Published in final edited form as:

Semin Cancer Biol. 2018 August ; 51: 36–49. doi:10.1016/j.semcancer.2017.12.004.

Epigenetic biomarkers in gastrointestinal cancers: The current state and clinical perspectives

Hege Marie Vedeld[a,b,c], **Ajay Goel**[d], **Guro E. Lind**[a,b,c,*]

[a]Department of Molecular Oncology, Institute for Cancer Research, Oslo University Hospital, Norwegian Radium Hospital, Oslo, Norway

[b]K.G. Jebsen Colorectal Cancer Research Centre, Oslo University Hospital, Oslo, Norway

[c]Centre for Cancer Biomedicine, Faculty of Medicine, University of Oslo, Oslo, Norway

[d]Center for Gastrointestinal Research, and Center for Translational Genomics and Oncology, Baylor Scott & White Research Institute and Charles A. Sammons Cancer Center, Baylor University Medical Center, Dallas, TX, USA

Abstract

Each year, almost 4.1 million people are diagnosed with gastrointestinal (GI) cancers. Due to late detection of this disease, the mortality is high, causing approximately 3 million cancer-related deaths annually, worldwide. Although the incidence and survival differs according to organ site, earlier detection and improved prognostication have the potential to reduce overall mortality burden from these cancers. Epigenetic changes, including aberrant promoter DNA methylation, are common events in both cancer initiation and progression. Furthermore, such changes may be identified non-invasively with the use of PCR based methods, in bodily fluids of cancer patients. These features make aberrant DNA methylation a promising substrate for the development of disease biomarkers for early detection, prognosis and for predicting response to therapy. In this article, we will provide an update and current clinical perspectives for DNA methylation alterations in patients with colorectal, gastric, pancreatic, liver and esophageal cancers, and discuss their potential role as cancer biomarkers.

Keywords

Biomarkers; Detection; DNA methylation; Gastrointestinal cancers; Prognosis

1. Introduction

Gastrointestinal (GI) cancers include malignancies arising in the esophagus, stomach, liver and bile ducts, gallbladder, pancreas, the small intestine, colon and rectum. Together they

*Corresponding author at: Department of Molecular Oncology, Institute for Cancer Research, Oslo University Hospital, Norwegian Radium Hospital, Oslo, Norway., hege.marie.vedeld@rr-research.no (H.M. Vedeld), Ajay.Goel@BSWHealth.org (A. Goel), guro.elisabeth.lind@rr-research.no (G.E. Lind).

Conflict of interest

None.

account for approximately 4.1 million new cases and 3 million deaths, annually worldwide [1]. Colorectal and gastric cancer are the two most common GI cancers worldwide, affecting 1.4 million and 952 000 people each year, respectively. Each cancer type leads to around 700 000 deaths per year. Liver cancer is also quite common with an incidence of 782 000 new cases, and the very high mortality rate causes ~746 000 deaths annually. Cancers of the pancreas (338 000) and esophagus (456 000) are relatively less common, but the poor overall survival (OS) in these two malignancies still make these cancer types as some of the leading causes of cancer-related deaths (330 000 and 400 000, respectively) [1]. Most patients with early-stage GI cancers are asymptomatic, leading to an increased risk for their diagnosis at advanced stages, where the treatment options are limited and patient outcomes are often poor. This clinical challenge underscores the need for identification and development of robust molecular markers that can facilitate reduced mortality rates, either through earlier detection and/or through better prediction of tumor response to specific therapies.

Epigenetic changes are common in all types of cancers, including GI cancers, and contribute to both cancer initiation and progression. ‘Epigenetics’ is defined as heritable changes in gene expression that do not cause permanent alteration of the underlying DNA sequences, and include *e.g.* DNA methylation, histone modifications and non-coding RNAs. The most commonly studied epigenetic alteration in cancer, including in GI cancer, is aberrant DNA methylation. DNA methylation is predominantly found at the 5′-position of cytosine residues (5mC or 5-methyl cytosine) followed by a guanine dinucleotide sequences (CpG). Some regions in the genome are characterized by a particularly high CpG content termed ‘CpG islands’, and are present in approximately 60% of human gene promoters [2]. Normally these CpG islands are unmethylated in normal cells, and allow active transcription of the gene involved. However, in cancer cells, these islands are frequently targeted for hypermethylation–an alteration that causes transcriptional repression of the associated gene, including tumor suppressors. Gene silencing by promoter hypermethylation occurs in the majority of cancer types, and occurs more frequently compared to genetic mutations. Since aberrant promoter DNA methylation may additionally be detected in various bodily fluids, including bile, feces, and blood, such markers are gaining a lot of attention as potential noninvasive cancer biomarkers for the early detection, prognosis and for predicting the treatment outcomes. Furthermore, DNA methylation is a highly stable mark that can easily be detected by PCR-based technologies, making it suitable for clinical use.

This review focuses on DNA methylation changes in GI cancers, including colorectal, gastric, esophageal, pancreatic, and liver cancers and their potential value as cancer biomarkers. Other epigenetic aberrations, including miRNAs, are outside the scope of this text and reviewed elsewhere [3–7].

2. Aberrant DNA methylation in colorectal cancer

2.1. Diagnostic biomarkers

Colorectal cancer develops in a step-wise manner through clear premalignant precursor adenomas by gaining increasingly more dysplastic features and eventually progressing to malignant carcinomas [8]. Colonoscopy, considered as the gold standard for screening

patients with colorectal cancer, has the potential to detect and remove these precursor lesions. The method is, however, invasive, expensive and further hampered by low compliance rates [9]. Fecal occult blood test (FOBT) and fecal immunochemical test (FIT), the most commonly used screening tests in Europe and other western countries have low sensitivity and specificity [10], mandating the need for improved biomarkers for detection of early stage colorectal cancers. In the colon, epigenetic alterations have been observed also in aberrant crypt foci [11] and in the pre-malignant adenomas [12], highlighting the clinical potential of exploiting aberrant promoter methylation for the early detection of colorectal adenomas and cancers. In this context, several blood and fecal-based DNA methylation biomarkers with clinical potential have been reported for colorectal cancer (reviewed in [13–16]).

2.1.1. Blood—Non-invasive tests, including blood-based assays, have the potential to improve overall patient compliance compared to colonoscopy. Indeed, this has also been shown for methylation of *SEPT9* [17], which is included in the FDA approved Epi proColon plasma test (Epigenomics AG, Berlin, Germany). The sensitivities for detecting cancer and advanced adenomas have been reported between 48 and 72%, and 11–22%, respectively, with specificities ranging from 80 to 95% [18,19]. Since positive test results have been observed in patients with other malignant and non-malignant conditions, clinical parameters should be evaluated along with the test results and suspected neoplasms should be confirmed by colonoscopy [20]. Although single markers have shown promise for cancer detection, biomarker panels might be more reliable considering tumor heterogeneity and technical challenges. In a recent prospective study the methylation of *BCAT1* and *IKZF1* was analyzed in plasma samples from > 2000 people, including 129 with cancer [21]. The two-marker blood test had a sensitivity and specificity of 66% and 94%, respectively. The sensitivity for advanced adenomas was, however, only 6%. The methylation frequency of these markers was reduced after surgery in several patients, motivating ClinicalGenomics to develop the Colvera™ test for monitoring tumor recurrence [21,22]. Two additional biomarker panels with high sensitivity and specificity for colorectal cancer have been detected [23,24]. The first panel (*CNRIP1*, *FBN1*, *INA*, *MAL*, *SNCA*, and *SPG20*) showed a combined sensitivity of 94% for colorectal cancers and 93% for adenomas, with an area under the ROC curve (AUC) of 0.98 and 0.97, respectively [23]. The second panel (*CDO1*, *DCLK1*, *SFRP1*, *ZNF331* and *ZSCAN18*) displayed a sensitivity of 95% for colorectal cancers and a specificity of 98% with an AUC of 0.98 [24]. Preliminary data showed that a selection of these markers performed well also in plasma and fecal samples, with a combined AUC 0.82 and 0.87, respectively [25]. The high AUC (0.93) for advanced adenomas in plasma samples underscore the promise of these markers for early detection of colorectal cancer.

