Promoter CpG island methylation markers in colorectal cancer: the road ahead

Despite increasing knowledge on the biology, detection and treatment of colorectal cancer (CRC), the disease is still a major health problem. Hypermethylation of promoter regions of genes has been studied extensively as a contributor in CRC carcinogenesis. In addition, it is the topic of many studies focusing on biomarkers for the early detection, prediction of prognosis and treatment outcome. Methylation markers may be preferred over current screening and test methods as they are stable and easy to detect. However, almost no methylation marker is currently being used in clinical practice, often due to a lack of sensitivity, specificity, or validation of the results. This review summarizes the current knowledge of hypermethylation biomarkers for CRC detection, progression and treatment outcome.

KEYWORDS: colorectal cancer ▪ DNA methylation ▪ early detection marker ▪ prediction marker ▪ prognostic marker

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Colorectal cancer (CRC) is the third most commonly diagnosed malignancy in men and women, behind lung and prostate, and lung and breast cancer, respectively [1]. Despite the overall improvements in CRC therapy, the disease remains a huge health burden with over 1 million cases worldwide and a disease specific mortality of approximately 50% in the developed world [2,3]. CRC is a heterogeneous disease, it is accepted that there are at least three distinct pathways through which a CRC can develop: the chromosomal instability pathway [4], the microsatellite instability (MSI) pathway and the CpG island methylation phenotype (CIMP) route [5,6]. The histology largely reflects the accumulation of genetic and epigenetic alterations that lead to unregulated growth of the intestinal epithelium [4,7–10]. From systematic genome-wide analyses of altered genetic and epigenetic alterations, several cancer candidate genes (CAN genes) have been identified that play a role in this disease. These genes are likely to be important in neoplastic development and are attractive for further research as biomarkers [11,12]. Although multiple molecular read outs of gene alterations exist, detecting epigenetic alterations is a particularly interesting approach for the noninvasive early detection of CRC and prediction of prognosis and response to treatment. Epigenetic alterations are defined as heritable changes in gene activity and expression that occur without alterations in the DNA sequence. Multiple epigenetic changes have been identified, which alone or in interplay with other alterations drive tumorigenesis and cancer progression. Promoter CpG island methylation is the most widely studied and best characterized epigenetic alteration in CRC [10], providing some of the most promising markers for early detection and prediction of prognosis or treatment response in CRC [13]. The inherent stability and the fixed position of acquired CpG methylation in the DNA of interest allows analysis in small amounts of nearly every tissue and cell type with simple and fast detection techniques based on PCR and/or sequencing technology [14,15]. This review summarizes the current state of the available promoter CpG island methylation markers for CRC and discusses the steps that need to be taken before these markers will truly be implemented into clinical practice.

Promoter CpG island methylation in CRC

Of the epigenetic alterations that regulate gene expression in CRC (DNA hypermethylation, DNA hypomethylation, post-translational histone modifications, chromatin looping, nucleosomal positioning and ncRNAs) promoter CpG island methylation is the most studied and well characterized modification [16]. DNA methylation is the postreplicative addition of a methyl group to the carbon 5-position of the cytosine, forming methyl cytosine, a reaction catalyzed by a family of enzymes called DNA methyltransferases (DNMTs). In mammalian cells, DNA methylation is observed in cytosines located 5′ to guanine, the so-called CpG dinucleotides. CpG dinucleotides are scattered throughout the genome, but cluster into so-called CpG islands at promoter regions of approximately a

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half of our genes [15]. Originally, CpG islands were defined as regions larger than 200 bp, forming clusters of CpG dinucleotides with a GC content of at least 50%. Currently, adapted criteria of CpG islands are in use [17]; however, a generally accepted definition for CpG islands is lacking. DNMTs catalyze the transfer of the methyl group from (*S*)-adenosyl-L-methionine to the cytosine of the CpG dinucleotide [18]. DNMT1 is responsible for maintenance of methylation patterns upon DNA replication, whereas DNMT3a and DNMT3b regulate *de novo* methylation [19]. These two members of the DNMT family are highly expressed in embryonic stem cells and downregulated in differentiated cells [20]. DNA methylation patterns in normal tissues are dependent on the activity of DNMTs, whose expressions are regulated at both the transcriptional and posttranscriptional level [21–23]. DNA hypermethylation is a gain of methylation in a locus originally unmethylated, which can cause repression of transcription [15,24]. It often occurs at specific regulatory sites in the promoter region and may have a tissue-, aging- or tumor-specific pattern [25–28]. Recently, it has been described that DNA methylation can also occur at CpG island shores, which are regions of lower CpG density that lie in close proximity (~2 kb) of CpG islands, which are also closely associated with transcriptional inactivation [29,30]. DNA methylation can inhibit gene expression by various mechanisms. Methylated DNA can promote the recruitment of methyl-CpG-binding domain (MBD) proteins. MBD family members in turn recruit histone modifying and chromatin-remodeling complexes to methylated sites [20,31]. DNA methylation can also directly inhibit transcription by blocking the recruitment of DNA binding proteins from their target sites [32]. Promoter CpG island methylation of tumor suppressor genes is recognized as a potent and prevalent way for inactivation of tumor suppressor genes [33–35]. Promoter CpG island methylation has been described in almost every tumor type and is often referred to as the third pathway in the Knudson model for inactivating tumor suppressor genes in cancer [15]. The recognition that a distinct subset of CRCs display significantly more promoter methylation than others has led to the introduction of the concept of CIMP [36,37]. In 1999, Toyota *et al.* identified a set of CpG islands that are methylated in cancer, including CRC, but not in normal gut epithelial cells. Since then, multiple CpG island sets to define CIMP have been described [37–42]. Specific pathological, clinical and molecular features, such as older age, female gender, poor tumor differentiation, proximal location of the tumor, *BRAF* mutations, *KRAS* mutations and wild-type *TP53* distinctly characterize CIMP-associated CRCs [39,41,43,44]. However, it is still unclear whether tumors that display CIMP phenotype are truly a unique molecular subgroup of tumors or a group of tumors that are on the extreme part of a normal distribution with regard to aberrant DNA methylation [36,42]. For a recent review on CIMP, see Hughes *et al.* [6].

Promoter CpG island methylation markers in CRC: the current status

Biomarkers are indicators of normal or abnormal biological processes. Specific changes in pathologies, biochemistries, genetics and epigenetics can provide comprehensive information on the nature of any particular disease. A good biomarker should be precise and reliable and be able to make a distinction between normal condition and disease, but also between different diseases. It is believed that biomarkers could have great potential in predicting the probability of disease, recognizing the disease at an early stage, and setting standards for the development of new therapy to treat cancer [45,46].

