, including methylcytosine binding protein 2 (MECP2), MBD1, MBD2, MBD3, MBD4 and Kaiso, have been identified in mammals. Furthermore, it has been shown that nucleosome remodeling complex (NuRD) can methylate DNA by interacting with DNA methylation binding protein MBD2, which directs the NuRD complex to methylate DNA and that DNA can be actively demethylated by processes involving base excision repair (BER) enzymes[3334, 35]. These and other recent findings have established that DNA cytosine methylation is a critical component of epigenetic gene regulation.

In cancer, including all gastrointestinal cancers, global DNA hypomethylation is observed as well as aberrant regional DNA hypermethylation. DNA hypermethylation has been extensively studied in virtually all cancers and is believed to contribute to cancer formation via repression of tumor suppressor gene expression[36–38]. DNA hypomethylation is also a hallmark feature of cancer, however, its functional role in cancer formation is less well understood. It has been suggested to contribute to cancer formation via the induction of genomic instability, inducing the expression of parasitic elements of DNA or by inducing the expression of oncogenes or cancer germline genes, but definitive evidence for an active causal (ie “driver”) role in cancer formation is lacking at this time[39–42]

Chromatin Alterations And Histone Modifications

Histones are a family of small basic proteins that include H2A, H2B, H3, and H4 and that have a globular domain and a flexible charged NH2 terminus known as the histone tail, which protrudes from a protein complex called a nucleosome. A nucleosome is an octomer of histone proteins and encompasses ~146 bp of DNA wrapped around this octomer. These octamers consist of two subunits of each of the core histone proteins[43]. Histone modifications influence chromatin structure, which plays an important role in gene regulation and carcinogenesis[44, 45]. In addition, there are histone variants that provide an additional layer of regulation, including H2A.Z, MacroH2A, H2A-Bbd, H2AvD, H2A.X, H3.3, CenH3, and H3.4[46].

Chromatin is a highly ordered B-form structure consisting of repeats of nucleosomes connected by linker DNA and exists in two distinct conformation states: heterochromatin, which is densely compacted and transcriptionally inert, and euchromatin, which is decondensed and transcriptionally active[47].(Figure 2B). Chromatin consists of DNA, histones, and non-histone proteins condensed into nucleoprotein complexes and functions as the physiological template of all eukaryotic genetic information[48]. Regulation of gene expression occurs through posttranslational modifications of the histone tails provided by covalent modifications, such as acetylation, methylation, phosphorylation, ubiquitination, sumoylation, proline isomerization, and ADP ribosylation[49–52]. Posttranslational

modifications to histone tails govern the structural status of chromatin and resulting transcriptional status of genes within a particular region of DNA. Euchromatin is characterized by high levels of acetylation and trimethylated H3K4, H3K36 and H3K79. On the other hand, heterochromatin is characterized by low levels of acetylation and high levels of H3K9, H3K27 and H4K20 methylation[53, 54]. These modifications are reversible and are controlled by a group of enzymes that include histone acetyltransferases (HATs) and deacetylases (HDACs), methyltransferases (HMTs) and demethylases (HDMs), kinases, phosphatases, ubiquitin ligases and deubiquitinases, SUMO ligases and proteases, which add and remove such modifications[52, 55]. These histone modifiers generally act in complexes, such as the repressive Polycomb (PcG) and activating Trithorax (TrxG) group complexes, which counterbalance each other in the regulation of genes important for development and have been implicated in cancer[56].

ATP-dependent chromatin-remodeling complexes are responsible for sliding of the nucleosomes, as well as insertion and ejection of histone octamers, which are processes that are important for transcriptional repression and activation, and other important cellular functions such as DNA replication and repair. There are also remodeling complexes that can be divided into four families: SWI/SNF, CHD (chromodomain and helicase-like domain), ISWI, and INO80.

With regards to cancer, histone modification alterations, alterations in chromatin structure and deregulation of the genes that regulate histone modifications and chromatin structure have been found to occur commonly in many cancer types, including gastrointestinal cancers[11]. These alterations appear to have cancer specific signature patterns and have potential to be used as cancer biomarkers. There is also interest in developing epigenetic therapies for cancer, although these have not been shown to be particularly effective in the treatment of gastrointestinal cancers to date[57, 58].

EPIGENETIC ALTERATIONS AND COLORECTAL CANCER

The current paradigm of the molecular pathogenesis of CRC is that colon neoplasms can arise through a variety of discrete molecular pathways that are driven predominantly by different primary mechanisms, including chromosomal instability (CIN), microsatellite instability (MSI), epigenetic instability (e.g. CpG Island Methylator Phenotype (CIMP) subclass of CRC), altered tumor microenvironments (e.g. CMS4 subclass of CRC) and altered metabolic states (e.g. CMS3 subclass of CRC)[59–61].

With specific regards to the epigenetic alterations found in colorectal polyps and CRC, aberrant DNA methylation is the best understood class of epigenetic alterations in CRC and affects both the formation of colon polyps as well CRCs. The discovery of a molecular subclass of CRCs defined as having a “CpG island methylator phenotype (or CIMP)” in 1999 was a significant advance in our understanding of the molecular mechanisms that orchestrate colorectal tumor formation and revealed the role of aberrant DNA methylation, in particular, as an important epigenetic alteration in CRC[62, 63]. CIMP CRCs arise predominantly from sessile serrated lesions and exhibit unique clinicopathological and molecular features, including a predilection for proximal location in the colon, female

gender, poor and mucinous histology, the presence of frequent *KRAS* and *BRAF* mutations and frequent MSI due to biallelic *MLH1* methylation.[62, 64–6667, 6837, 63].

DNA alterations in colon neoplasms include both hypermethylation and hypomethylation of DNA. DNA hypermethylation can silence tumor-suppressor genes via effects on promoters and noncoding DNA elements (e.g. enhancers, etc.)[69, 70]. Global DNA hypomethylation is commonly observed in most cancers, including CRC. It is believed to influence CRC development by inducing chromosomal instability, global loss of imprinting and super-enhancer activation[70–73]. Genome-wide hypomethylation generally occurs within repetitive transposable DNA elements such as the LINE-1 or short interspersed nucleotide elements (SINE, or Alu) sequences[74–76]. LINE-1 hypomethylation inversely associates with MSI and/or CIMP[76, 77]. Furthermore, a number of studies have demonstrated that a high degree of LINE-1 hypomethylation correlates with worse patient survival[78–81]. One hypothesis is that hypomethylation of LINE/SINE sequences may induce inadvertent activation of potential proto-oncogenes[82], which implies that LINE-1 hypomethylation has a functional role in CRC formation[83].

Aberrant DNA methylation in the “traditional” and “serrated” polyp pathways

As stated earlier, there are multiple pathways for the formation of colon polyps and CRC, and sessile serrated lesions (SSLs) as well as adenomas have malignant potential[60, 84–86]. It is of note that the terminology for serrated polyps has recently changed and that the term SSLs refers to lesions previously called sessile serrated adenomas or sessile serrated polyps[87]. There are at least 2 or 3 recognized serrated polyp pathways that begin with different precursor lesions: hyperplastic polyps, serrated polyps, and serrated adenomas, the malignant potential of these precursors varies significantly[64]. Notably, recent findings suggest that in addition to microenvironment factors, genetic and epigenetic alterations that occur at low frequency in the normal colon mucosa are a major factor involved with whether the initial lesion in CRC formation is an adenoma vs. SSL[88–90], The bulk of epigenetic alterations appear to arise during polyp formation, which is unlike genetic alterations, which occur predominantly after the polyp initiation[90].