2.1.2. Feces—Colonocytes from tumors are constantly shed into the lumen, and identification of aberrant methylation in fecal samples represents a good source for cancer specific detection of colorectal cancer. Aberrant methylation of VIM was included in the first commercial fecal test for colorectal cancer detection (ColoSure, LabCorp) [26]. The sensitivities for cancers and adenomas have been reported between 33 and 81% and 15–45%, respectively, with specificities ranging from 82 to 100% [26,27]. The FDA approved

Cologuard (Exact Sciences) evaluates methylation of *BMP3* and *NDRG4*, mutant *KRAS*, β-actin and hemoglobin. In a large population based study (n ~ 10 000), the fecal test reached a sensitivity of 92% for cancers and 42% for advanced precancerous lesions with a specificity of 87% [28]. Importantly, the sensitivity for advanced precancerous lesions was higher for Cologuard compared to FIT alone [28]. An observational cross-sectional cohort study was recently initiated in the Netherlands, aiming at including 4000 individuals for evaluating Cologuard and FIT as alternative screening methods for colorectal cancer [29].

2.2. Prognostic and predictive biomarkers

The tumor-node-metastasis (TNM) classification system is used for colorectal cancer staging, to predict prognosis and for deciding treatment. Patients within the same TNM stage do however, show high variability when it comes to both survival and response to therapy. Proper selection of patients within stages that may benefit from chemotherapy and/or targeted therapy may increase survival and treatment efficacy, and simultaneously reduce the toxicity and cost of such treatments. Abnormal CpG island methylation is a source for prognostic and predictive information. Examples of such markers are listed in Table 1 and reviewed in [13].

A subset of colorectal cancers (15–20%) develop through the CpG island methylator phenotype (CIMP) pathway and are characterized by particularly high levels of aberrantly methylated genes [30,31]. Although it has been suggested that CIMP confers improved survival [32], most studies report that CIMP is associated with a worse prognosis [33–36], including two recent meta-analyses [37,38]. Additionally, a recent study, analyzing more than 1000 tumor samples, found that CIMP was associated with inferior survival, both across all patients and specifically within patients with MSS and MSS *BRAF* mutated tumors. The finding remained significant in multivariate analyses adjusted for stage [39]. Furthermore, CIMP has been suggested to have predictive value, although opposing findings have been reported. Some studies have shown that patients with CIMP positive colorectal cancers display better survival when treated with 5-FU based adjuvant chemotherapy in addition to surgery [40,41], or that these patients may have a survival benefit when treated with irinotecan in addition to 5-FU [42]. However, others have reported that CIMP is not predictive of response towards adjuvant chemotherapy, or that chemotherapy does not increase survival in patients with CIMP tumors [43–45]. It has furthermore been reported that patients with CIMP positive tumors experienced a worse survival when treated with chemotherapy compared to surgery alone [46].

As illustrated in Table 1, some genes with prognostic potential have also been suggested to possess predictive value. For instance, Perez-Carbonell et al. reported that patients with *IGFBP* methylation had an improved disease free survival (DFS), and further that stage II and III patients with high levels of *IGFBP3* methylation did not seem to benefit from adjuvant 5-FU-based chemotherapy [47]. Methylation of *HPP1* in blood has also, in addition to being associated with worse survival, been suggested as a potential marker for the identification of patients who have likely not benefitted from a combination chemotherapy with bevacizumab [48,49]. Furthermore, patients with MSI tumors, normally caused by epigenetic silencing of *MLH1*, have been shown to have either no benefit or to

have increased mortality when treated with 5-FU [50]. Similarly, Ebert et al. observed a strong association between *TFAP2E* methylation and lack of response towards 5-FU based chemotherapy, by analyzing four independent patient cohorts with primary rectal cancer and metastatic colorectal cancers [51]. Methylation may also predict improved response towards chemotherapy. For instance, a significantly higher proportion of patients with *WNT5A* methylation responded to 5-FU-based chemotherapy compared to patients with an unmethylated promoter [52]. Also *MGMT* methylation has been reported to associate with better response and improved median progression free survival (PFS) in metastatic colorectal cancer patients treated with alkylating agents [53].

2.3. Summary and perspectives

A decrease in colorectal cancer death rates has been observed in western countries the last years [54,55], largely explained by improvements in early detection. However, current screening strategies are not optimal considering cost, invasiveness and compliance, and less invasive screening methods are warranted. Non-invasive DNA methylation biomarkers have shown great promise for colorectal cancer detection, including the two FDA approved tests Epi proColon (blood) and Cologuard (feces). Both tests do, however, have room for improvements when it comes to sensitivity and specificity. Several other DNA methylation biomarkers have also been suggested, including biomarkers panels, but validation in independent and large population based series are needed.

When it comes to prognostication and prediction of treatment response, reliable DNA methylation biomarkers for colorectal cancer are still lacking. Several independent studies have, however, shown that colorectal cancer patients with CIMP have an inferior survival. Once a consensus marker panel to identify CIMP is defined and routinely analyzed with a quantitative method, it is likely that CIMP can provide valuable information about prognosis and potentially guide treatment.

3. Aberrant DNA methylation in gastric cancer

3.1. Diagnostic biomarkers

The incidence of gastric cancer has declined in most western countries, but remains one of the most common causes of cancer-related deaths in Asia [1]. The most important approach to reduce the mortality in these patients is by early detection and curative resection of malignant lesions. In Asian countries, including Korea and Japan where the incidence of gastric cancer is particularly high, screening programs, including endoscopy and barium-meal have been implemented [56]. These methods are however, not optimal considering availability, cost, and participation rate [56].

Infection with Heliobacter pylori (H.pylori) and Epstein-Barr virus (EBV) are considered as major risk factors for gastric cancer. Both infections are associated with increased levels of promoter DNA methylation [57–59]. As is the case in colorectal cancer, promoter DNA methylation of several tumor suppressor genes have been identified in premalignant stages of gastric carcinomas [60,61], highlighting their suitability as biomarkers for early cancer detection.

3.1.1. Blood—A number of hypermethylated genes with potential for early detection have been identified in blood specimens from gastric cancer patients (reviewed in [5,62,63]). In a study from 2002, Lee et al. reported frequent methylation of *CDH1* (57%), *DAPK* (48%), *GSTP1* (15%), *CDKN2A (p15*, 56%), and *CDKN2A (p16*, 52%) in sera from 54 cancer patients, but in none of the 30 age-matched serum samples from individuals without cancer [64]. Combining the markers further increased the sensitivity (83%) without compromising the specificity (100%) [64]. Since this initial study, methylation of *CDH1* [65,66] and *CDKN2A* [65–67] have been confirmed as potential biomarkers for gastric cancer detection in blood samples. Another example is *RNF180*, with a sensitivity of 76% (150/198) and specificity of 100% (23/23) in tissue samples [68]. Further analyses in 32 plasma samples confirmed a high sensitivity (56–63%) and specificity (91–100%) [68]. Bernal et al. identified frequent promoter methylation (95%) of *RPRM* in plasma samples from 43 gastric cancer patients, but rarely in the 31 controls (10%), suggesting *RPRM* also as a potential biomarker for early detection of gastric cancer [69].

3.1.2. Gastric washes/gastric juice and feces—Gastric washes represent an alternative source for detecting aberrant DNA methylation is gastric cancer. By analyzing the methylation levels of six genes (*ADAM23*, *GDNF*, *MINT25*, *MLF1*, *PRDM5*, *RORA*) in gastric washes from 20 cancer patients and 48 controls Watanabe and colleagues found that a combination of the markers *MINT25*, *PRDM5* and *GDNF* achieved a high sensitivity (95%) and specificity (92%). Among individual genes, *MINT25* displayed the highest sensitivity (90%) and specificity (96%) [70]. Recently, it was reported that *BARHL2* methylation in gastric washes had promising potential for detection of gastric cancer with significantly higher methylation frequency in the 128 analyzed cancer samples compared to the 32 control samples [71]. Additionally, the methylation levels detected in the cancer patients decreased significantly after endoscopic resection, suggesting that *BARHL2* methylation could be also useful for monitoring tumor recurrence. Furthermore, promoter methylation of *BARHL2* in exosomal DNA from gastric juice could discriminate between cancer (n = 20) and controls (n = 10) with a sensitivity and specificity of 90% and 100%, respectively [71].