In cancer, a biomarker might be either a molecule secreted by the tumor or it can be a specific response of the body to the presence of cancer. Genetic, epigenetic, proteomic, glycomic and imaging biomarkers can be used for the diagnosis of cancer (early detection markers), prediction of the course of the disease (prognostic markers) or prediction of treatment response (predictive markers) or dosage (pharmacodynamics biomarkers) [47–50].

For CRC, no clinically established epigenetic biomarkers for early detection, prognosis or prediction of treatment response are available yet. In Tables 1 & 2, we summarize the most promising methylation markers for early detection, prognosis and prediction of treatment response that have been identified so far in CRC.

Early detection markers

CRC identified at early stages is easily treatable and can often be cured by surgical resection of the involved tissue: cure rates (5-year postdiagnosis) are >90% for early-stage disease and only 5% for advanced disease [51]. Currently, colonoscopy (the gold standard) and fecal occult blood test are used for early detection; the former is highly sensitive but invasive and costly, while the latter is easy to use but less sensitive, especially for

Table 1. Sensitivity and specificity of early detection markers in fecal or serum DNA†.

Gene	Sensitivity, % (n)			Specificity, % (n)	Functional evidence in CRC		Author (year)	Ref.
	Hyperplastic polyps	*Adenoma*	*CRC*	*CRC*	In vitro	In vivo		
Fecal DNA								
Vimentin			46 (43/94)	90 (20/198)	n	n	Chen *et al.* (2005)	[56]
			73 (29/40)	87 (16/122)			Itzkowitz *et al.* (2007)	[58]
			88 (35/40)‡	82 (22/122)‡			Itzkowitz *et al.* (2007)	[58]
			81 (34/42)	82 (43/241)			Itzkowitz *et al.* (2008)	[57]
			86 (36/42)‡	73 (65/241)‡			Itzkowitz *et al.* (2008)	[57]
			77 (63/82)	83 (62/363)			Itzkowitz *et al.* (2008)	[57]
			83 (68/82)‡	82 (65/363)‡			Itzkowitz *et al.* (2008)	[57]
		45 (9/20)	41 (9/22)	95 (2/38)			Li *et al.* (2009)	[59]
SFRP1		100 (7/7)	84 (16/19)	86 (2/14)	n	n	Zhang *et al.* (2007)	[66]
SFRP2	38 (3/8)	70 (7/10)	94 (2/52)	93 (2/25)	n	n	Huang *et al.* (2007)	[62]
	46 (6/13)	33 (2/6)		100 (0/6)			Oberwalder *et al.* (2008)	[64]
	42 (11/26)	62 (21/34)	87 (60/69)	93 (2/30)			Wang *et al.* (2008)	[63]
	33 (15/46)	46 (29/63)	84 (142/169)	54 (29/63)			Tang *et al.* (2011)	[84]
HIC1		31 (4/13)	42 (11/26)	100 (0/32)	y [155]	y [154]	Lenhard *et al.* (2005)	[67]
CDKN2A		31 (9/29)		84 (3/19)	n	n	Petko *et al.* (2005)	[68]
MGMT		48 (14/29)		72 (5/18)	n	n	Petko *et al.* (2005)	[68]
MLH1		0 (0/29)		90 (2/19)	n	n	Petko *et al.* (2005)	[68]
CDKN2A/MGMT/ MLH1	40 (4/10)	55 (16/29)		72 (7/25)			Petko *et al.* (2005)	[68]
ATM/APC/ MGMT/ hMLH1/HLTF/ SFRP2/GSTP1		68 (20/30)	75 (15/20)	90 (3/30)			Leung *et al.* (2007)	[69]
NDRG4			61 (17/28)	93 (3/45)	y	n	Melotte *et al.* (2009)	[70]
			53 (25/47)	100 (0/30)			Melotte *et al.* (2009)	[70]
GATA-4			71 (20/28)	84 (7/45)	y	n	Hellebrekers *et al.* (2009)	[71]
			51 (24/47)	93 (2/30)			Hellebrekers *et al.* (2009)	[71]

†*Sensitivity was determined in fecal or serum samples from patients with CRC, colorectal adenomas or hyperplastic polyps. Specificity was determined in healthy control subjects or persons with a negative colonoscopy assessment.*
‡*Combination marker test utilizing methylated vimentin in combination with DNA integrity assay.*
§*Combination marker test including mutant* KRAS*, the β-actin gene and quantity of hemoglobin (by the porphyrin method).*
¶*Stage I–III CRC.*
#*Adenomas >1 cm.*
††*Adenomas <1 cm.*
‡‡*Stage I CRC.*
§§*Stage II CRC.*
CRC: Colorectal cancer; FIT: Fecal immunochemical test; n: Not validated; y: Validated.

Table 1. Sensitivity and specificity of early detection markers in fecal or serum DNA† (cont.).

Gene	Sensitivity, % (n)			Specificity, % (n)	Functional evidence in CRC		Author (year)	Ref.
	Hyperplastic polyps	*Adenoma*	*CRC*	*CRC*	In vitro	In vivo		
Fecal DNA (cont.)								
TFPI2		43 (3/7)	73 (8/11)	100 (0/12)	n	n	Glöckner *et al.* (2009)	[72]
			89 (23/26)	79 (9/45)			Glöckner *et al.* (2009)	[72]
		21 (4/19)	76 (36/47)	93 (2/30)			Glöckner *et al.* (2009)	[72]
OSMR			38 (26/69)	95 (4/81)	n	n	Kim *et al.* (2009)	[73]
BMP3, NDRG4, vimentin, TFPI2§		82 (18/22)	87 (26/30)	90 (5/46)			Ahlquist *et al.* (2012)	[85]
		63 (84/133)#	91 (27/30)¶					
		54 (51/94)††	85 (214/252)	90 (29/293)			Ahlquist *et al.* (2011)	[75]
PHACTR3			55 (12/22)	95 (4/78)	n	n	Bosch *et al.* (2011)	[74]
		32 (6/19)	66(29/44)	100 (0/30)			Bosch *et al.* (2011)	[74]
PHACTR3 and FIT		33 (7/24)	95 (19/20)	94 (3/48)			Bosch *et al.* (2011)	[74]
Serum/blood DNA								
APC			6 (3/49)	100 (0/41)	n	y [153]	Leung *et al.* (2005)	[76]
hMLH1			43 (21/49)	98 (1/41)	n	n	Leung *et al.* (2005)	[76]
HLTF			34 (17/49)	98 (1/41)	y [152]	n	Leung *et al.* (2005)	[76]
ALX4			83 (25/30)	70 (9/30)	n	n	Ebert *et al.* (2006)	[77]
TMEFF2			65 (87/133)	69 (56/179)	n	n	Lofton-Day *et al.* (2008)	[78]
NGFR			51 (68/133)	84 (29/179)	n	n	Lofton-Day *et al.* (2008)	[78]
SEPT9			69 (92/133)	86 (25/179)	n	n	Lofton-Day *et al.* (2008)	[78]
			72(24/33)	90 (3/33)			Tänzer *et al.* (2010)	[82]
		14 (3/22)	60 (18/30)	89 (5/49)			Ahlquist *et al.* (2012)	[85]
			90 (45/50	88 (11/94)			Warren *et al.* (2011)	[83]
NEUROG1			52 (14/27)‡‡	91 (4/45)	n	n	Herbst *et al.* (2011)	[79]
			64 (45/70)§§					
SFRP2	2 (1/46)	6 (4/63)	67 (113/169)	94 (4/63)	n	n	Tang *et al.* (2011)	[84]