The adenoma to CRC pathway is initiated by alterations in the WNT signaling pathway, most commonly *APC* mutations, and subsequent progression is associated with alterations that affect the MAPK and TP53 pathways (e.g. *KRAS* and *TP53* mutations and methylation of genes that regulate these pathways). The SSL to CRC sequence is primarily characterized by activation of the mitogen activated protein kinase (MAPK) pathway by oncogenic mutations in *BRAF* and *KRAS*. Serrated polyps that carry mutant *BRAF* are also commonly CIMP and can progress to microsatellite stable or unstable CRC, depending on whether epigenetic inactivation of the mismatch repair protein MLH1 occurs[84] In contrast, traditional serrated adenomas more commonly activate the MAPK pathway via *KRAS* mutations, carry *RSPO* fusion transcripts, and typically have a low CIMP status. Unlike classic adenomatous polyps, sessile serrated polyps and traditional serrated adenomas do not typically have genetic alterations in *APC* or *CTNNB1*[84,91], and appear to activate WNT signaling late in the polyp to CRC sequence via aberrant DNA methylation of SFRP family genes such as *CDX2, MCC*[92, 93]. SSLs also more commonly employ epigenetic alterations to disrupt other

CRC pathways and processes, including the p53 signaling pathway (*IGFBP7*)[94] and cell cycle control proteins (*CDKN2A*)[95].

Clinical Applications Of DNA Methylation In The Prevention And Management Of CRC

There is considerable enthusiasm for the use of cancer related molecular alterations for the prevention and management of a variety of cancers, including the majority of GI cancers. Aberrantly methylated DNA biomarkers have proven the most robust and successful clinically used molecular markers to date. Another epigenetic feature, chromatin alterations, is an emerging class of promising biomarkers that will likely be in clinical use in the near future[96]. For the past two decades, we have witnessed a tremendous effort on the development of DNA-methylation based biomarkers in the prevention and management of CRC, and some of them are now available for clinical use (Figure 3, Table 1). The earliest application of methylated gene biomarkers in CRC clinical care occurred over a decade ago and was the use of methylated *MLH1* for determining the likelihood of MSI CRC being sporadic vs. hereditary in origin[97]. Methylated *MLH1* and *BRAF* mutations arise almost exclusively in sporadic MSI CRC and are used in the clinic to identify CRC patients who should be considered for genetic testing.

Screening/Early detection

Because epigenetic alterations are much more frequent than genetic mutations in polyps[98], they have greater potential as diagnostic biomarkers for the detection of colonic polyps and cancers, which is evident by the fact that the currently clinically approved screening assays for CRC, the ColoGuard assay (Exact Sciences) and EpiProcolon (Epigenomics) are based on methylated DNA. The greater potential for methylated DNA based alterations and perhaps other epigenetic alterations to detect colon polyp and early stage CRC in circulating DNA has also led to the development of blood-based assays for CRC screening that are currently under clinical investigation or that are available clinically. (See section below.)

Stool-based biomarkers—Specific biofluids, such as blood (plasma or serum) and feces are the most common analytes used in CRC screening tests. Since the initial discovery by Sidransky and colleagues of mutant *KRAS* in fecal specimens from patients with CRC[99], numerous studies have supported using fecal DNA for potential screening assays for the early detection of CRC.

To date, a large number of hypermethylated genes including *APC*, *ATM, BMP3*, *CDKN2A*, *SFRP2, GATA4*, *GSTP1, HLTF, MLH1, MGMT, NDRG4, RASSF2A, SFRP2, TFPI2*, *VIM, WIF1* as well as others, have been analyzed in fecal DNA for the early detection of CRC[100–107]. Of this large list of potential epigenetic based CRC screening markers, methylated *VIM, BMP3,* and *NDRG4* have been shown to be robust and accurate enough to be approved for clinical use. Methylated *VIM* was the first stool-based epigenetic biomarker approved for the early detection of CRC and was marketed under the name ColoSure™, (Lab Corp, Burlington, NC)[108, 109].

Subsequent to the ColoSure assay, a next generation stool based multi-target (MT) stool DNA assay was developed and FDA approved in 2014. This assay is a stool DNA based

assay that detects methylated *BMP3*, methylated *NDRG4*, mutant *KRAS*, and occult hemoglobin (Cologuard® (Exact Sciences Corporation). In a large clinical trial of average risk individuals (the Deep C trial), this MT stool DNA assay was compared to the FIT assay and to colonoscopy (N=9989)[110], and showed an overall sensitivity of 92% (95% CI, 83–97.5%) for CRC and 93% (95% CI 83.8–98.2%) for stage I-III CRC, compared to sensitivity of FIT at 74% (95% CI, 61.5–84%) and 73% (95% CI, 60.3–83.9%), respectively (p=0.002). For advanced adenomas and sessile serrated polyps, the sensitivity of the test increased proportionately with lesion size and grade. The molecular assay was significantly more sensitive than FIT for advanced adenomas: 42% (95% CI, 38.9–46%) vs. 24% (95% CI, 20.8–27%), respectively, for those ≥1 cm and 66% vs 43% for those ≥2 cm (p< 0.001). Sessile serrated polyps ≥1 cm were detected at a rate of 42% for the molecular assay compared 5% for FIT (p< 0.001); In this study the test specificity for detection of CRC and advanced pre-cancers was 87%[110].

Since 2014, >3 million MT stool DNA assays have been conducted to date leading to a real-world use experience of this assay, which has similar performance to that seen in the Deep C trial[111]. In addition, recent studies have assessed the performance of the MT stool DNA assay in noncompliant patient populations and found improved compliance in this group[112]. The assay has been also assessed in African Americans, who have a higher risk for CRC than the general population, and was found to have equivalent performance to that seem in Caucasian patients[113]. Importantly, at its current price-point, cost-effectiveness studies have had conflicting results of the utility of the MT stool DNA assay compared to FIT tests[114, 115].

Blood-based biomarkers—Due to accessibility and high patient acceptance, blood is invariably regarded to be the most ideal analyte for cancer biomarkers. The majority of blood-based biomarkers for cancer to date have been proteins or glycoproteins (e.g. PSA, CEA, CA-125, etc.). Recent studies have demonstrated the potential of circulating tumor DNA (ctDNA) for cancer detection and management, and there are now “liquid biopsy” assays being used in the clinical care of cancer patients[116, 117]. For CRC, somatic tumor-derived mutations in ctDNA are promising markers for the early detection of recurrent cancer, for monitoring treatment response, and for prognosis[118–121]. However, they have not been shown to be detected at a high frequency in patients with advanced polyps and early stage CRC using current technology, which likely reflects the very low frequency of these mutations in the plasma (6.6ng/ml blood, <0.1–0.01% ct DNA)[98, 122–124]. Methylated DNA and chromatin fragmentation patterns, in contrast can be found in patients with advanced polyps and early stage CRC more commonly. This is likely a consequence of the high frequency of epigenetic alterations in colon polyps and early CRC. Consequently, methylated DNA has been detected in higher proportions of patients with early stage CRC patients compared to DNA mutations, which has led to their assessment as blood-based early detection assays[125, 126].