Finally, *RASSF2* and *SFRP2* promoter methylation has been found in 57% of fecal samples from gastric cancer patients (n = 21), with 89% specificity [72]. The same genes were also positive for colorectal cancer (75%), indicating that the assay could be useful for detection of both cancer types.

3.2. Prognostic and predictive biomarkers

The TNM staging system is used to predict prognosis and to decide treatment for gastric cancer patients. Similar to colorectal cancer, this pathological system is suboptimal and better markers for predicting prognosis and response to specific drugs are needed.

Promoter hypermethylation of several genes have been reported to be associated with worse prognosis in gastric cancer (Table 2 and reviewed in [63,73,74]). For instance, *BCL6 B* methylation was analyzed in 309 patients with gastric cancer from two different cohorts, and in 20 normal tissue specimens [75]. Combining the two cohorts, 55% of the

cancer patients displayed *BCL6 B* promoter methylation. No methylation was detected in the normal samples, confirming its tumor specificity. It was further shown that *BCL6 B* methylation was an independent predictor of worse OS [75]. Methylation of either *BNIP3* or *DAPK* was shown to confer a significantly poorer OS and PFS in a study including 80 tissue samples from gastric cancer patients. Methylation was additionally predictive of lower response to 5-FU based chemotherapy [76]. Kato et al. noted that methylation of *DAPK* together with *TMS1*was associated with poor prognosis, and also lower response towards 5-FU among patients with recurrent disease [77]. Recently, it was reported that methylation of *NDRG4* was predictive for poor prognosis among Chinese gastric cancer patients. Validation in the TCGA data which included 357 gastric patients, did however, imply an improved prognosis for patients with *NDRG4* promoter methylation, suggesting that different ethnicities, therapies, and methylation detection methods could influence both the methylation frequencies and survival [78]. Additional examples of methylated genes that confer improved prognosis include *BMP4* methylation, which also caused sensitization to cisplatin [79], IGF2 methylation [80], and methylation of *MLH1* [81].

Several of the markers reported as potential markers for detection of gastric cancer have also been suggested to possess prognostic or predictive value. Methylation of *CDH1*, both in preoperative peritoneal washes [82], serum [83], and tissue samples [84] has for instance been reported to be significantly associated with worse survival. In addition to showing that *XAF1* was frequently methylated in a cancer-specific manner, in the serum from gastric cancer patients, Ling et al. reported that patients with *XAF1* promoter methylation had significantly poorer survival compared to those with an unmethylated promoter. *XAF1* methylation in sera was additionally associated with tumor recurrence [85]. Methylation of *RPRM* was reported to predict both poor survival among patients with advanced gastric cancer, and poor response towards 5-FU and cisplatin [86]. Also *CDKN2A* has been suggested both as a marker for detection, and as a potential marker for poor prognosis. Shi et al. analyzed 119 tumor samples for promoter methylation of *e.g. CDKN2A*, and found that methylation of this gene, in addition to *MGMT*, *RASSF2* and *FLNc*, was associated with poor survival, both across all samples and when only tumors without residual disease were included [87]. Methylation of *CDKN2A* has further been reported to be significantly associated with improved survival in patients treated with 5-FU [88].

CIMP has also been identified in gastric cancer [89]. In contrast to colorectal cancer, most studies suggest a lower mortality for patients with CIMP positive gastric cancer [81,90], although an unfavorable prognosis has also been reported [91,92]. A meta-analysis by Zong et al., which included 12 studies and 1000 patients, concluded that CIMP was not a prognostic marker for gastric cancer [93]. Most other studies included much fewer samples than this, making it difficult to infer true clinical potential of these biomarkers.

3.3. Summary and perspectives

The majority of the patients with gastric cancer have regional or distant metastases at the time of diagnosis [54]. The failure to detect cancer at a stage where clinical intervention is effective obviously contributes to the high gastric cancer mortality. Although screening programs have been implemented in some high-risk countries, the available methods are

not optimal. Several DNA methylation biomarkers with high sensitivity and specificity for non- or minimally invasive detection of gastric cancer have been identified. However, the number of samples analyzed for these markers is a limiting factor, and validation of candidate markers in larger and independent samples series is needed. This is also the case for prognostic and predictive candidate DNA methylation biomarkers.

4. Aberrant DNA methylation in esophageal cancer

4.1. Diagnostic biomarkers

Esophageal cancer is a highly fatal malignancy. There are two main classes of histopathologically distinct esophageal cancer, esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). ESCC accounts for about 90% of cases worldwide [94] and is especially prevalent in Eastern Asia and Southern and Eastern Africa, whereas EAC, associated with an increasing incidence, is more commonly seen in Western countries [95].

4.1.1. Esophageal adenocarcinoma—EAC is usually diagnosed at a late stage, with few treatment options, explaining the underlying reason for high mortality rates associated with this disease. The cancer can develop from the premalignant Barrett's esophagus [96], and risk stratification of EAC in individuals with Barrett's esophagus is an area of active research. *CDKN2A* was one of the first genes to be identified as methylated during the neoplastic progression in Barrett's esophagus [97,98]. By analyzing multiple clinical specimens from a handful of patients, Eads and colleagues demonstrated that hypermethylation of *CDKN2A*, along with promoter methylation of *APC* and *ESR1* was present in large contiguous fields [99]. These researches further found significantly higher incidences of hypermethylation in tissues from patients with dysplasia or cancer, compared to patients with no further progression of their disease, confirming that abnormal DNA methylation represents a clinical tool in stratifying Barrett's esophagus patients at risk of developing cancer [100].

From targeted analyses, several additional genes have been suggested as biomarkers for predicting disease progression in Barrett's esophagus patients [101–105]. An eight-gene biomarker panel, including *CDKN2A*, *RUNX3*, *HPP1*, *NELL1*, *TAC1*, *SST*, *AKAP12*, and *CDH13*, was validated in a multicenter, double-blind, tissue-based study. The panel predicted approximately half of the Barrett's esophagus-associated EACs analyzed, with a high specificity [106]. A recent 27 K methylation array based study identified an alternative panel of four DNA methylation biomarkers: *SLC22A18*, *PIGR*, *GJA12* and *RIN2*. The markers were validated in a retrospective cohort and subsequently in a multicenter study. The biomarker panel achieved an area under the ROC curve of 0.988 to distinguish Barrett's esophagus and EAC patients, and was able to stratify patients from the prospective cohort into distinct risk groups [107].

4.1.2. Esophageal squamous cell carcinoma—An integrated genomic characterization of ESCC and EAC from the TCGA research network recently demonstrated that these two subtypes could be differentiated not only by histology but also by their

molecular features, including the DNA methylation levels [108]. Interestingly, ESCC seem to resemble squamous carcinomas of other organs more than they do with EAC.

CDKN2A was one of the first genes to be identified as methylated also in ESCC [109]. Analyzing *CDKN2A* and 13 additional gene promoters, Guo and colleagues could illustrate accumulation of promoter methylation through the histological progression from squamous dysplasia to ESCC [110], underscoring the potential of using DNA methylation biomarkers for early detection of ESCC. In line with this, Ishii et al. confirmed, from analyses of 14 gene promoters, that ESCC patients had accumulated low levels of DNA methylation already in the non-neoplastic esophageal epithelium. The frequency of methylation increased through intraepithelial neoplasia to advanced ESCC; hence most likely contributing to the pathogenesis of this malignancy [111]. The genes with the highest methylation frequencies in ESCC cases were *MGMT* (80%) and *SFRP1* (64%) [111]. DNA methylation in ESCC has not been as extensively studied as is the case in EAC; however *RARB2* (up to 70%) [112] and *FHIT* (up to 69%) [113] are examples of genes reported to be frequently methylated. Interestingly, studies that have analyzed both EAC and ESCC seem to find significantly lower methylation frequencies of specific genes in the latter group ((*RPRM*) [114], *APC* [115]).

Finally, using a bead-array analysis of more than 800 cancer-related genes Lima et al. identified 37 CpG sites that were differentially methylated between ESCCs and surrounding tissues. Of these, *TFF1* was methylated both in ESCC and surrounding tissue in contrast to healthy esophageal mucosa, suggesting that it could be an early event and potential biomarker for ESCC [116].