†*Sensitivity was determined in fecal or serum samples from patients with CRC, colorectal adenomas or hyperplastic polyps. Specificity was determined in healthy control subjects or persons with a negative colonoscopy assessment.*
‡*Combination marker test utilizing methylated vimentin in combination with DNA integrity assay.*
§*Combination marker test including mutant* KRAS, *the* β*-actin gene and quantity of hemoglobin (by the porphyrin method).*
¶*Stage I–III CRC.*
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††*Adenomas <1 cm.*
‡‡*Stage I CRC.*
§§*Stage II CRC.*
CRC: Colorectal cancer; FIT: Fecal immunochemical test; n: Not validated; y: Validated.

Table 2. Summary of methylation markers with prognostic significance for colorectal cancer patients.

Gene	Tissue	Methylation, % (n)	Prognostic significance	Functional evidence in CRC		Author (year)	Ref.
				In vitro	*In vivo*		
ID4	Cell lines	100 (3/3)	Tumor grade and poor OS	n	n	Umetani *et al.* (2004)	[99]
	Adenoma	0 (0/13)				Umetani *et al.* (2004)	[99]
	Primary tumor	53 (49/92)				Umetani *et al.* (2004)	[99]
	Liver metastasis	73 (19/26)				Umetani *et al.* (2004)	[99]
MGMT	Primary tumor	43 (20/47)	Lower aggressiveness	n	n	Krtolica *et al.* (2007)	[101]
p16	Primary tumor	51 (24/47)	Lower aggressiveness	n	n	Krtolica *et al.* (2007)	[101]
PTPRD	Cell lines	50 (3/6)		n	n	Chan *et al.* (2008)	[11]
	Primary tumor	50 (10/20)	Metastasis			Chan *et al.* (2008)	[11]
	Primary tumor†	76 (38/51)	Gender and			Mokarram *et al.* (2009)	[107]
	Primary tumor†	76 (38/51)	MSI-H tumors			Mokarram *et al.* (2009)	[107]
	Primary tumor	50 (10/20)	n.a.			Yi *et al.* (2011)	[108]
RET	Cell lines	50 (3/6)		n	n	Chan *et al.* (2008)	[11]
	Primary tumor	11 (2/20)	Metastasis			Chan *et al.* (2008)	[11]
	Primary tumor†	37 (19/51)	n.a.			Mokarram *et al.* (2009)	[107]
	Primary tumor†	41 (21/51)	n.a.			Mokarram *et al.* (2009)	[107]
	Primary tumor	11(2/20)	n.a.	n	n	Yi *et al.* (2011)	[108]
CHFR	Primary tumor‡	63 (52/82)	Worse OS/RFS			Tanaka *et al.* (2011)	[100]
IGFBP3	Primary tumor	25 (5/20)	Worse OS	n	n	Yi *et al.* (2011)	[108]
EVL	Primary tumor	60 (12/20)	Worse OS	n	n	Yi *et al.* (2011)	[108]
CD109	Primary tumor	33 (7/20)	Worse OS§	n	n	Yi *et al.* (2011)	[108]
FLNC	Primary tumor	30 (6/20)	Worse OS§	n	n	Yi *et al.* (2011)	[108]

†*Primary tissue from all cancer stages.*
‡*Primary tissue from stage II and III.*
§*Simultaneous methylation of* IGFBP3, EVL, CD109 *and* FLNC *was associated with a worse survival in stage II CRCs.*
CRC: Colorectal cancer; MSI-H: Microsatellite instability-high; n: Not validated; n.a.: No statistically significant association found; OS: Overall survival; RFS: Recurrence-free survival; y: Validated.

CRC precursor lesions [52–54]. Therefore, novel sensitive and specific noninvasive biomarkers for the early detection of CRC are of great interest. Recent advances in genomics and proteomics have expanded the opportunities to detect novel biomarkers and offer new approaches for cancer screening. Over recent years, several DNA methylation markers have been proposed as useful early detection markers for CRC (Table 1). Bosch *et al.* have recently reviewed molecular biomarkers and methylation markers for the early detection of CRC [55]. Promoter CpG island methylation of vimentin has emerged as a potential marker for early detection of CRC. Aberrant vimentin methylation was detected in fecal DNA from CRC patients, but rarely in fecal DNA of normal control patients [56]. Three other independent studies confirmed these findings [57–59]. Itzkowitz *et al.* were able to increase sensitivitiy and specificity by combining a DNA integrity assay (DIA) with the detection of methylated vimentin. Sensitivity and specificity of methylated vimentin in fecal DNA of 40 CRC patients and 122 normal control subjects were 73 and 87%, respectively, whereas sensitivity and specificity in combination with DIA were 88 and 82%, respectively [58]. In an independent validation study, the same group obtained similar results (sensitivity: 86%; specificity: 73%) with a simplified and improved DIA [57]. Combination analysis of mutations in the *KRAS* and *APC* genes and vimentin methylation revealed a sensitivity of 58% in fecal DNA of 19 CRC patients and a specificity of 84% in feces of 75 normal subjects [60]. Analysis of combined methylation of *MGMT*, *MLH1* and vimentin in fecal DNA resulted in a sensitivity of 75% in 60 CRC patients and 60% in 52 patients bearing an adenoma, whereas the specificity was 86% in 37 control subjects. Vimentin is currently the only fecal DNA test, which is commercially available in the USA under the name ColoSure™. ColoSure is a single-marker test that detects methylated vimentin. The at-home test requires that patients collect and mail one whole stool sample. However, the analytic

sensitivity and specificity of the ColoSure test are not yet known [61].