To date, several potential blood-based diagnostic methylation biomarkers have been identified for CRC detection, including *ALX4*[127], *APC*[107], *CDKN2A*[105], *HLTF*[128], *HPP1*[129], *MLH1*[128], *MGMT*[107], *NEUROG1*[130], *NGFR*, *RASSF2A*[107], *SFRP2*, *VIM*[107], and *WIF1*[107], *B4GAT1*[131], *BCAT1, IKZF1*[132]; *SFRP1, SDC2,* and *PRIMA1*[126]. The best

studied methylated DNA blood-based biomarkers to date are *mSEPT9, mSDC2*, and a combination of *mBCAT1* and m*IKZF1*, which is marketed under the name Colvera (Clinical Genomics). They have been shown to consistently detect CRC in serum or plasma samples and have high potential to eventually be used in the clinic for monitoring for recurrent CRC[133125,134,135]. Other methylated DNA biomarkers are under active investigation for the early detection of recurrent CRC and for screening for CRC.

Currently, the most established methylated DNA blood biomarker is methylated Septin 9 (*SEPT9*), which belongs to the gene family that encodes a group of GTP-binding[136] and filament-forming proteins[137] involved in cytoskeletal formation. Lofton-Day and colleagues first identified methylated *SEPT9 (mSEPT9)* as a non-invasive diagnostic biomarker for CRC reporting a 69% sensitivity and 86% specificity[138]. However, a subsequent prospective CRC screening trial (PRESEPT) showed lower sensitivity for CRC (48.2% at 91.5% specificity)[138]. Subsequent studies validated the clinical significance of m*SEPT9* as a potential biomarker for CRC screening, but the FDA stance on the test is that it is only recommended in people who refuse to undergo other CRC screening tests, because of its low sensitivity for CRC (52–72%) compared to other CRC screening assays[125]. It is commercially-offered as a blood-based screening test in various assays including EpiproColon® 1.0 (Epigenomics, Seattle, WA), ColoVantage® (Quest Diagnostics, Madison, NJ) and RealTi*me m*S9 (Abbott Laboratories, Des Plaines, IL) and has been approved for CRC screening by the Chinese FDA. A major issue of m*SEPT9* as a CRC screening assay, is its low sensitivity for the detection of advanced adenomas (11%), underscoring the need for further improvement of this test if it is to be used for population-based screening of colorectal neoplasia. A recent study demonstrated that the methylated *SEPT9* assay was superior to fecal immunochemical (FIT) at detecting CRC neoplasms, but in this study both approaches were suboptimal for diagnosing patients with advanced adenomas[139].

In summary, the data to support the clinical use of CRC screening biomarkers for the early detection of recurrent CRC suggests methylated DNA plasma-based markers have high potential to eventually be used in the clinic for the early detection of recurrent CRC. It is less clear if they will be effective CRC screening markers. The most promising CRC epigenetic alterations under evaluation at this time for CRC screening are based on a combination of biomarkers that include methylated DNA and chromatin fragmentation patterns. These combination biomarker assays are being assessed in ongoing CRC screening trials at this time [Circulating Cell-free Genome Atlas study (NCT02889978; GRAIL); Session VCTPLO2, CT021 - Prediction of cancer and tissue of origin in individuals with suspicion of cancer using a cell-free DNA multi-cancer early detection test, and the ECLIPSE trial of the Lunar-2 assay (Kim, AACR 2020 abstract #916; Guardant Health)]. There appears to be potential for a robust biomarker panel of methylated genes to be be developed into a clinically accurate CRC screening method in the near future.

Epigenetic Prognostic Biomarkers

Currently, the most accurate means for assessing CRC patient prognosis is based on the pathological staging and specific histologic features of the tumor. However, the heterogeneity of survival times in patients with the same stage of CRC is well known, which

highlights the need for a more accurate system for determining CRC patient prognosis. Multiple large and sufficiently powered clinical studies with independent external validation cohorts have demonstrated the feasibility of using specific methylated DNA signatures for developing prognostic biomarkers in CRC[11].

Among all epigenetic biomarker candidates, CIMP status is the most promising prognostic indicator for CRC patients to date. CIMP-positive cancers correlate with an overall unfavorable prognosis[81, 140–143]. Rijnsoever and colleagues showed in a cohort of 206 stage III CRC patients that CIMP-positive status associated with poor survival[144]. Another independent study analyzed more than 600 CRC patients and also found that CIMP associated with poor prognosis in MSS (microsatellite stable) CRC patients[145]. Some studies suggested that poor prognosis in CIMP-positive CRCs is from coexisting V600E *BRAF* mutations[146, 147], however, in addition to CIMP, MSI status remains an important confounding factor that likely underlies the difference in prognosis of CIMP-positive MSS vs. MSI cancers[148]. These data highlight that the prognosis of patients with CIMP CRCs is affected by the MSI status of the tumor.

In addition to hypermethylation of various genes/loci, growing evidence suggests that DNA hypomethylation status associates with the prognosis of CRC patients. Ogino and colleagues have reported a correlation between LINE-1 hypomethylation and poor survival in prospective cohort studies of CRC patients[78]. Subsequent studies not only validated this association for LINE-1 hypomethylation and CRC prognosis[79–81], but also identified other potential genes that correlate with adverse outcomes[149–154].

In aggregate, these studies provide evidence that aberrantly methylated DNA loci have potential for use as prognostic biomarkers for CRC; however, further investigation is required to develop clinically reliable, standardized assays in order for these assays to be used in clinical care.

Epigenetic Predictive Biomarkers for Response to Treatment

Despite recent advances in the development of cancer therapeutics, the currently used chemotherapeutic drugs have modest efficacy for advanced CRCs, especially when used without consideration for molecular subtypes. It is now well recognized that molecular markers in CRC, such as mutant *RAS* (*KRAS, NRAS*) family genes and microsatellite instability, have clinical utility for targeting anti-EGFR (i.e. prediction of resistance to EGFR blockade) and immune checkpoint blockade therapies, respectively[155]. There is an unmet need for predictive biomarkers that can be used to target cytotoxic and targeted therapies to those CRC patients most likely to benefit.

Over the last decade, a number of aberrantly methylated genes have been assessed as predictive biomarkers for CRC patients undergoing various chemotherapeutic regimens. The majority of these studies have not progressed beyond Phase I/II discovery phase and thus will not be discussed further in this article. CIMP as a predictive marker has been intensively studied for more than a decade; however, the best done studies to date have yielded conflicting results on its use for predicting response to 5-fluorouracil (5-FU)[144156157]. Thus, at this time, CIMP is not used clinically for directing 5-FU based therapy. More recently,

prospective studies assessing CIMP as a predictive marker for adjuvant irinotecan and oxalplatin have shown modest prognostic effects for overall survival in stage III, MSS, and CIMP-positive CRCs with the addition of irinotecan to adjuvant 5FU and leucovorin and in stage III CIMP-positive CRCs treated with 5FU, leucovorin, and oxaliplatin (FOLFOX-4)[158159]. These studies suggest promise for the use of CIMP as a prognostic and possibly predictive marker and also highlight the need for additional studies of the interaction between CIMP status and therapeutic response to various treatments.