4.1.3. Blood—Although several DNA methylation biomarkers have been suggested for esophageal cancer, only few of them have been analyzed in a noninvasive manner in blood. Methylation of *APC* has been found in the plasma from 25% (13/52) of the EAC patients and in 6% (2/32) of ESCC patients [115]. Furthermore, *CDKN2A* methylation has been found in 23% (7/31) of ESCC patients harboring a methylated primary tumor [117]. Among four additional genes analyzed by Li and coworkers in serum from 45 ESCC patients and 15 controls, *CDH1* achieved the highest AUC value of 0.822 followed by *DAPK* (0.800), *RASSF1A* (0.778), and *RARB* (0.567) [118]. Zhai and coworkers used methylation arrays to identify hundreds of differentially methylated loci between cell-free circulating DNA from a limited number of EAC and Barrett's esophagus patients. The results have so far not been validated by an independent method or in larger clinical series [119].

4.2. Prognostic and predictive biomarkers

DNA methylation biomarkers with prognostic value for esophageal cancer are generally scarce and often analyzed in small samples series (n < 100; reviewed in [100]). Suggested biomarkers are *APC* [115,120], *TAC1* [121], and *NELL1* [122]. In EAC, promoter methylation of multiple genes has additionally been suggested as a predictor of poor prognosis [123]. In ESCCs, *FHIT* has been analyzed in a larger cohort (n = 257), and aberrant promoter methylation was significantly associated with a poor prognosis for early stage (I &II) cancers (multivariate HR = 5.81, $P = 0.009$) [124]. Finally, in a recent study

CDO1 methylation was suggested as an independent prognostic factor for ESCC patients (multivariate HR = 2.00, $P = 0.03$) based on analyses of 169 cancer samples [125].

4.3. Summary and perspectives

Because of late detection, only 11–18% of the patients with esophagus cancer survive 5 years [126]. DNA methylation biomarkers present in premalignant or early stage cancer could contribute to earlier detection thereby reducing the mortality. However, at present, only a small number of the identified biomarker candidates for esophagus cancer have been analyzed in non-invasive material, and the few studies that have been performed are limited by few samples. The same goes for prognostic and predictive biomarkers. As such, before epigenetic biomarkers become clinically useful in esophagus cancer, more and larger studies are needed, preferentially with the use of quantitative methods.

5. Aberrant DNA methylation in pancreatic cancer

5.1. Diagnostic biomarkers

Pancreatic ductal adenocarcinomas (PDACs), the most prevalent subtype of pancreatic cancer, evolves through non-invasive precursor lesions, most commonly pancreatic intraepithelial neoplasias (PanINs) [127]. These lesions typically grow slowly, and may take between 10 and 15 years to develop into malignant lesions that can further metastasize [128], providing an ideal opportunity for the early detection and curative treatment of affected patients. The microscopic PanINs are usually not visible by pancreatic imaging, and other markers, such as CA19–9, is inadequate as its level may also be elevated in benign diseases [127,129]. This highlights the need for biomarkers that can both reliably identify the high-grade PanINs (with high probability of malignancy) and facilitate further differentiation between malignant and benign disease. Interestingly, several epigenetically silenced genes have been detected in the non-invasive PanINs, including *NTPX2*, *SARP2*, *RPRM*, and *LHX1* [130].

5.1.1. Pancreatic juice—Pancreatic juice contains exfoliated cells from all parts of the pancreas, and represents a good source for detecting aberrations in the pancreatic ductal epithelium. Indeed, several genes commonly methylated in PDACs are also detectable in pancreatic juice of patients with invasive pancreatic cancer. Methylation of ≥2 of the fives genes *CCND2*, *TFPI2*, *PENK*, *NPTX2*, and *FOXE1* has been shown to distinguish between patients with cancer (n = 11) and individuals without neoplasia (n = 64), including chronic pancreatitis, with high sensitivity (82%) and specificity (100%) [131]. In another study, by using reduced representation bisulfite sequencing (RRBS) several differentially methylated regions (DMRs) between cancer and benign and normal control samples were identified [132]. These regions were both technically (by methylation specific PCR or MSP) and biologically validated, before tested by quantitative MSP (qMSP) assays in a pilot study of 102 pancreatic juice samples from 61 patients with cancer, 22 with chronic pancreatitis and 19 with normal pancreas. The AUC values for methylation of *CD1D*, *KCNK12*, *CLEC11A*, *NDRG4*, *IKZF1*, and *PKRCB* for detection of cancer compared with normal pancreas and chronic pancreatitis ranged from 0.83–0.92 and 0.73–0.92, respectively. The most promising gene, *CD1D*, could discriminate between pancreatic cancer and normal tissues

with a75% sensitivity and 95% specificity. Compared to patients with chronic pancreatitis, the specificity was 91% [132]. Several additional aberrantly methylated genes have been identified in pancreatic juice from cancer patients, including *NPTX2* (67%), *SARP2* (46%), and *CLDN5* (42%) [133], *PENK* (67%) and *CDKN2A* (11%) [134], and *APC* (71%) [135].

5.1.2. Blood—Genes aberrantly methylated in cell free DNA in plasma represent another potential source for non-invasive pancreatic cancer detection. By analyzing patients with PDAC (n = 95), and non-malignant controls (chronic pancreatitis, n = 97, and healthy controls, n = 27), a diagnostic prediction model was generated, including age > 65, and promoter methylation of *BMP3*, *RASSF1A*, *BNC1*, *MESTv2*, *TFPI2*, *APC*, *SFRP1* and *SFRP2*. The model displayed an AUC of 0.86, with a sensitivity of 76% and a specificity of 83% for pancreatic cancer [136]. By analyzing 42 serum samples from patients with pancreatic cancer and 26 samples from healthy controls, methylation of *BNC1* and *ADAMS1* could detect cancer with a sensitivity of 79% and 48%, respectively [137]. The specificity was also high; 89% for BNC1 and 92% for *ADAMTS1*. Combining the two markers resulted in a sensitivity of 81% and a specificity of 85%. Interestingly, the genes were frequently methylated in tissue specimens from PanINs (*ADAMST1*: 25%, *BNC1*: 70%) and stage I invasive cancers (*ADAMST1*: 63%, *BNC1*: 97%), high-lightening their potential as early detection markers. Furthermore, the combination of *ADAMTS1* and *BNC1* methylation showed superior sensitivity compared with CA19–9 [137]. Analyzing > 300 blood samples from cancer patients and 300 control samples in a test and validation series by Illumina arrays, Pedersen et al. identified several differently methylated sites between cancer and normal samples, which potentially could aid in the detection of pancreatic cancer [138].

5.1.3. Brush samples—Finally, aberrant methylation for pancreatic cancer detection has also been observed in brush samples. Parsi et al. analyzed the methylation levels of *TFPI2*, *NPTX2* and *CCND2* in biliary brush samples from 41 patients with PDAC, 10 patients with biliary tract cancer, and from 66 individuals with benign biliary tract diseases. At least one methylation positive gene was detected in 73% of patients with pancreatic cancer, in 80% of patients with biliary tract cancer, and in 14% of patients with a benign stricture, concluding that the detection of aberrantly methylated genes in endoscopic brush samples are promising tools to differentiate benign and malignant biliary strictures [139].

5.2. Prognostic and predictive biomarkers

Since most pancreatic cancer patients present with advanced disease (> 80%), where the prognosis is poor regardless of the treatment, less focus has been put on trying to identify biomarkers with prognostic and predictive potential. Still, some methylation candidates have been proposed. In a study analyzing promoter methylation in whole-blood samples from 30 pancreatic cancer patients, methylation of the two genes *TNFRSF10C* and *ACIN1* was found to be significantly associated with shorter OS ($P = 0.023$ and $P = 0.012$, respectively) [140]. By analyzing 87 primary cancers, Sato et al. observed that patients with *RPRM* methylation had a significantly shorter OS compared to those without methylation (univariate HR = 2.2, $P = 0.009$). In multivariate analyses, adjusted for tumor size and differentiation, patients with *RPRM* methylation still tended to have poorer prognosis,

although the results were not statistically significant (HR = 1.8, $P = 0.07$) [141]. It has been further shown that patients with hypermethylated *MUC1* and *MUC4* had significantly improved survival compared to patients with hypomethylated promoters [142]. Finally, by analyzing 11 samples with RRBS Thomson and colleagues identified increased methylation in the promoter region of three genes (*FAM150A*, *ONECUT1*, and *RASSF10*), which strongly correlated with worse survival [143].