The *SFRP* genes were also studied as novel early detection markers in fecal DNA. Three independent studies identified *SFRP2* promoter CpG island DNA methylation as potential early detection marker in fecal DNA of patients with CRC with a sensitivity ranging from 77 to 94% and a specificity ranging from 77 to 93% [62,63]. Furthermore, Oberwalder *et al.* found that the methylated *SFRP2* promoter CpG island can also be detected in feces of patients with colorectal adenomas and hyperplastic polyps [64]. A more recent study by Nagasaka *et al.* investigated methylation of *SFRP2* and *RASSF2* in fecal DNA obtained from CRC patients. *SFRP2* had a sensitivity of 63% and a specificity of 92% in 84 CRC patients and 113 control subjects, respectively. *RASSF2* was detected in 45% of the CRC patients and in 5% of the control subjects [65]. Promoter CpG island methylation of *SFRP1* has also been extensively studied as a methylation marker in CRC tissue. Yet, only one group studied the methylation status of *SFRP1* in fecal DNA resulting in a sensitivity of 84% and specificity of 86% in 19 patients with CRC and in 14 healthy control subjects, respectively. In addition, the *SFRP1* promoter was found to be methylated in all seven tested adenoma patients [66]. A high specificity of the *HIC1* gene methylation was also seen in a study with fecal DNA of CRC and colorectal adenoma patients. Nonetheless, in both the study populations, the sensitivity was relative low with 42% of 26 CRC patients and 31% in 13 adenoma patients. However, specificity of *HIC1* was 100% in 32 control subjects [67].

Other reported methylation markers in fecal DNA include the *CDKN2A*, *MGMT* and *MLH1*. Promoter CpG methylation frequencies observed for these genes are 31, 48 and 0% of 29 individuals with adenomas and in 16, 27 and 10% of individuals with no detectable polyps. However combination of these markers obtained sensitivity of 55% in 29 patients with adenomas and 40% in ten patients with hyperplastic polyps. Thereby, specificity was relatively high (72%) in fecal DNA from 25 healthy individuals [68]. The performance of a combination of methylation markers, namely *ATM, APC, HLTF*, *GSTP1*, *MGMT, MLH1* and *SFRP2* were tested by Leung *et al.* They observed a sensitivity of 75% in fecal DNA from 20 CRC patients, whereas the sensitivity for detected methylation in fecal DNA of 30 patients with adenomas was 68%. The specificity in 30 healthy controls was 90% [69]. We recently described the *NDRG4* gene promoter CpG island methylation as a promising early detection marker in fecal DNA in two independent series, yielding a sensitivity of 53–61% (of 47 and 28 CRC patients, respectively) and a specificity of 93–100% (of 45 and 30 control subjects, respectively) [70]. In addition, we identified the transcription factor *GATA4* to be a novel biomarker for the detection of CRC. *GATA4* methylation could be used as an early detection marker for CRC with a sensitivity of 51–71% found in 28 and 47 CRC patients, and a specificity from 84–93%, obtained in 45 and 30 control subjects, respectively [71]. Glöckner *et al.* reported promoter CpG island methylation of *TFPI2* in three independent populations of CRC patients (n = 11, 26, 47) and healthy controls (n = 12, 45, 30) and found a sensitivity of 73–89% and specificity of 79–100%. *TFPI2* DNA promoter methylation was also studied in fecal DNA of patients with colorectal adenomas (n = 7, 19) and resulted in a sensitivity of 21–43% and a specificity of 93–100% [72]. The *OSMR* has been found to be methylated in fecal DNA of 69 CRC patients with a sensitivity of 38% and a specificity of 95% in fecal DNA of 81 control samples [73]. Bosch *et al.* selected *PHACTR3* from 18 candidate genes, as a possible early detection methylation marker in fecal DNA, yielding a sensitivity of 55–66% (of 22 and 44 CRC patients, respectively), a sensitivity of 32% of 19 patients with advanced adenomas and a specificity of 95–100% in 78 and 30 control subjects. Combination of *PHACTR3* DNA methylation with the widely used fecal immunochemical test (FIT) resulted in an overall increase in sensitivity in patients with adenomas (33% of 24 patients) and in patients with CRC (95% of 20 patients). Specificity in 48 control subjects was 94% [74]. Recently, Ahlquist *et al.* perfomed a large multicenter study incorporating a panel of methylation markers, namely vimentin, *NDRG4*, *TFPI2* and the *BMP* gene, in combination with *KRAS* mutations, β-actin as a reference gene, and hemoglobin. A sensitivity of 85% in fecal DNA of 252 CRC patients and 63% of 133 patients with adenomas (>1 cm) and a specificity of 90% in 293 controls was achieved by quantitative allele-specific real-time target and signal amplification technique [75].

As a blood-based test for CRC detection, not depending on stool collection, could have the potential for better patient compliance, many groups are nowadays focusing on the potential of DNA methylation markers in blood DNA. Leung *et al.* analyzed methylation of

APC, *MLH1* and *HLTF* in serum samples from 49 patients with CRC and reported a sensitivity of 6, 43 and 34%, respectively and a specificity of 98, 98 and 100%, respectively in 41 control samples [76]. Ebert *et al.* found that *ALX4* was frequently methylated in serum of patients with different gastrointestinal cancers, with a sensitivity of 83% in 30 colon cancer patients and a specificity of 70% in 30 control subjects [77]. *TMEFF2*, *NGFR* and *SEPT9* were selected out of 56 CAN genes as possible markers for the early detection of CRC in blood DNA [78]. This panel had a sensitivity of 56, 51 and 69%, respectively, whereas specificities were 69, 84 and 86% in plasma DNA of 133 CRC patients and 179 control patients, respectively [78]. Recently, *NEUROG1* has been identified as a possible diagnostic methylation marker in serum DNA. By detecting methylated *NEUROG1,* Herbst *et al.* were able to distinguish between patients with stage 1 CRC (n = 27) and stage 2 CRC patients (n = 70), with sensitivities of 52 and 64%, respectively. Specificity in 45 control subjects was 91% [79]. *SEPT9* has been studied as potential early detection marker in serum for CRC [80–82]. Recently, Warren *et al.* has found a sensitivity of 90% in 50 serum samples of CRC patients and a specificity of 88% in 94 healthy control samples [83].