Although mostly still in the early phase of development, some promising single gene pharmaco-epigenetic biomarkers have been identified in various cancers with methylated *MGMT* for directing temozolamide treatment of gliomas being the best established to date[160]. A recent study showed the feasibility of using hypermethylated Transcription Factor AP-2 Epsilon (*TFAP2E*) as a predictive biomarker for response to 5-FU based chemotherapy in CRC patients[161]. Furthermore, DNA methylation microarray profiling of oxaliplatin sensitive vs. resistant CRC cell lines revealed that oxaliplatin-resistant cells exhibited hypermethylation of the BRCA1 interactor *SRBC* gene; which was subsequently shown to associate with poor progression free survival (PFS) in CRC cohorts treated with oxaliplatin although the results of the initial study have not been replicated to date[162]. The results of these early phase studies using methylated genes as predictive markers and the ability to develop reliable assays for methylated genes is expected to continue to drive the investigation of methylated genes as response predictors for CRC therapy.

Epigenetic CRC Risk Biomarkers: “Field Cancerization” and ‘Epigenetic Drift”:

The concept of “field cancerization” (or field effect) was first proposed in 1953 by Slaughter et al[163]. Field cancerization is characterized by the occurrence of genetic and epigenetic alterations in histologically normal-appearing tissues, and is believed to lead to an increased risk for synchronous or metachronous primary tumors. While genetic alterations are common in CRC cells, these are believed to be rare in normal cells. In contrast, some studies suggest somatic epigenetic dysregulation occurs not only in cancer tissues, but also in non-cancerous and pre-neoplastic tissues. Considering that epigenetic alterations could contribute to the early events predisposing to malignant transformation, these studies suggest epigenetic events are potentially more promising somatic CRC risk markers (aka field cancerization markers) than are gene mutations. Methylation changes in tumor-suppressor genes occurs more frequently in the normal colonic mucosa of CRC patients than healthy controls[164], suggesting that they may be one of the earliest events that predispose normal mucosa to tumorigenic transformation in CRC[165]. Furthermore, loss of the insulin-like growth factor-II (*IGF2*) gene imprinting occurs at a higher frequency in the normal mucosa adjacent to cancer tissue, compared to normal mucosa in patients without CRC[166], emphasizing the potential of loss of *IGF2* imprinting to be a biomarker to identify patients at greater risk for CRC development. Other studies have revealed that both hypermethylation of tumor-suppressive genes such as *SFRP, ESR1, MYOD, EVL*, and *MGMT*, as well as LINE-1 hypomethylation in normal colonic mucosa correlates with an increased risk of CRC compared to patients without these traits[167–171] (Grady, personal communication; Yu et al, under review)

It is of particular interest that accumulating evidence supports that the landscape of DNA methylation can be modified as a “function of age”. DNA methylation has been proposed to result from a gradual stochastic age-dependent dysregulation caused by a combination of external environmental factors and internal spontaneous random errors in the maintenance of methylation. This process of age-dependent alterations in methylation is defined as “epigenetic drift”[172]. Interestingly, such age-associated DNA methylation often targets the promoters of tumor-suppressive genes[173, 174]. In monozygotic twins, epigenetic divergence with age suggests the underlying epigenetic drift may in part help explain the disease discordance[175–177]. Since this new concept closely relates to the “field effect”, identification of biomarkers that overlap both the “epigenetic drift” and “field effect” in colorectal mucosa may allow development of next-generation biomarkers for determining risk for CRC development.

Histone modification alterations in CRC: potential for use as biomarkers.

Altered histone modifications are commonly found in CRCs[178] and associate with altered chromatin activity states and gene expression through effects on gene promoters and noncoding regulatory elements[179, 180]. Some studies have demonstrated potential for histone modifications to be CRC biomarkers, however, due to the technical limitations of assays that assess the post-translational histone modification state, it has been difficult to reliably determine the histone modification state in primary cancer tissues and to develop tests that are sufficiently robust to be used in clinical care. Global alterations of specific-histones in primary tissues have been the focus for biomarker development in CRC. Studies of H3K4me2, H3K9ac, and H3K9me2 alterations detected by immunohistochemical staining in liver metastases suggest that low H3K4me2 expression levels correlate with poor prognosis[181]. Additionally, other studies in CRC suggest that histone modifications, such as acetylation of H3 lysine 56 and di- or tri-methylation of H3 lysine 9 and 27, have potential to be prognostic markers in CRC.[178, 181–185]. Similarly, studies of histone modifications in circulating nucleosomes have identified reduced levels of H3K9me3- and H4K20me3 as potential diagnostic biomarkers for CRC[186, 187]. However, thus far, these studies are all proof of concept Phase I biomarker studies. Further research is needed to determine whether any of these modifications will be clinically useful.

EPIGENETIC ALTERATIONS IN BARRETTS ESOPHAGUS AND ESOPHAGEAL ADENOCARCINOMA

Barretts Esophagus (BE) is specialized small intestinal metaplastic epithelium of the esophagus and is a precursor to esophageal adenocarcinoma (EAC). It has increased dramatically in the last 40 years and results in roughly 20,000 deaths/year in the U.S. Most, if not all, EAC originates in BE through a metaplasia-dysplasia-carcinoma sequence whereby BE progresses to low-grade dysplasia followed by high-grade dysplasia and then intramucosal carcinoma and invasive carcinoma[189, 190]

As with CRC, genetic and epigenetic alterations occur in BE and EAC and play an important role in the pathogenesis of EAC[191]. Epigenetic alterations identified in BE and EAC include DNA methylation alterations, histone alterations, aberrant expression of noncoding RNAs

and chromatin alterations[191–193]. The best studied class of epigenetic alterations in BE and EAC is secondary to DNA methylation and found in the majority of BE and EAC cases[194192, 195, 196]. Factors that influence DNA methylation in the esophagus include aging, smoking and obesity[197, 198]. Hypermethylated genes shown in BE and EAC include known tumor-suppressor genes, such as *APC, CDKN2A (p16INK4a), RUNX3, MGMT, CDH1*, and *SFRP* family members among others[195]. A subset of the hypermethylated genes are believed to play a causal (termed "driver") role in driving the formation of EAC, while most appear to be BE and EAC specific passenger alterations, which do not functionally induce EAC formation distinguishing them from driver genes[192, 195]. Aberrant methylation of classic tumor suppressor genes such as *CDKN2A* and *MGMT* has been correlated with loss of mRNA and protein expression in the metaplasia-dysplasia-carcinoma sequence of BE to EAC[199, 200].

Recently, Yu et al identified four methylation subtypes of EAC and BE through genome-wide DNA methylation profiling[192]. The four methylation subtypes were identified through the use of a recursively partitioned mixture model (RPMM) and included High Methylator (HM), Intermediate Methylator (IM), Low Methylator (LM), and Minimal Methylator (MM) subtypes, which were defined by the overall methylated CpG burden and pattern of methylation alterations. The high methylator subtype (HM) had more activating events in *ERBB2* compared to the other subtypes, which suggested a unique dependence on epidermal growth factor (EGF) signaling in HM BE and HM EAC, and also a higher global mutation load. This EGF signaling pathway dependence of the HM subtype appears to arise from both oncogenic ERBB2 and through the epigenetic silencing of the tyrosine phosphatase non-receptor 13 (*PTPN13*), which is specific to the HM subtype. Subsequent studies have supported the observation of molecular subclasses of BE and EAC[194].