About 75% of patients treated with gemcitabine, the first- line treatment for pancreatic cancer patients, demonstrate no response to such treatments. Tan et al. generated 30 pancreatic cancer xenografts from surgically resected primary carcinomas and treated them with gemcitabine. Methylation analyses (the Golden Gate methylation cancer panel I) identified two genes, *GSTM1* and *ONECU*, that were differentially methylated between the 10 xenografts that responded and the 20 that were non-responsive to treatment. The finding was validated in 12 pancreatic cancer cell lines treated with gemcitabine, suggesting that hypermethylation of the two genes may sensitize patients to this type of drug treatment [144].

5.3. Summary and perspectives

Pancreatic cancer is considered a largely incurable disease, with a 5-year survival rate of 9% [54]. The poor prognosis is mainly caused by cancer detection after metastases, where effective treatment options are lacking. Identifying robust biomarkers for earlier detection thus have a large potential impact if successful, as earlier detection could enable surgical resection of the tumor and thereby reduce mortality from pancreatic cancer. Although some epigenetic biomarkers have been reported for pancreatic cancer detection, including *CD1D*, their accuracy is not optimal, underscoring that more research is needed. This is also the case for potentially prognostic and predictive DNA methylation biomarkers in pancreatic cancer. Such markers are therefore not anticipated to reach the clinic in the near future.

6. Aberrant DNA methylation in liver cancer

6.1. Diagnostic biomarkers

Hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) are the two most frequent primary liver cancers, accounting for > 80% of the cases. Most liver cancers (~85%) occur in Asia, and can largely be explained by the continent's high rate of chronic infections with hepatitis B- (HBV) and C- viruses (HCV), which are known risk factors for HCC and CCA [145–147]. For CCA, primary sclerosing cholangitis (PSC) is a common predisposing condition, with a lifetime risk of CCA between 5 and 10% [148]. Distinguishing between benign biliary strictures and malignant changes are difficult in PSC patients, and in up to 37% of cases, CCA is not detected until laparotomy is performed in connection with intended liver transplantation or at autopsy [149]. CA19–9 is a commonly used biomarker for CCA, but is elevated in > 30% of patients with PSC [148]. Serological tests, including α-fetoprotein (AFP), are commonly used diagnostic biomarkers for HCC, but may show positive test results in other malignancies and in healthy pregnant women [150]. For both HCC and CCA, the diagnosis is challenging due to the non-specific clinical presentation,

resulting in late detection [147]. With more sensitive and specific biomarkers increased number of patients could potentially qualify for curative treatment, including surgery [151].

6.1.1. Brush, bile and blood samples

6.1.1.1. Cholangiocarcinoma (CCA): Aberrant DNA methylation has been observed both in patients with PSC and in CCA precursor lesions (biliary intraepithelial neoplasia; BiIN), emphasizing the suitability of such markers for early detection of CCA [152]. As previously mentioned (section 5.1), the methylation levels of *TFPI2*, *NPTX2* and *CCND2* could detect biliary tract cancers (n = 10) from biliary brush samples with a sensitivity of 80% and a specificity of 86% compared to patients with benign liver conditions [139]. The usefulness of brush samples to differentiate between benign and malignant biliary strictures has further been confirmed by Andresen and colleagues. In an initial study analyzing tissue specimens from 39 CCA patients and 54 samples from individuals with non-malignant liver disease, a biomarker panel comprising *CDO1*, *DCLK1*, *SFRP1* and *ZSCAN18* was identified with high sensitivity (87%) and specificity (100%) for detection of CCA [153]. These genes, in addition to several other markers, were further validated in biliary brush samples comprising a test series (15 CCAs, 20 PSC controls) and a validation series (34 CCAs and 34 PSC controls) [151]. Across both series, a four marker panel (*CDO1*, *CNRIP1*, *SEPT9*, and *VIM*) could detect CCA with a sensitivity of 85% and a specificity of 98%, with an AUC of 0.944. Importantly, the biomarker panel outperformed standard brush cytology, underscoring the potential to improve detection of CCA, especially among patients with PSC [151].

Bile may also be suitable for CCA detection. Shin et al. reported that methylation of the marker panel *CCND2*, *CDH13*, *GRIN2B*, *RUNX3*, and *TWIST1* achieved 70% sensitivity in a training set (n = 20), 74% sensitivity in the first validation set (n = 33), and 83% sensitivity in the second validation set (n = 24), with 100% specificity for all sets (total n = 48) [154]. Finally, promoter hypermethylation of *SHOX2* and *SEPT9* was shown to display a sensitivity of 45% and a specificity of 99% when analyzed in plasma from 20 CCA patients and 100 controls [155].

6.1.1.2. Hepatocelluar carcinoma (HCC): As early as 1999, it was shown that promoter methylation of *CDKN2A* was detected in plasma/serum of HCC patients (n = 22, 59% sensitivity) with a 100% specificity, including 38 patients with chronic hepatitis/cirrhosis and 10 healthy controls [156]. In addition to *CDKN2A*, several genes, mainly in tissue specimens but also in blood samples, were found to be frequently methylated in HCC (reviewed in [150,157–160]). A recent metaanalysis highlighted six genes (*CDH1*, *CDKN2A*, *GSTP1*, *RASSF1A*, *RUNX3* and *WIF1*) which, across more than three studies, were significantly hypermethylated in serum from HCC compared to normal samples [161]. In addition, a marker panel consisting of *APC*, *GSTP*, *RASSF1A* and *SFRP1* has been evaluated in plasma samples from 72 HCC patients, 37 patients with benign liver disease and 41 samples from normal controls. The panel achieved a sensitivity of 93% and a specificity of 82%, with an AUC of 0.933. Compared to benign controls, the sensitivity, specificity and AUC were 85%, 82% and 0.877, respectively [162]. Finally, using a genome wide approach in 62 HCC patients, several DMRs regions were identified between tumor

and adjacent non-tumor tissues. For a subset of the markers, pyrosequencing of 38 plasma samples was performed resulting in a combined sensitivity of 87% for HCC detection [163].

6.2. Prognostic and predictive biomarkers

The survival of patients with liver cancer is generally poor due to late disease detection where effective treatment options are lacking. As such, incidence often mirrors death. Better ways of predicting prognosis and response to *e.g.* liver transplantation and surgery could allocate resources to those that most likely would benefit from such treatments.

6.2.1. CCA—Based on 36 methylation probes (Illumina HM450) a methylation signature was generated that could predict poor survival among patients with CCA both in a training set (n = 221; multivariate HR = 13.35, $P < 0.001$) and in a validation set (n = 83, $P = 0.01$) [164]. Furthermore, the signature could accurately predict tumor recurrence in the training set (multivariate HR = 5.8, $P < 0.001$). By analyzing 79 patients with CCA, Lee and colleagues found that methylation of *APC*, *CDKN2A* and *TIMP3* was significantly associated with worse OS in univariate analyses [165]. Methylation of *DAPK* was in another study shown to be an interdependent predictor of poor survival while analyzing 37 patients with biliary tract carcinomas (multivariate HR = 8.71, $P = 0.024$) [166].

6.2.2. HCC—In line with CCA, genome-wide methylation studies have been used to identify subgroups of HCC patients with poor prognosis [167,168]. Single genes have also been proposed to have prognostic value, including methylation of *RASSF1* in plasma (n = 72), which was shown to be an independent prognostic factor for OS (multivariate HR = 3.26, $P = 0.003$) [162]. Similarly, hypomethylation of S100A8 was significantly associated with both worse OS (multivariate HR = 1.71, $P < 0.05$) and PFS (multivariate HR = 1.77, $P < 0.05$), analyzing 52 HCC tissue samples [169]. Finally, SOCS3 methylation was recently reported to predict treatment response and prognosis in 246 HCC patients receiving transarterial chemoembolization (TACE) treatment (multivariate HR = 3.44, $P < 0.001$) [170].