As described above, multiple studies have reported methylation markers as tools to screen for CRC in fecal or serum DNA. However, whether methylation markers in fecal or serum DNA are more favorable to screen for CRC is still a matter of debate. Tang *et al.* investigated *SFRP2* methylation in fecal, as well as serum DNA of patients bearing nonadenomatous polyps (n = 46), adenomas (n = 63) or CRC (n = 169). Sensitivity of methylated *SFRP2* in fecal DNA was higher in all patient groups (33% in patients with polyps; 46% in patients with adenomas; 84% in patients with CRC) compared with sensitivity of *SFRP2* in serum DNA (2% in patients with polyps; 6% in patients with adenomas; and 67% in patients with CRC). However, specificity of methylated *SFRP2* was higher in serum DNA of 63 control subjects (94%), than in their fecal DNA (54%) [84]. In another study, the sensitivity of a multimarker test in fecal DNA was compared with methylated *SEPT9* in serum. A recent study by Ahlquist *et al.* showed that a combination of methylation markers (*BMP3, NDRG4,* vimentin and *TFPI2*) with mutated *KRAS*, the β-actin gene and quantity of hemoglobin, had higher sensitivities in stool samples compared with methylated *SEPT9* in their matched serum samples of 22 patients with adenomas (82 and 14%, respectively) and 30 patients with CRC (87 and 60%, respectively). Specificity in both tests was relatively high, with 90% in the fecal DNA-based test (five out of 46) and 89% of methylated *SEPT9* in serum (five out of 49) [85].

Prognostic markers

The prognosis of CRC is influenced by many factors, which are both patient and tumor related [7–9,86]. Currently, the tumor–node–metastasis (TNM) system, an internationally accepted classification system describing the extent of infiltration of the tumor through the bowel wall, presence or absence of lymph node metastasis and metastasis beyond the affected specific bowel region, is the main tool to provide information on prognosis and to determine treatment protocols [87,88]. The presence of lymph node metastasis, within this TNM classification, currently remains the most significant prognostic factor. Specific histological factors, obtained from the resected colon specimen, have also proved to have an additional prognostic effect in specific TNM stages. These factors include histological grade, margins, histological type (including special subtypes), host immune response, tumor border configuration/budding, isolated tumor deposits, number of harvested lymph nodes, perineural invasion, lymphovascular invasion and morphological clues to MSI [5,89–92]. Nevertheless, this information cannot sufficiently predict the prognosis of single cancer cases [7,86,93].

The mainstay treatment of CRC is surgery and depending on the pathological stage (and specific histological and patient factors) adjuvant chemotherapy is administered [8]. Low-risk patients (stage 1 and low-risk stage 2) do not receive adjuvant therapy because it is thought that these patients have a good prognosis. Nevertheless, 20–30% of these patients will die within 5 years after diagnosis as a result of CRC [94]. In addition, there is considerable variation in the course of the disease and the treatment response within the same stage. Clinicians are often not able to accurately predict which patients are at high risk for recurrence and might benefit from chemotherapy. Prognostic markers identifying patients at risk of recurrence could be an improvement in the current patient management and support clinicians in the decision-making process of which patients should receive adjuvant treatment.

Over the last years, researchers have focused on identifying prognostic biomarkers within

primary tumor tissue. The carcinoembryonic antigen (CEA) is one of the earliest studied biomarkers in CRC for its potential as a prognostic biomarker [95] and postoperative CEA has indeed been shown to be a prognostic factor after resection of colorectal liver metastases [96]. A meta-analysis revealed that CEA is highly specific, but lacks sensitivity to predict CRC recurrence [97]. In addition, for patients with stage 2 CRC, the Oncotype DX® Colon Cancer assay (Genomic Health, Inc) is now commercially available as a prognostic marker test, and the ColoPrint assay (Agendia BV) is currently in the validation phase [98]. Both tests make use of gene-expression profiling, enabling the identification of differentially expressed genes in CRC patients with distinct clinical outcomes. As it remains to be observed how the Oncotype DX Colon Cancer assay and the ColoPrint assay will be implemented in daily clinical practice, the search for novel biomarkers improving prognosis of stage 2 CRC patients is still essential.

Methylation has also been suggested to play a role in determining CRC prognosis. We recently summarized the most promising findings of genetic and epigenetic alterations as prognostic and predictive methylation markers for CRC [12]. Umetani *et al.* found that *ID4* is frequently methylated in CRC and methylation was associated with tumor grade and a poorer overall survival [99]. However, this finding could not be replicated by another study [100]. Simultaneous methylation of *p16* and *MGMT* was statistically significantly associated with a lower aggressiveness of the tumor. Only 27% of 11 patients with simultaneous *p16* and *MGMT* methylation showed a detectable occurrence of metastasis and/or death, compared with 67% of 33 of patients without simultaneous methylation [101]. Previous studies on *MGMT* methylation have suggested the suitability of *MGMT* as a prognostic marker in CRC, however the prognostic significance was inconsistent [102–105]. It has been reported that loss of *MGMT* is associated with MSI low CRCs, which are known to have a poorer prognosis [102,106].

Combination studies of microarray data and methylation characteristics of eight CAN genes with decreased expression revealed that *RET* and *PTPRD* gene methylation were associated with clinical characteristics, such as metastasis [11] but this was not replicated in other studies [107,108]. Promoter CpG methylation of *CHFR* was also found to be associated with survival and was claimed to be an independent predictor for tumor recurrence [100]. Yi *et al.* identified *IGFBP3* and *EVL* out of six extracellular matrix pathway genes with prognostic potential for CRC [108]. Methylation of these genes was significantly associated with worse survival in three series (n = 147, 72 and 558). Moreover, simultaneous DNA methylation of *IGFBP3* and *CD109* was associated with worse survival for stage 2 CRCs. The methylation status of two global DNA methylation markers (LINE-1 and Alu) and nine loci (*MINT1, MINT2* and *MINT31, p16, hMLH1, p14, SFRP1, SFRP2* and *WNT5A*) was studied in tissue samples from sporadic CRC to describe the role of CIMP in predicting recurrence and disease-free survival in resected stage 3 CRC. The study revealed that DNA methylation is a useful biomarker of recurrence in CRC [109]. In addition, Ward *et al.* showed that DNA methylation in CIMP-associated genes was related to a poor outcome in CRC, but this prognostic effect was lost in methylated tumors with MSI [110]. By defining three subgroups (no-CIMP, CIMP-low and CIMP-high) in 582 colon adenocarcinoma patients, Barault *et al.* have found a shorter 5-year survival time in microsatellite-stable patients with CIMP-low or CIMP-high status. No difference in survival was observed between the three CIMP groups in MSI colon cancer patients [111].