Of relevance to biomarker discovery, a large number of genes and loci have been identified as high frequency targets of aberrant methylation in BE and EAC[192]. Although the functional significance of these methylated genes is still not clear, these DNA methylation events have proved to have potential as biomarkers of BE, as discussed below. The published studies to date suggest aberrant DNA methylation is a common molecular mechanism that mediates the development of esophageal cancer and that aberrantly methylated genes and loci are potential screening and surveillance biomarkers for BE and EAC.

METHYLATED DNA BIOMARKERS FOR BARRETTS ESOPHAGUS SCREENING AND SURVEILLANCE

Barretts Esophagus screening markers

Genetic and epigenetic alterations occurring in Barretts esophagus (BE) and early stage EAC are potential biomarkers for use in cancer care and prevention BE and EAC. Studies over the last 3 years have shown methylated DNA biomarkers to be the most promising class of BE and EAC biomarkers to date. Of particular interest is the recent development of a "molecular cytology" assay for methylated *VIM* in DNA samples from esophageal cytology brushings obtained during endoscopies of 322 individuals, divided into training and validation cohorts[201]. The assay showed 91% sensitivity for detecting BE, BE with dysplasia, and EAC

at 93% specificity, with essentially identical results obtained in both the training and validation cohorts[201].

This assay was further refined with the addition of a second methylated gene, *CCNA1,* and used on samples collected with an FDA approved swallowable balloon based device (the Esocheck device, Lucid Diagnostics) for obtaining targeted non-endoscopic brushings of the distal esophagus[201]. which improved the sensitivity to 95% (at 91% specificity) for BE, BE and dysplasia and EAC cases and for detecting 96% of BE with dysplasia and 96% of EAC[201]. A methylated DNA panel (tradename EsoGuard) based on these markers run on Esocheck collected samples is currently being further validated in a nationwide multi-center clinical trial and is undergoing commercial development.

Additional potential BE markers have been identified and validated by others include *B3GAT2* and *ZNF793*, that are aberrantly methylated in BE. Clinical validation studies confirmed *B3GAT2* and *ZNF793* methylation levels were significantly higher in BE samples (median 32.5% and 33.1%, respectively) than in control tissues (median 2.29% and 2.52%, respectively; $P < 0.0001$ for both genes) and that gene-specific MethyLight assays could accurately detect BE ($P < 0.0001$ for both) in endoscopic brushing samples with m*ZNF793* having a sensitivity of 70% and specificity of 100% for BE[202]. These markers show promise to further improve the performance of a methylated gene panel for BE screening.

In addition to the Esocheck device, other swallowable cytology collection devices are being assessed and currently being evaluated for use in BE screening assays, such as the ‘Cytosponge’ and “Esophacap” devices, which are both swallowed capsules that degrade in the stomach to release a sponge tethered to a string[203–205]. Unlike the Esocheck device, these devices sample the entire esophagus and oropharynx, which increases the potential to impair biomarker performance. Using a Cytosponge based assay, Chettouh et al discovered and assessed hypermethylated *TFPI2, TWIST1, ZNF345* and *ZNF569* as potential BE screening markers. Methylated *TFPI2* was shown to achieve the best sensitivity in both the pilot and validation Cytosponge cohorts (85% and 79%, respectively, AUC 0.88)[206].

In summary, these studies have established that methylated DNA is a potential new biomarker class that will enable practical non-endoscopic screening and early detection of BE, an approach with potential to reduce the steadily increasing mortality from EAC. Table 2 below summarizes BE screening markers used for BE early detection that have been evaluated in clinical cohorts. (Table 2)

Barretts Esophagus Surveillance And Risk Prediction Biomarkers

BE is associated with approximately 4X increased risk of EAC, which has led to the recommendation that patients with BE undergo regular endoscopic surveillance in order to prevent or detect EAC at its earliest stage[207]. However, only 0.1–0.3% of people/year with BE will progress to high-grade dysplasia or EAC, thus, a biomarker (or biomarker panel) that can accurately risk stratify high risk patients with BE who are likely to progress from those low risk BE patients who are unlikely to develop EAC is needed[208]. Such a marker could potentially spare the great majority of individuals with a diagnosis of BE from the cost, inconvenience, and risks of regular endoscopic surveillance. Being placed in a ‘low-

risk' group might also reduce the feelings of anxiety about developing EAC that have been shown to be associated with a diagnosis of BE[209]. The search for accurate risk stratification markers for BE is an area of intense investigation but has not yielded any markers have proven adequate to be used in the clinical setting. Immunostaining assays for p53 and aneuploidy appear to have the highest likelihood for eventual adoption into clinical care at this time[207].

With regards to epigenetic risk prediction biomarkers, a retrospective study comparing BE patients who progressed to HGD or EAC to those who did not using hypermethylated *CDKN2A* (OR 1.74, 95% CI 1.33 – 2.20), *RUNX3* (OR 1.80, 95% CI 1.08 – 2.81), and *HPP1* (OR 1.77, 95% CI 1.06 – 2.81), has shown an association with an increased risk of progression. Age, BE segment length, and hypermethylation of other genes (*TIMP3, APC,* or *CRBP1*) were not found to be independent risk factors with this assay[210]. A follow-up study using these same epigenetic markers in combination with three clinical parameters (gender, BE segment length (SL), and pathologic assessment) demonstrated this multi-parameter method could stratify BE patients into high, intermediate, and low risk for progression to HGD or EAC. This tissue based assay has not been adopted into routine clinical use to date[211]. In a later iteration of this approach, this risk assessment tool was expanded to include additional genes previously shown to be hypermethylated in BE and/or EAC to generate an eight-marker risk-of-progression panel. In a retrospective analysis of 145 people with stable BE that did not progress to EAC vs. 50 people who did progress to EAC, this panel predicted progression with a sensitivity of ~50% when the specificity was set at 90%[212]. None of these candidates have advanced to phase III or IV biomarker trials. In summary, studies to date have demonstrated the potential for methylated DNA biomarkers to be risk prediction markers, but further studies are needed.

LIVER CANCER: EPIGENETIC ALTERATIONS

HCC arises secondary to a variety of etiologic factors, including predominately hepatitis B virus (HBV), hepatitis C virus (HCV), fatty liver disease (NAFLD), alcohol, and genotoxins. The HCCs that arise in the setting of these factors do so through a progressive pathway from premalignant cirrhosis-related premalignant nodular lesions, which include both regenerative and dysplastic nodules, to HCC[213, 214]. These factors induce genomic and epigenomic alterations as well as inflammatory cytokines that appear to mediate the formation and progression of HCC, with prominent genetic alterations including *TERT* promoter and *TP53* mutations, and common epigenetic alterations including *RASSF1A* and *SOCS1*. The epigenetic alterations arise early in the pre-neoplastic cirrhotic phase of HCC carcinogenesis, precede many of the genetic alterations, and associate with a field effect in the cirrhotic liver[215–217]. Assessment of the clonal evolution of HCC has revealed that the epigenetic arise and evolve independent of the genetic alterations[216]. Furthermore, pre-malignant patterns of epigenetic alterations occur in some nodular lesions and are linked to the proliferative and dysplastic capacity of these nodules[216].