6.3. Summary and perspectives

Most HCC and CCAs are detected at a stage where curative options are limited and the prognosis is poor. Identifying accurate biomarkers for early detection, especially among patients that are at high risk of developing these cancers, still remains an unmet clinical need. Several interesting epigenetic biomarkers have been suggested for liver cancer detection, including a biomarker panel consisting of *CDO1*, *CNRIP*, *SEPT9* and *VIM* for detection of CCA in biliary brush samples. These biomarkers do however, require validation, preferentially in blood. Although bile and biliary brush samples are interesting sources for detection of epigenetic biomarkers, the potential risk of pancreatitis during collection of such material underscore the need for non-invasive analyses.

Only a small percentage of patients with HCC and CCA are fit for surgery or liver transplantation. Biomarkers to better identify patients most likely to benefit from such treatment are naturally attractive. Although some epigenetic biomarkers for this purpose have been suggested, they are far from entering the clinic.

7. Conclusions and perspectives

Epigenetic changes are common in gastrointestinal cancers, and are frequently found in pre-malignant conditions and early stage cancers in different biological fluids, rendering such aberration quite promising for early cancer detection. Fig. 1 summarizes some of these promising DNA methylation biomarkers. Of note, few epigenetic biomarkers have so far been implemented in the clinic. Suboptimal methods and design of biomarker discovery studies, selective and incomplete reporting combined with small samples sizes and lack of independent validation, have been highlighted as some of the explanations for the discrepancy between the number of reported promising biomarkers and those that are in fact implemented in the clinic [171]. Regarding validation of potential biomarkers, the use of sensitive technologies, proper unbiased optimization and standardization of commonly used methods is critically important [172]. In light of this, and based on the limited sample series analyzed so far and the general lack of independent validation, non-invasive biomarkers for most of the GI cancer types are far from being adopted for clinical applications. The exception is colorectal cancer, where the two DNA methylation-based tests Epi ProColon and Cologuard have been approved by the FDA for non-invasive cancer detection. The use of non-invasive material, especially blood, for detection is attractive from a compliance perspective. Blood is furthermore easily accessible, and may give a better picture of the tumor heterogeneity compared to a single biopsy [173], which is especially important considering prognostic and predictive biomarkers. Such tests, however, do have the potential of detecting any lesion, irrespective of location, which may represent a pitfall for a diagnostic test. As evident from Fig. 1, several genes are hypermethylated in more than one GI cancer type, including the Epi ProColon *SEPT9*, which has also been reported by Epigenomics themselves [20]. For improved cancer-type specificity of a diagnostic test, adding tissue specific markers is one of the options. Another alternative is site-specific sampling, such as feces for colon, gastric washes for gastric, bile for bile duct, and pancreatic juice for pancreatic cancer detection. Several promising biomarkers have been identified using this approach, including Cologuard for detection of colorectal cancer.

Currently, no epigenetic biomarker has been approved for prognostic or for predicting response to treatment in any of the GI cancers. However, with increasing application of genome-wide methylation analyses, identification of additional methylation markers are anticipated. Such changes may in the future play a major role in GI cancer detection, for determining prognosis and for predicting response to specific treatments. Proper study-design, quantitative methods and large samples series with independent validation are, however, important factors to succeed in the discovery and development of clinically relevant biomarkers.

Acknowledgements

This work was supported by grants from the South-Eastern Norway Regional Health Authority (to G.E. Lind), the Research Council of Norway (project number 239961, to G.E. Lind), and the Centre of Excellence funding scheme (project number 179571). This work was also supported by the CA72851, CA181572, CA184792, CA187956 and CA202797 grants from the National Cancer Institute, National Institute of Health; RP140784 from the Cancer Prevention Research Institute of Texas; grants from the Sammons Cancer Center and Baylor Foundation, as well as funds from the Baylor Scott & White Research Institute, Dallas, TX, USA awarded to A.G.

Abbreviations

AUC	area under the ROC curve
CCA	cholangiocarcinoma
EAC	esophageal adenocarcinoma
ESCC	Esophageal squamous cell carcinoma
FDA	US Food and Drug Administration
FIT	fecal immunochemical test
FOBT	fecal occult blood test
GI	gastrointestinal
HCC	hepatocellular carcinoma
MSP	methylation specific PCR
OS	overall survival
PDAC	pancreatic ductal adenocarcinoma
PFS	progression free survival
PSC	primary sclerosing cholangitis
qMSP	quantitative methylation specific PCR
ROC	receiver operating characteristics curve

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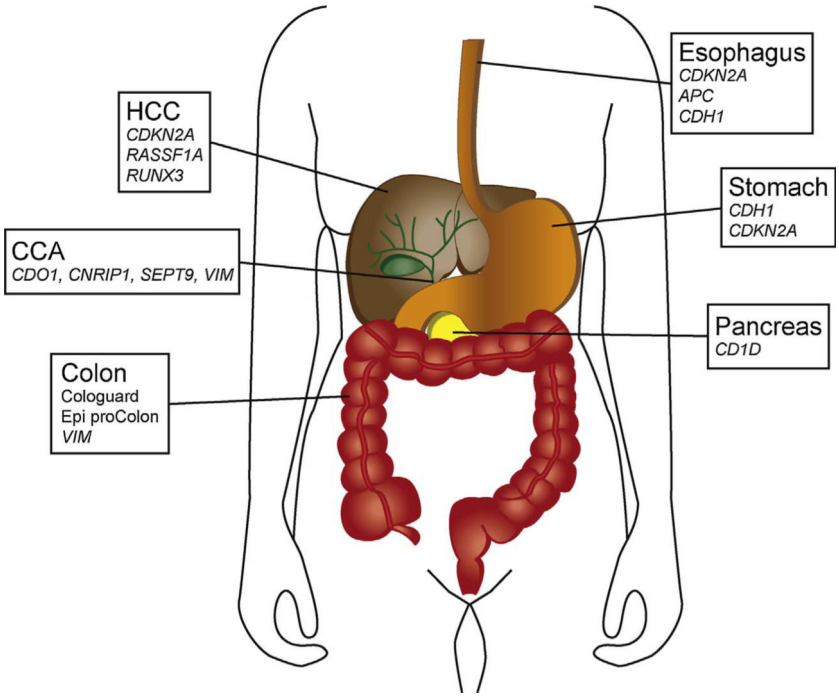

Fig. 1.

Potential non-or minimally-invasive DNA methylation biomarkers for the detection of different gastrointestinal cancers. FDA approved markers are marked in bold, validated markers or markers that are reported to be frequently methylated in larger non-invasive patients series are in normal typing. Smaller sample series have been analyzed for CCA, esophageal-, HCC and pancreatic- cancer compared to colorectal and gastric cancer. Abbreviations: CCA, cholangiocarcinoma; HCC, hepatocellular carcinoma.

Table 1

Prognostic and predictive DNA methylation biomarkers for colorectal cancer.

Gene /panel	Material	Cancer samples	Stage	Effect of methylation	Survival / Response	Method	Ref
APC1A, CDKN2A, RASSF1A	Tissue	111	I-IV	Poor survival	*RR = 2.20, *P* = .037	Pyrosequencing	[174]
AGBL4, FLI1, TWIST1	Plasma	353	I-IV	Poor DFS	HR= 2.00, *P* = .001	MALDI-TOF	[175]
					*HR = 0.495, *P* = .495		
CDKN2A	Tissue	902	I-IV	Poor OS	HR = 1.36, *P* = .004	qMSP	[176]
					*HR = 1.03, *P* = .78		
CDKN2A	Tissue	111	I-IV	Poor survival	*P* = .036	Pyrosequencing	[174]
					**P* = .065		
CDKN2A, KRAS mut	Tissue	86	I-III	Poor OS	*HR = 3.0, *P* = .036	MSP	[177]
CDKN2A, NEUROG	Tissue	497 (FOLFOX)	II-III	Worse OS	*HR = 2.89, *P* = .002	qMSP	[178]
CDX2	Tissue	72	I-IV	Poor OS	*HR = 3.02, *P* = .030	MSP	[179]
CIMP	Tissue	1035	I-IV	Poor OS	*HR = 1.89, *P* < .001	qMSP	[39]
				Poor TTR	*HR = 1.86, *P* < .001		
CIMP	Tissue	206	I-IV	Poor OS	*P* = .029	qMSP	[33]
					*CIMP not included		
CIMP	Tissue	734	I-IV	Poor OS	*P* = .009, **P* = .451	qMSP	[34]
				Poor DFS	*P* = .008, **P* = .337		
CIMP	Tissue	190 (cohort 1)	I-IV	Poor CSS	*HR = 1.84, *P* > .05	qMSP	[35]
		574 (cohort 2)			*HR = 1.10, *P* > .05		
CIMP	Tissue	272 (MSS)	I-IV	Poor relative survival	*HR = 2.90, *P* = .001	MSP	[36]
CIMP	Tissue	649	I-IV	Improved CSS	*HR = 0.44, *P* < .05	qMSP	[32]
CIMP	Tissue	24 (CIMP+)	II-III	5-FU improved survival	*P* = .022	qMSP	[40]
		100 (CIMP−)		5-FU no survival benefit	*P* = .988		
CIMP	Tissue	67 (CIMP+)	III	5-FU improved survival	*P* = .002	MSP	[41]
		140 (CIMP−)		5-FU no survival benefit	*P* = .60		