Predictive markers

Next to answering the question of which patients should be treated, it is also still unclear why some patients respond to therapy whereas others do not. A total of 30–40% of patients receiving adjuvant treatment after tumor resection do not benefit from standard therapy (5-fluoruoracil [5-FU] ± oxaliplatin or irinotecan) and suffer from potentially harmful side effects instead [112–114]. Currently, it is hard to accurately predict which CRC patients will benefit from adjuvant chemotherapy. As adjuvant cancer therapy imposes a huge burden on patient as well as society and healthcare system, it is essential to identify patients who will benefit from adjuvant therapy, sparing others needless toxicity and the financial burden of chemotherapy that will not work [115,116].

Currently, only mutational status of *KRAS* is used as a biomarker in CRC therapy decision-making but even this biomarker cannot accurately predict which patients will respond to treatment [117–119]. MSI status in CRC has been associated with 5-FU nonresponse in an adjuvant setting [120,121]. Two studies have reported, that the combination of 5-FU with irinotecan might be beneficial for CRC patients bearing

MSI [122,123]. However, MSI status as a predictor for treatment response remains controversial and inconclusive [124]. It has been reported from several studies, that CIMP may be of prognostic as well as predictive importance for CRC patients. Ogino *et al.* studied the relationship between CpG island methylation and the clinical outcome in 30 CRC patients with a CIMP panel according to Weisenberger [39]. The presence of CIMP (either ≥9/13 or ≥7/13 methylated markers) in CRC patients was associated with a worse prognosis [12,125]. A correlation between the presence of CIMP and treatment effects of 5-FU are contradictory [125,126]. Contradictory results are mainly due to a not standardized definition of CIMP. Nevertheless, a recent study by Jover *et al.* has shown that CIMP positive CRCs, defined by the Weisenberger panel, do not benefit from 5-FU-based adjuvant chemotherapy. CIMP positive, stage 2–3 CRC patients, who did not receive adjuvant 5-FU-based chemotherapy, had longest disease-free survival, whereas CIMP positive CRC patients treated with adjuvant chemotherapy showed shorter time of disease-free survival [127]. Thus, CIMP as a predictive factor needs still to be investigated. Hypermethylation of the *MGMT* promoter might also be useful as a predictive marker in CRC. It has been reported that loss of *MGMT* function through DNA methylation is thought to sensitize cells to the effects of alkylating agent-based chemotherapy [128]. MGMT is able to remove mutagenic and cytotoxic adducts from O^6-guanine in DNA [129]. Thus, *MGMT* expression could predict sensitivity or resistance to alkylating agents. Promoter CpG methylation of the *WRN* gene has been associated with longer survival of 45 CRC patients treated with irinotecan [130]. In addition, *WRN* hypermethylation seems to be associated with mucinous differentiation in CRC, independent of MSI status and CIMP [131].

Although many claims have been made on the possible roles of numerous genes, all these possible methylation markers for CRC need to be validated in larger cohorts before they are applicable for clinical use. Many results seem to be promising, but the numbers of samples within the study populations were relatively low in almost every study. This is most likely due to limited access to tissue samples. Moreover, as for all CRC methylation markers, studies are often not replicated using similar methodologies resulting in conflicting results. In the following section, we summarize suggestions for a better identification of novel epigenetic biomarkers.

Considerations to epigenetic biomarker development

The last decade has yielded an extensive map of aberrant DNA methylation events in cancer cells, particularly for the hypermethylated CpG islands of tumor suppressor genes [15,20]. DNA methylation plays a well-established role in cancer and is relatively stable and easy to detect [15]. In addition, as discussed in this review, CpG island hypermethylation can be used as a tool to detect cancer cells in several types of biological fluids and tissue biopsies [132,133] and many studies started using this easily accessible biological material in translational and clinical settings [134]. New powerful techniques can now even detect minimal amounts of aberrant DNA methylation in tissue, blood or other biological materials, [135] indicating the promise of tumor specific methylation patterns for the diagnosis, prognosis, and prediction of therapy outcome in cancer patients.

Current epigenetic biomarker development is mainly based on a candidate marker approach. Within this approach, after initial identification, potential candidate markers are first tested in cell lines and/or in a small set of patient samples to determine the abundance of these markers in specific tissues. Next, candidate markers are tested in a larger patient population to quantify the results and to assess reproducibility. Finally, if necessary, the procedure of the assay is optimized [136]. However, these candidate marker approaches are prone to overlook possible relevant biomarkers as the initial candidate selection might not have contained the best candidate gene.

Genome-wide approaches, such as high-throughput sequencing methods [137], can potentially revolutionize the diagnosis and treatment of cancer as the unbiased nature of the technique reduces the possibility to overlook promising biomarkers. Whole-genome profiling technologies facilitate the assessment of differential methylation aberrations on a whole-genome scale with a locus-specific resolution. These techniques have enabled the integration of epigenetic data with genomic profiles, improving our ability to study genetic and epigenetic alterations in cancer and to select possible biomarkers. These approaches are likely to discover disease-specific epigenetic alterations within new genomic regions, including those that are located within well-known candidate regions such as CpG islands or promoter regions [13]. However, this advancement in biomarker development also gives rise to new challenges, such as the correct

interpretation of large amounts of data and the implementation of multiple-testing statistical procedures. Recently, a systematic approach to biomarker development has been described to overcome many of these challenges [13]. This systematic approach consists of three key goals: to maximize the genomic coverage in the early stages of the search by using genome-wide experimental methods, for example, the use of enzymes sensitive to cytosine methylation [138]; to utilize computational methods for identifying and optimizing a small number of promising candidate biomarkers; and to validate selected biomarkers in large cohorts using highly targeted assays [13,139]. Hereafter, genomic regions can be selected as possible biomarkers, which still need to be tested at their specific CpG sites of interest. To date, a number of experimental approaches to detect DNA methylation have been developed and optimized. Methylation-specific PCR [140] and MethyLight [141] are able to detect the methylation status of several CpGs, whereas bisulphite pyrosequencing [142], methylation-sensitive single nucleotide primer extension [143], combined bisulphite restriction analysis [144] and mass spectrometry [145] provide information of quantitative DNA methylation at individual CpGs.