Some of these genomic and epigenomic alterations are also known to occur preferentially or exclusively in the setting of specific causes, with distinct liver cancer epigenomes found in HCV and alcohol related HCC[216]. HBV-induced HCCs carry a high proportion of *TP53*

mutations and lack *TERT* promoter mutations. Furthermore, HBV integration sites appear to be common in HBV mediated HCC and to affect candidate oncogenes, such as *TERT* and *MLL4* (most common), *CCND1* and *GLI2*, as well as others[218]. In contrast, HCV mediated HCC display significantly increased frequencies of *CDKN2A* promoter silencing and *TERT* promoter mutations[218]. Some of the epigenetic alterations have been shown to affect the clinical behavior of the cancer as well as patient prognosis[219, 220].

Methylated DNA Biomarkers For HCC Screening And Surveillance

As noted for CRC and EAC, methylated DNA (mDNA) alterations have properties that make them uniquely suited for development into molecular markers for HCC. In evidence of their potential as HCC biomarkers, a 6-marker circulating free methylated DNA (cf mDNA) marker panel yielded an AUROC of 0.96 with HCC detection sensitivity of 95% and specificity or 92% in a recent study[221]. Although selection and other biases almost certainly inflated the marker panel performance reported in this study, based on these encouraging results this panel is being further assessed and is undergoing external validation phase 2 studies in a variety of different patient populations.

GASTRIC CANCER EPIGENOMICS

Pangenomic analysis of gastric cancer (GCA) has revealed molecular subtypes of gastric cancer that vary based on mutation patterns, DNA methylation, and biological pathway dependence. Among several efforts to classify gastric cancer, The Cancer Genome Atlas (TCGA) and the Asian Cancer Research Group (ACRG) have proposed gastric cancer subtypes based on gene expression, microsatellite instability (MSI) and DNA methylation[222]. The TCGA identified four distinct subtypes: the Epstein Barr virus (EBV) subtype, which is enriched for tumors carrying this virus; the MSI subtype; the chromosomal instability (CIN) subtype; and the genome stable subtype. EBV subtype GCAs account for ~9% of GCA and exhibit distinct genome-wide increases in DNA methylation that is CIMP-like. EBV-CIMP gastric cancers possess the highest levels of DNA methylation seen among all cancer types[222, 223]. The MSI subtype (~22% of GCA) is defined by a classic microsatellite unstable genome, often is a consequence of aberrant methylation of the DNA mismatch repair gene, *MLH1*. MSI GCAs have a very high tumor mutation burden and have a hypermethylated CIMP epigenomic state, although to a lower extent than seen in the EBV subtype GCAs. The chromosomal instability (CIN) subtype cancers (~50% of GCAs) are defined by aneuploidy, and have relatively lower levels of methylation, as does the genome-stable subtype (~20% of GCAs), which are largely genetically stable. In the genome stable subtype, epigenetic alterations appear to be the primary mechanism for altering oncogene and tumor suppressor gene activity[74].

Environmental factors such as infectious agents, chronic inflammation, diet, physical activity, age and smoking have been correlated with changes in the GCA methylome[224]. Helicobacter pylori mediated inflammation is associated with regional DNA hypermethylation and hypomethylation of Alu repeat elements in human gastric mucosa[225, 226], and eradication of H. pylori has been associated with complete or partial reversal of methylation at cancer-related genes such as *CDH1, MGMT* and *COX2*[227–229].

Importantly, although H. pylori eradication results in decreased DNA methylation, the DNA methylation levels do not revert to those observed in uninfected states, suggesting long lasting effects from H pylori infection on the gastric mucosa, even after it is eliminated. By contrast, EBV-associated hypermethylation is thought to be caused by direct pathogen infection rather than inflammation-related intermediate pathways, possibly by EBV modulation of host DNMT1 activity and downregulation of the TET2 demethylase[224, 230]. Notably, CIMP can also occur in GCA that are EBV and MSI negative and account for ~26% of the total CIMP group in the TCGA cohort,[222]. The precise mechanism underlying the acquisition of CIMP in this subset of CIMP GCAs is not known at this time.

Epigenetic alterations in GCA can alter the activity of tumor suppressor genes and oncogenes. The most robust evidence to date with regards to the role of aberrantly methylated genes in GCA has been shown for *CDKN2A/p16*, which is methylated in all EBV subtype GCAs; *MLH1*, which is methylated in the majority of MSI GCAs and mediates the MSI phenotype; and *CDH1*, which is methylated commonly in diffuse GCAs and in GCAs arising in the Hereditary Diffuse Gastric Cancer syndrome[36, 231] Notably, studies of the aberrant methylation of *CDH1* provided some of the first evidence of the functional importance of DNA hypermethylation in cancer as a bona fide mechanism for biallelic inactivation of tumor suppressor genes[36]

Methylated DNA Biomarkers For GCA Prevention, Screening And Prognosis

The application of epigenetics to the management of gastric cancer has been assessed in early phase translational studies, which have demonstrated that aberrantly methylated genes may be used as risk markers, early detection markers, and prognostic markers for GCA. Ushijima and colleagues have carried out a comprehensive series of studies demonstrating that aberrantly methylated genes that arise in the normal stomach likely do so as a consequence of H pylori induced methylation and indicate a field cancerization effect associated with a 2–3X increased risk for GCA[232, 233]. DNA methylation markers to detect gastric cancer have also been identified in plasma, serum, gastric juice and fecal samples from patients with gastric cancer, albeit with varying specificity and sensitivity[234–236]. Patients with CIMP GCAs show superior survival in some cohort studies, suggesting the potential for CIMP to be used as a prognostic marker[237]. Regrettably, none of these assays have been subjected to clinical studies sufficient to determine whether they are suitable for use in the clinic.

PANCREATIC CANCER AND EPIGENETIC ALTERATIONS

The role of epigenetic alterations in pancreatic adenocarcinoma (PANCA) is less thoroughly understood compared to other GI cancers. Recent studies have begun to provide insight into the epigenomics of PANCA. Subtypes of PANCA have been identified though analysis of DNA methylation, copy number, lncRNA, miRNA, and aberrant protein expression[238]. In an assessment of 150 patients with PANCA in the TCGA, unsupervised clustering of DNA methylation data for high-purity samples revealed two major subgroups (termed H1 and H2). The H1 cluster (n = 41) had more extensive DNA hypermethylation than the H2 cluster (n = 35). Unlike with colorectal and gastric cancer, no CIMP subtype has been found to date in

PANCA. Others have investigated the TDGC PANCA datasets and have identified three possible sub-groups based on methylation patterns, somatic mutations and copy number alterations, histologic features, and stage features, but not on gene expression[239].

With regards to the functional consequence of epigenetic alterations in PANCA, in the TCGA study, integrated analysis of the DNA methylation and mRNA expression data revealed 98 genes that appear to be silenced by DNA methylation, including genes that have been implicated in the development of other cancers but not previously reported to be altered in PANCA. Presumably epigenetically silenced genes worth mention include *ZFP82*, a suspected tumor suppressor gene, *PARP6*, *DNAJC15, BRCA1* and *MGMT*[238]. The application of aberrantly methylated genes to clinical care for PANCA patients, either as biomarkers or as therapeutic targets is in early development, and its translational potential is unclear at this time[240–242].