Gene /panel	Material	Cancer samples	Stage	Effect of methylation	Survival / Response	Method	Ref
CIMP	Tissue	615	III	Poor OS	HR = 1.36, *P* = .044	qMSP	[42]
					**P* = .031		
CIMP	Tissue	145 (CIMP+)	III	IFL (vs 5-FU/LV) improved OS	HR = 0.62, *P* = .07	qMSP	[42]
		470 (CIMP−)		IFL (vs FU/LV) worse OS	HR = 1.38, *P* = .049		
CIMP	Tissue	12 (CIMP+)	II-III	5-FU worse DFS	*P* = .023	MSP	[46]
		38 (CIMP−)		5-FU no survival benefit	*P* = .146		
CIMP/*MLH1*	Tissue	115	II	Poor DFS	*HR = 3.16, *P* = .011	qMSP	[180]
				Poor OS	*HR = 4.70, *P* = .002		
CREB1	Tissue	33 (rectum)	II-III	Predictive of lower response to radiation	*P* = .031(histology)	qMSP	[181]
DIRAS1	Tissue	146	I-IV	Poor OS	*P* = .012	MSP	[182]
FOXE1	Tissue	396	I-IV	Poor OS	*P* = .008	Illumina HM450	[183]
HLTF	Serum	103	IV	Poor OS	*HR = 1.8, *P* = .04	qMSP	[49]
HLTF	Serum	77	I-IV	Poor OS	RR = 3.0, *P* = .008	qMSP	[184]
HOPX	Tissue	170	III	Poor DSS	*HR = 1.40, *P* = .035	qMSP	[185]
HPP1	Plasma	467	IV	Poor OS	HR = 1.86, *P* < .05	qMSP	[48]
HPP1	Plasma (after therapy initiated)	467	IV	Predictive of non-responders to bevacizumab comb therapy	AUC = 0.77, NPV = 97.7 (RECIST)	qMSP	[48]
HPP1	Serum	103	IV	Poor OS	*HR = 1.6, *P* < .05	qMSP	[49]
HPP1	Serum	77	I-IV	Poor OS	RR = 5.1, *P* = .001	qMSP	[184]
HPP1, HLTF	Serum	77	I-IV	Poor OS	*RR = 3.4, *P* = .007	qMSP	[184]
IGFBP3	Tissue	34 (training)	II	Improved RFS	*HR = 6.46, *P* = .012	qMSP	[186]
		81 (validation)			*HR = 2.40, *P* = .029		
IGFBP3	Tissue	425	II-III	Improved DFS	*HR = 0.49, *P* < .01	Pyrosequencing	[47]
IGFBP3	Tissue	89 (methylated)	II-III	Chemotherapy no survival benefit	*P* = .20	Pyrosequencing	[47]
		157 (unmethylated)		Chemotherapy survival benefit	*P* = .040		
KISS1	Tissue	100 (validation 1)	I-III	Poor DSS and OS	*P* = .034 (DSS), *P* = .015 (OS)	MSP, BGS	[187]
		190 (validation 2)			*P* = .030 (DSS)		

Gene /panel	Material	Cancer samples	Stage	Effect of methylation	Survival / Response	Method	Ref
MGMT	Plasma	49 (TMZ)	IV	Improved PFS	$P = .008$	MethylBEAMing	[53]
MGMT	Tissue	68	IV	Improved response to dacarbazine	DCR = 44% (methylated), DCR = 6% (unmethylated), $P = .012$	MSP	[188]
MGMT	Tissue	61 (training)	IV	Improved response to alkylating agents	PPV = 0.67, NPV = 0.89 (RECIST)	MethylBEAMing	[53]
		21 (validation)			PPV = 0.5, NPV = 0.67 (RECIST)		
MGMT	Tissue	111	I-IV	Improved survival	*RR = .36, $P = .023$	Pyrosequencing	[174]
MLH1	Tissue	195	I-IV	Improved OS	*HR = .56, $P = .01$	MS-MLPA	[189]
PCDH10, SPARC, UCHL1	Tissue	71 (5-FU/LV)	II	Poor DFS and OS	$P = .069$ (DFS), $P = .139$ (OS)	qMSP	[190]
		72 (surveillance)		Improved DFS and OS	$P = .031$ (DFS), $P = .003$ (OS)		
RARb2	Tissue	73	I-IV	Poor OS	$P = .026$	MSP	[191]
RET	Tissue	233 (series 1)	II	Poor OS	*HR = 2.46, $P < .05$	MSP, pyrosequencing	[192]
		231 (series 2)			*HR = 1.13, $P > .05$		
		294 (series 3)			*HR = 1.91, $P < .05$		
SEPT9	Serum (after 1y follow-up)	137	II-III	Poor CSS	*HR = 2.69, $P < .05$	qMSP	[193]
SFRP2	Tissue	77	I-IV	Poor OS	*HR = 5.15, $P = .041$	MSP	[194]
SHISA3	Tissue	120 (OS)	I-IV	Poor OS	*HR = 2.9, $P = .002$	Pyrosequencing	[195]
		83 (DFS)	II-III	Poor DFS	*HR = 4.0, $P = .003$		
SLFN11	Tissue	128	T1-T4/N0-N2	Poor OS	*HR = 0.44, $P = .022$	MSP	[196]
				Poor RFS	*HR = 0.44, $P = .023$		
SYK	Tissue	139	I-IV	Poor OS	*HR = 1.77, $P = .001$	MSP	[197]
SYNPO2	Tissue	31 (training)	I-IV	Poor DSS and OS	$P = 0.132$ (DSS)	MSP, BGS	[198]
		100 (validation 1)	II		$P = .046$ (DSS), $P = .031$ (OS)		
		48 (validation 2)	II-III		$P = .012$ (DSS), $P = .009$ (OS)		
TAC1	Serum (after 6 months follow-up)	144	II-III	Poor CSS	*HR = 4.12, $P \leq .001$	qMSP	[193]
				Poor DFS	*HR = 5.72, $P < .001$		
TFAP2E	Tissue	74 (cohort I)	IV	Lower response to 5-FU	$P < .001$ (RECIST)	qMSP	[51]
		36 (cohort II)	IV		$P < .001$ (RECIST)		
		42 (cohort III)			$P < .001$ (histology)		
		68 (cohort IV)			$P < .001$ (histology)		

Gene /panel	Material	Cancer samples	Stage	Effect of methylation	Survival / Response	Method	Ref
TFAP2E	Tissue	193	I-III	Improved OS	*HR = 2.24, *P* = .025	MS-HRM	[199]
				Improved RFS	*HR = 2.44, *P* = .025		
TFAP2E	Tissue	154 (chemotherapy)	II-III	Improved OS	*HR = 2.55, *P* = .017	MS-HRM	[199]
				Improved RFS	*HR = 2.36, *P* = .032		
WNT5A	Tissue	126 (treated with 5-FU)	I-IV	Improved PFS	*P* < .005	MSP	[52]
WRN	Tissue	88 (treated with irinotecan)	NA	Improved OS	*P* < .001	MSP	[200]

Abbreviations: BGS, bisulfite genomic sequencing; COBRA, combined bisulfite restriction analysis; DCR, disease control rate (= partial response+ stable disease); DFS, disease free survival; DSS, disease specific survival; HR, hazard ratio; MSP, methylation specific PCR; MSS, microsatellite stable; NA, not available; NPV. Negative predictive value; OS, overall survival; PFS, progression free survival; PPV, positive predictive value; qMSP, quantitative methylation specific PCR; RECIST, response evaluation criteria in solid tumors; RFS, recurrence/relapse-free survival; RR, relative risk; TTR, time to recurrence.