As transcriptional silencing does not require hypermethylation of the entire CpG island, identifiying the core regions regulating gene expression is essential for evaluation of the clinical value of DNA hypermethylation. For the majority of known methylation markers, the exact location of biologically and clinically relevant hypermethylation has not yet been identified [146]. Although promoter CpG islands are often located around the transcription start site they can also be found more upstream or downstream of the transcription start site. To assess the methylation status of a gene it is crucial to design appropriate primers for the biologically and/or clinically relevant region. If the core region containing clinically relevant methylation is identified, corresponding assays should be designed according to standardized procedures, as results can often not be compared due to the use of different assays [6,146]. Optimal primer design is critical to obtain reliable results. Although clinically relevant hypermethylation of a specific locus is not always associated with gene expression and might serve as a surrogate marker for functional hypermethylation of another locus, we expect that the best validated markers will be those for which good correlations between DNA methylation and gene expression exists.

Although these new technologies and new insights have enabled scientists to identify several promising biomarkers, the translation of basic research to clinical practice is a multistep process, which takes many years. Clinical translation of biomarkers is often difficult as biomarker identification studies suffer from problems, such as selection of the study design, interpretation of data and statistical issues. Furthermore, a biomarker has to fulfill particular requirements. For almost every biomarker, noninvasiveness, easy detection, stability within a particular cancer type, validation in a large population and cost–effectiveness are important requirements to become widely accepted in the clinic [147–151]. In addition, before a biomarker can be suitable for implementation in clinical practice, the biological role of the biomarker also needs to be elucidated using both *in vitro* and *in vivo* experiments. Not fulfilling one of these requirements hampers the introduction of the marker in the clinic. We recently described that available evidence on potential prognostic or predictive biomarkers is too scarce and many questions remain unanswered [12]. Current methylation biomarker discovery is often limited to the detection of aberrant DNA methylation patterns in cases and controls (early detection markers) or in groups of cases (prognostic and predictive markers). Information on the biological role of a potential biomarker could aid the selection of relevant methylation markers. A marker may be extremely powerful in the early detection of CRC or to predict treatment outcome but often we poorly understand its biology and its role in pathogenesis. If methylation of a biomarker is related to its biological function, for example through promoter silencing, the process of pathogenesis may become more coherent and would therefore make the biomarker more likely to be a true biomarker. In addition, understanding the biological function of a biomarker or the underlying pathway may help to select additional biomarkers thereby increasing sensitivity and specificity. A recent meta-analysis on early detection methylation markers in fecal DNA revealed, that these markers are currently not accurate enough to be used alone [152]. Moreover, research on biomarkers is susceptible to publication bias and false-positive results, due to small population and effect sizes and initial studies often show promising results that cannot be replicated in later studies [153–155]. Studying the biological role of a biomarker could lead to a better understanding of these contradictory results. To date, only a few studies have validated the biological role of possible methylation markers in CRC (**Tables 1 & 2**) [70,71,156–159].

Most markers currently do not reach a sufficient level of evidence to be recommended in clinical practice [116,160]. The major challenge in the identification and development of epigenetic biomarkers will be the integration of all the above-mentioned requirements, accounting for possible issues with techniques or data interpretation and its functional interpretation in conjunction with clinical outcomes and epidemiology. Therefore, we suggest that biomarker discovery requires close collaboration between researchers with expertise in statistics, bioinformatics, molecular biology and clinical medicine.

Conclusion & future perspective

In the past decade, a number of potential biomarkers for early detection, prognosis and prediction for CRC have been identified. Despite a great need for biomarkers, the use of methylation markers in the clinic is limited. The main reason is a lack of validating data of these potential methylation markers. New genome-wide technologies and data from large-scale

Executive summary

Background

- Despite increasing knowledge on colorectal cancer (CRC), the disease remains a major health burden with over 1 million cases worldwide and a disease specific mortality of approximately 50% in the developed world.
- Multiple epigenetic changes have been identified, which alone or in interplay with other alterations drive tumorigenesis and CRC progression.
- Promoter CpG island methylation is the most studied and best characterized epigenetic alteration in CRC.

Promoter CpG island methylation in CRC

- DNA methylation is the postreplicative addition of a methyl group to the carbon 5-position of the cytosine, a process that can lead to gene silencing.
- Promoter CpG island methylation of tumor suppressor genes is recognized as a potent and prevalent way for inactivation of tumor suppressor genes.
- The recognition that a distinct subset of CRCs display significantly more promoter methylation than others has led to the introduction of the concept of CpG island methylator phenotype (CIMP).
- Specific pathological, clinical and molecular features, such as older age, female gender, poor tumor differentiation, proximal location of the tumor, *BRAF* mutations, *KRAS* mutations and wild-type *TP53* distinctly characterize CIMP-positive CRCs.

Promoter CpG island methylation biomarkers in CRC: the current status

- No clinically established biomarker for early detection, prognosis or prediction of treatment response is available yet.
- Methylation markers in fecal/serum/blood DNA are of great interest for the early detection of CRC and extensive studies have led to the commercially available ColoSure™ test. However, most identified biomarkers lack validation and often inconsistent results have been obtained.
- The tumor–node–metastasis (TNM) system, an internationally accepted classification system, is the main tool to provide information on prognosis and to determine treatment protocols, but lacks precision.
- Prognostic markers identifying patients at risk of recurrence could be an improvement in the current patient management and support clinicians in the decision-making process of which patients should receive adjuvant treatment.
- CIMP may be of prognostic as well as predictive importance for CRC patients. It has been reported that CIMP was associated with a poor prognosis in CRC patients. However, CIMP as a predictive factor still needs to be investigated.
- Several promising methylation markers predicting the prognosis and treatment response of CRC patients have been studied. Nevertheless, study populations were often low and results could not be replicated.

Considerations to epigenetic biomarker development

- Epigenetic biomarker development is challenging and often suffers from a lack of utilization of resources.
- Epigenetic biomarker development requires expertise on biomarker characteristics, an appropriate technique to identify a reliable biomarker, a technique which is applicable in the daily clinical use, expertise in analyzing and interpreting the results and expertise on the biological function of the biomarker itself.
- For the implementation of an epigenetic biomarker, clinical and biological validation is highly recommended.

Conclusion & future perspective

- Validation of potential biomarkers in multiple population-based screening studies, cohort studies and randomized clinical trials is required to assess the clinical value and significance of a biomarker.
- Combinations of epigenetic markers and epigenetic markers combined with molecular (e.g., gene or protein) markers or well-established methods (e.g., TNM or fecal immunochemical test) may increase clinical applicability.
- Knowledge on the biological function of a biomarker might be important to be taken into account in a systematic approach to ensure optimal assessment of the biomarker.

genome-wide studies might help uncover the clinically relevant relationships between genetics, epigenetics and tumor behavior of biomarkers. Validation of potential biomarkers in multiple population-based screening studies, cohort studies and randomized clinical trials is required to assess the clinical value and significance of a biomarker. The sensitivity and specificity of a methylation marker may vary in different studies. Therefore, studying panels of markers or combinations of methylation markers with well established CRC markers or current screening methods, will be the subject of future CRC management. Establishing a sufficient level of evidence is necessary to make epigenetic biomarkers applicable in daily clinical procedures. One of the major challenges in the implementation of novel biomarkers is the interdependency of the discovery of the biomarker and the clinical testing of the corresponding treatment and these can only move forward in parallel. Therefore, knowledge on the biological function of a biomarker should be taken into account in a systematic approach of biomarker development.