CONCLUSIONS

In summary, epigenetic alterations are common in pre-malignant and malignant tumors of the gastrointestinal tract and have been found in all cancers arising in the gastrointestinal tract to date. These alterations not only effect the initiation and progression of gastrointestinal cancer but also appear to be robust biomarkers that can be used for the early detection of pre-malignant conditions, such as colon polyps and BE, as well as early stage cancers. Recent studies have led to FDA approved colon cancer screening assays based on methylated DNA and it is likely that additional screening and surveillance assays for CRC as well as BE and EAC will be available in the near future.

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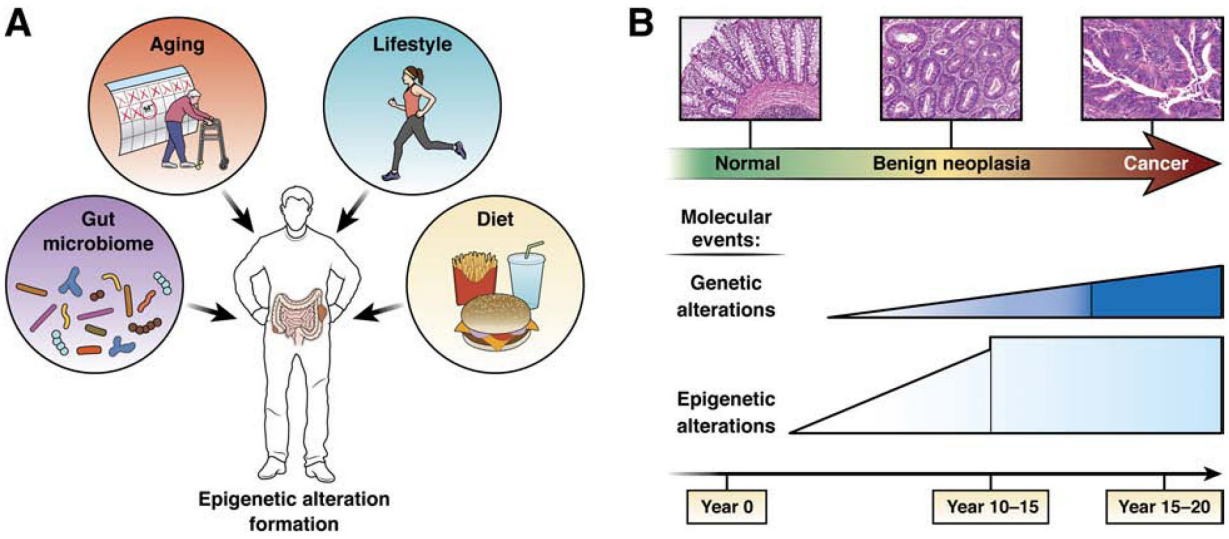

Figure 1:

Schematic diagram showing factors that influence epigenetic alteration formation and the timing of epigenetic alteration formation in GI tract cancer formation.

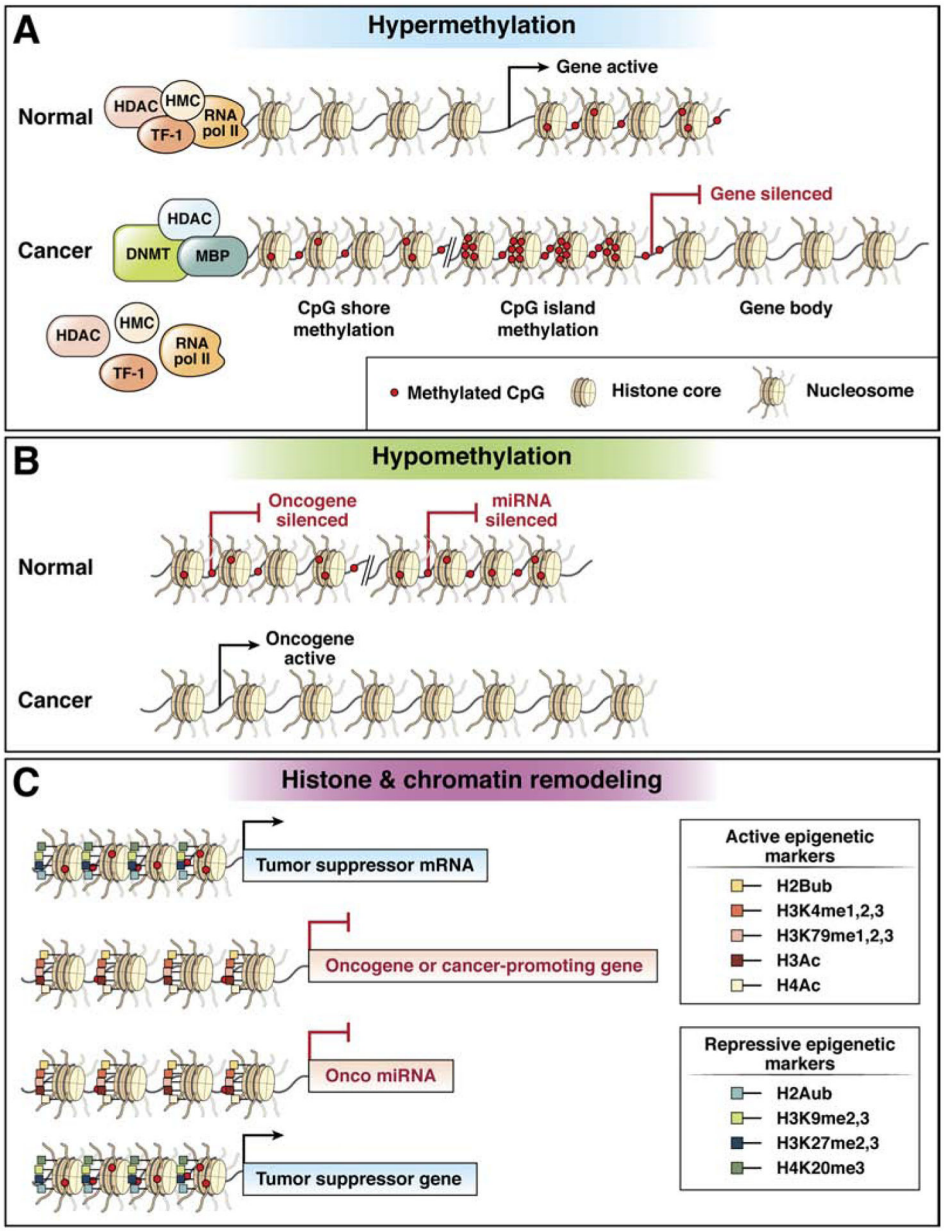

Figure 2A and B:

Schematic diagram of DNA hypermethylation and DNA hypomethylation in cancer. (TF-1=transcription factor 1, TF-2=transcription factor 2, TF-3=transcription factor 3, RNA pol II=RNA polymerase II, HDAC=histone deacetylase complex, DNMT=DNA methyltransferase, MBP=methyl binding protein, Onco miRNA=oncogenic microRNA. CpG shores are the regions immediately flanking CpG islands (CGI), and they can be methylated just as CpG islands can be. The consensus definition of a CpG shore is up to 2kbp away from the CpG island. miRNA silencing refers to hypermethylation of microRNA loci that silence miRNA expression, which can suppress tumor suppressor miRNAs or increase the expression of inactivated oncogenes that are the targets of the miRNAs

Figure 2C: Schematic diagram of DNA chromatin structure and histone modification states that affect conformation of chromatin. (H2A=histone 2A, H2B=histone 2B, H3=histone 3, H4=histone 4, ub=ubiquitylated, me=methylated, Ac=acetylated, K=lysine. Onco miR is an oncogenic microRNA.)