*Multivariate.

Table 2

Prognostic and predictive DNA methylation biomarkers for gastric cancer.

Gene /panel	Material	Cancer samples	Stage	Effect of methylation	Survival/Response	Method	Ref
APC	Serum	79	I-IIIAB	Poor OS	*HR = 4.6, *P* = .046	MSP	[201]
APC, CDH1	Serum	58	I-IV	Poor survival	*P* = .0006	qMSP	[202]
ASC/TMS1	Tissue	200	T1-T4/N0-N3	Poor OS	*HR = 0.536, *P* = .001	MSP	[203]
BCL6B	Tissue	208 (cohort 1)	I-IV	Poor OS	*RR = 2.14, *P* = .001	COBRA, BGS	[75]
		101 (cohort 2)			*RR = 1.85, *P* = .02		
BNIP	Tissue	80	IV or recurrence	Poor OS	*P* = .031	MSP	[76]
BNIP, DAPK	Tissue	80	IV or recurrence	Lower response to 5-FU	*P* = .003 (RECIST)	MSP	[76]
BNIP, DAPK	Tissue	80	IV or recurrence	Poor OS	*P* = .001	MSP	[76]
				Poor PFS	*P* = .002		
CACNA2D3	Tissue	53	Advanced[a]	Poor survival	*OR = 4.38, *P* = .002	MSP, BGS	[204]
CDH1	PPW	92	I-IV	Poor DFS	RR = 333, *P* < .001	qMSP	[82]
CDH1	Serum	97	I-IV	Poor OS	*P* < .05	MSP	[83]
CDH1	Tissue	73	II-III	Poor DFS	*RR = 2.28, *P* < .001	MSP	[84]
				Poor OS	*RR = 1.94, *P* = .004		
CDKN2A	Tissue	119	I-IV	Poor survival	*P* < .0001	MSP	[87]
CDKN2A	Tissue	38 (chemotherapy)	I-IV	Improved DFS	*RR = 0.093, *P* = .043	MSP	[88]
CDO1	Peritoneal washes	102	I-IV	Poor OS	*P* = .0004	qMSP	[205]
				Poor RFS	*P* = .005		
CHFR	Tissue	12	Advanced[a] or recurrence	Improved response to TXL or TXT	86% PR or NC (CHFR+), 20% PR or NC (CHFR−)	MSP	[206]
CIMP	Tissue	68	I-IV	Improved OS	*P* = .069	COBRA	[81]
CIMP/*MLH1*	Tissue	68	I-IV	Improved OS	**P* = .031	COBRA	[81]
DAPK	Tissue	81	I-IV	Poor OS	*P* = .045	MSP	[77]
DAPK	Tissue	80	IV or recurrence	Lower response to 5-FU	*P* = .012 (RECIST)	MSP	[76]
DAPK	Tissue	80	IV or recurrence	Poor PFS	*P* = .007	MSP	[76]
DAPK and TMS1	Tissue	81	I-IV	Poor OS	*P* < .001	MSP	[77]
				Poor RFS	*P* < .0001		

Gene /panel	Material	Cancer samples	Stage	Effect of methylation	Survival/Response	Method	Ref
DAPK and TMS1	Tissue	43 (5-FU)	IV or recurrence	Poor OS	$P = .083$	MSP	[77]
				Poor TTR	$P = .008$		
DAPK1	Tissue	141	I-IV	Poor OS	$P = .04$	qMSP	[207]
					*OR = 1.13, $P > .05$		
DKK2	Tissue	92	I-IV		*HR = 2.05, $P = .041$	Pyrosequencing	[208]
DLEC1	Tissue	148	I-IV	Poor RFS	*HR = 2.43, $P = .025$	qMSP	[209]
FLNc	Tissue	119	I-IV	Poor survival	$P = .03$	MSP	[87]
GFRA3	Tissue	24	I-IV	Poor OS	*$P = .01$	Illumina HM27K	[210]
IGF2	Leukocytes	299	I-IV	Improved OS	*HR = 0.42, $P = .001$	qMSP	[80]
MGMT	Tissue	79	I-IV	Poor DFS	$P < .02$	MSP	[211]
MGMT	Tissue	119	I-IV	Poor survival	$P = .02$	MSP	[87]
MINT2	Serum	92	I-IV	Poor DFS	*RR = 4.11, $P < .05$	qMSP	[212]
	PPLF				*RR = 3.26, $P < .05$		
MLH1	Tissue	68	I-IV	Improved OS	$P = .026$	COBRA	[81]
NDRG4	Tissue	110	I-IV	Poor OS	*HR = 1.887, $P = .020$	qMSP, pyrosequencing	[78]
PAX5	Tissue	460	T1-T4/N0-N3	Poor OS	*HR = 1.39, $P = .005$	BGS	[213]
PAX5	Tissue	187	I-IV	Poor OS	*RR = 2.10, $P = .01$	MSP, BGS	[214]
PAX6	Tissue	141	I-IV	Poor OS	$P < .001$	qMSP	[207]
					*OR = 1.51, $P > .05$		
PCDH10	Tissue	104	I-IV	Poor OS	*RR = 1.73, $P = .039$	COBRA	[61]
PCDH10	Tissue	471	T1-T4/N0-N3	Poor OS	*HR = 1.41, $P = .011$	BGS	[215]
RARb	Tissue	141	I-IV	Poor OS	$P < .001$	qMSP	[207]
					*OR = 2.04, $P > .05$		
RASSF10	Serum	82	I-IV	Poor OS	*OR = 13.96, $P < .001$	BGS	[216]
				Poor DFS	*OR = 13.11, $P < .001$		
RASSF1A	Tissue	141	I-IV	Poor OS	$P = .004$	qMSP	[207]
					*OR = 1.72, $P > .05$		
RASSF2A	Tissue	119	I-IV	Poor survival	$P = .01$	MSP	[87]

Gene /panel	Material	Cancer samples	Stage	Effect of methylation	Survival/Response	Method	Ref
RKIP	Tissue	76	I-IV	Poor survival	*OR = 0.22, *P* = .042	MSP	[217]
RNF180	Plasma	32	I-IV	Poor CSS	*RR = 2.13, *P* = .02	qMSP	[68]
RPRM	Tissue	49 (5-FU + cisplatin)	IV or recurrence	Poor DSS	*P* = .05	qMSP	[86]
RPRM	Tissue	68	Advanced[a]	Poor DSS	*HR = 2.148, *P* = .026	qMSP	[86]
SPARC	Tissue	185	I-IV	Poor OS	*RR = 2.75, *P* < .001	MSP	[218]
TIMP3	PPW	92	I-IV	Poor DFS	*HR = 1.72, *P* < .001	qMSP	[219]
TIMP3	Serum	92	I-IV	Poor DFS	*HR = 1.55, *P* = .03	qMSP	[219]
XAF1	Serum	202	I-IV	Poor DFS	*HR = 5.71, *P* < .001	qMSP	[85]

Abbreviations: BGS, bisulfite genomic sequencing; COBRA, combined bisulfite restriction analysis; DSS, disease specific survival; HR, hazard ratio; MSP, methylation specific PCR; NC, no change; OR, odds ratio; OS, overall survival; PPLF, preoperative peritoneal lavage fluid; PPW, preoperative peritoneal washes; PR; partial response; qMSP, quantitative methylation specific PCR; RECIST, response evaluation criteria in solid tumors; RFS, relapse/recurrence free survival; RR, relative risk; TTP, time to progression; TXT, Taxotere; TXL, Taxol.

[a]Invading muscuaris propria.

*Multivariate.