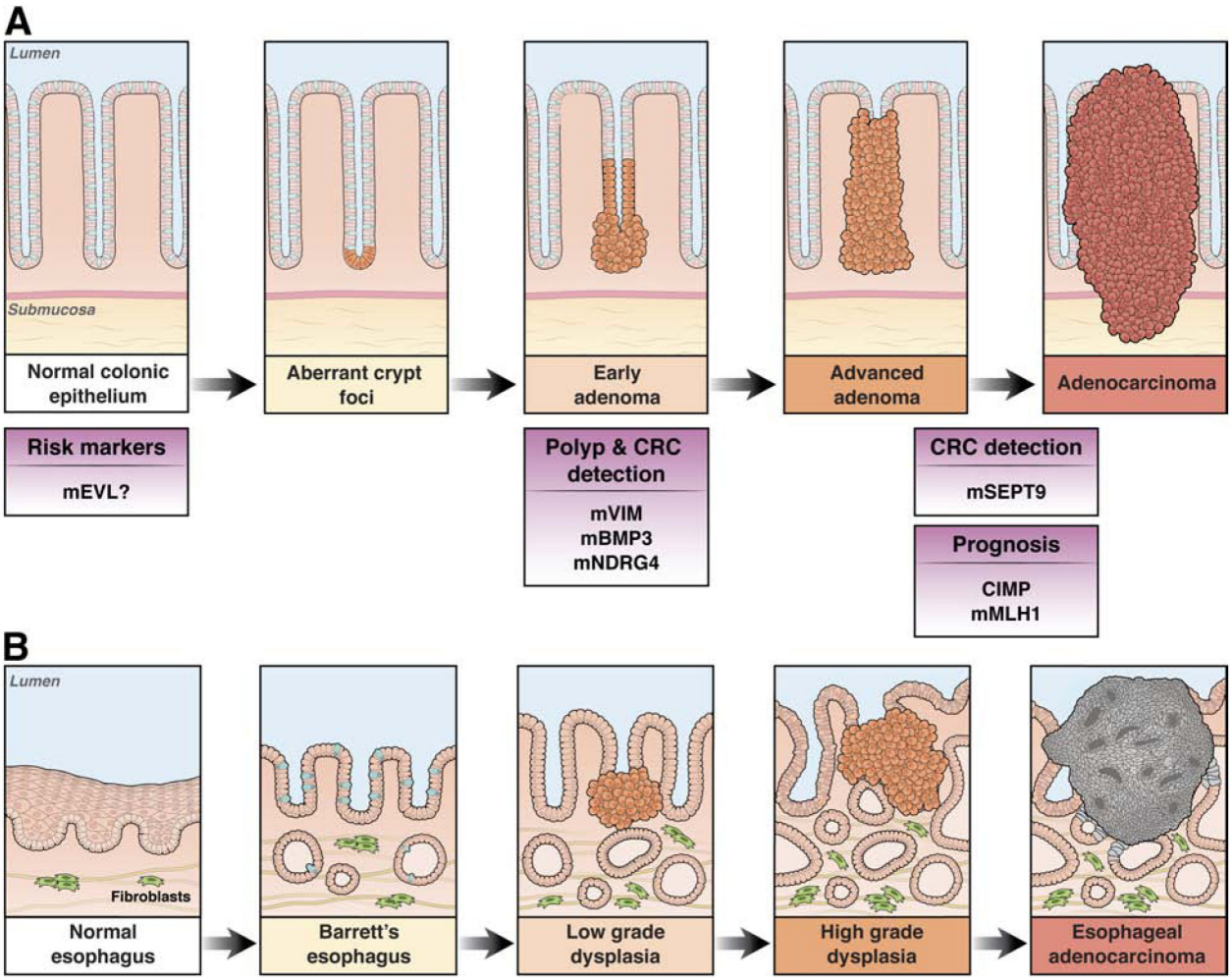

Figure 3:

Diagram showing the polyp-to-CRC progression sequence and associated methylated gene alterations that have been shown to be potential or clinically used polyp and CRC detection markers or prognostic markers. The prefix "m" designates the methylated gene. *mEVL* and *mMLH1* are used as tissue based biomarkers, whereas *mVIM, mNDRG4,* and *mBMP3* are stool based biomarkers. *mSEPT9* is a blood-based biomarker.

Table 1:

Validated DNA-methylation biomarkers for colorectal cancer

Clinical Use	Biomarkers	Available Commercial Assays	Study Design of Major Trials with Assay	Ref.
Stool-based CRC screening	*mVIM,*	ColoSure™	Case (N=42) control (N=241) study	109
	mBMP3 and *mNDRG4*	Cologuard® (detects mutant *KRAS,* and includes a FIT test)	Prospective cohort based clinical trial in screening population (N=9989)	110
blood-based diagnostic marker	*mSEPT9*	EpiproColon® 1.0; ColoVantage®; RealTime mS9	Multiple trials: 1)Prospective cohort based clinical trial in screening population (N=7941) (Church);2) Case-Control study (N=269) (deVos); 3) Case-Control study (N=312) (Lofton-Day, 2008)	125 135,138,139
	mBCATI mIKZFI	Colvera	Cross-sectional study (N=220)	133
Tissue-based prognostic markers	*CIMP* panel	NA	Multiple trials: **1** Case-Control study from 2 phase I/II clinical trials (N=31)(Ogino, 2007): **2** Case-Control study from phase 3 clinical trial (N=615) (Shiovitz, 2014);3) Observational cohort study (N=2050)(Phipps, 2015)	140, 142, 159, 188
Diagnostic tool to screen for Lynch syndrome	*mMLHI*	MLH1 Hypermethylation analysis	**1** Cross sectional study (N=1066)	67

Table 2:

Validated Barretts Esophagus early detection markers

BE early detection marker	Method	Study Design	AUC	Sensitivity	Specificity
mVIM and *mCCNA1*	bsNSG (Esocheck device)	Case-control Validation cohort (N=86)[201]		90%	92%
mB3GAT2	methyLight PCR (endoscopic brushings)	Case-control Validation cohort (N=66)[202]	0.95	80%	86%
mZNF793	methyLight PCR (endoscopic brushings)	Case-control validation cohort (N=66)[202]	0.96	80%	93%
mTFPI2	methyLight PCR (cytosponge)	Case-control validation cohort (N=278)[206]	0.88 (0.84–0.91	82%	96%
mTWIST1	methyLight PCR (cytosponge)	Case-control validation cohort (N=278)[206]	81% (0.77–0.86)	70%	93%

IHC=immunohistochemistry, bsNSG=bisulfite next generation sequencing