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References

Papers of special note have been highlighted as:
▪ of interest
▪▪ of considerable interest

1 Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer statistics, 2009. *CA Cancer J. Clin.* 59(4), 225–249 (2009).

2 Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int. J. Cancer* 127(12), 2893–2917 (2010).

3 Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J. Clin.* 61(2), 69–90 (2011).

4 Morris EJ, Maughan NJ, Forman D, Quirke P. Who to treat with adjuvant therapy in Dukes B/stage II colorectal cancer? The need for high quality pathology. *Gut* 56(10), 1419–1425 (2007).

5 Jass JR. Classification of colorectal cancer based on correlation of clinical, morphological and molecular features. *Histopathology* 50(1), 113–130 (2007).

6 Hughes LA, Khalid-De Bakker CA, Smits KM *et al.* The CpG island methylator phenotype in colorectal cancer: progress and problems. *Biochim. Biophys. Acta* 1825(1), 77–85 (2012).

▪ **Systematic review on the CpG island methylator phenotype.**

7 George B, Kopetz S. Predictive and prognostic markers in colorectal cancer. *Curr. Oncol. Rep.* 13(3), 206–215 (2011).

8 Mutch MG. Molecular profiling and risk stratification of adenocarcinoma of the colon. *J. Surg. Oncol.* 96(8), 693–703 (2007).

9 Walther A, Johnstone E, Swanton C, Midgley R, Tomlinson I, Kerr D. Genetic prognostic and predictive markers in colorectal cancer. *Nat. Rev. Cancer* 9(7), 489–499 (2009).

10 Kondo Y, Issa JP. Epigenetic changes in colorectal cancer. *Cancer Metastasis Rev.* 23(1–2), 29–39 (2004).

11 Chan TA, Glockner S, Yi JM *et al.* Convergence of mutation and epigenetic alterations identifies common genes in cancer that predict for poor prognosis. *PLoS Med.* 5(5), e114 (2008).

12 Smits KM, Cleven a H, Weijenberg MP *et al.* Pharmacoepigenomics in colorectal cancer: a step forward in predicting prognosis and treatment response. *Pharmacogenomics* 9(12), 1903–1916 (2008).

13 Bock C. Epigenetic biomarker development. *Epigenomics* 1(1), 99–110 (2009).

▪▪ **Review outlining a systematic approach to epigenetic biomarker development.**

14 Esteller M, Fraga MF, Guo M *et al.* DNA methylation patterns in hereditary human cancers mimic sporadic tumorigenesis. *Hum. Mol. Genet.* 10(26), 3001–3007 (2001).

15 Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. *N. Engl. J. Med.* 349(21), 2042–2054 (2003).

16 Van Engeland M, Derks S, Smits KM, Meijer GA, Herman JG. Colorectal cancer epigenetics: complex simplicity. *J. Clin. Oncol.* 29(10), 1382–1391 (2011).

▪ **Review providing a comprehensive overview on colorectal cancer (CRC) epigenetics.**

17 Takai D, Jones PA. Comprehensive analysis of CpG islands in human chromosomes 21 and 22. *Proc. Natl Acad. Sci. USA* 99(6), 3740–3745 (2002).

18 Vucic EA, Brown CJ, Lam WL. Epigenetics of cancer progression. *Pharmacogenomics* 9(2), 215–234 (2008).

19 Okano M, Bell DW, Haber DA, Li E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for *de novo* methylation and mammalian development. *Cell* 99(3), 247–257 (1999).

20 Esteller M. Epigenetic gene silencing in cancer: the DNA hypermethylome. *Hum. Mol. Genet.* 16(Spec No 1), R50–R59 (2007).

21 Jost JP, Bruhat A. The formation of DNA methylation patterns and the silencing of genes. *Prog. Nucleic Acid Res. Mol. Biol.* 57, 217–248 (1997).

22 Kim H, Park J, Jung Y *et al.* DNA methyltransferase 3-like affects promoter methylation of thymine DNA glycosylase independently of DNMT1 and DNMT3B in cancer cells. *Int. J. Oncol.* 36(6), 1563–1572 (2010).

23 Turker MS, Bestor TH. Formation of methylation patterns in the mammalian genome. *Mutat. Res.* 386(2), 119–130 (1997).

24 Merlo A, Herman JG, Mao L *et al.* 5′ CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. *Nat. Med.* 1(7), 686–692 (1995).

25 Baylin SB, Herman JG, Graff JR, Vertino PM, Issa JP. Alterations in DNA methylation: a fundamental aspect of neoplasia. *Adv. Cancer Res.* 72, 141–196 (1998).

26 Bird A. The essentials of DNA methylation. *Cell* 70(1), 5–8 (1992).

27 Costello JF, Fruhwald MC, Smiraglia DJ *et al.* Aberrant CpG-island methylation has non-random and tumour-type-specific patterns. *Nat. Genet.* 24(2), 132–138 (2000).

28 Esteller M. CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. *Oncogene* 21(35), 5427–5440 (2002).

29 Irizarry RA, Ladd-Acosta C, Wen B *et al.* The human colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores. *Nat. Genet.* 41(2), 178–186 (2009).

30 Doi A, Park IH, Wen B *et al.* Differential methylation of tissue- and cancer-specific CpG island shores distinguishes human induced pluripotent stem cells, embryonic stem cells and fibroblasts. *Nat. Genet.* 41(12), 1350–1353 (2009).

31 Lopez-Serra L, Esteller M. Proteins that bind methylated DNA and human cancer: reading the wrong words. *Br. J. Cancer* 98(12), 1881–1885 (2008).

32 Kuroda A, Rauch TA, Todorov I *et al.* Insulin gene expression is regulated by DNA methylation. *PLoS ONE* 4(9), e6953 (2009).

33 Hoque MO, Kim MS, Ostrow KL *et al.* Genome-wide promoter analysis uncovers portions of the cancer methylome. *Cancer Res.* 68(8), 2661–2670 (2008).

34 Momparler RL. Cancer epigenetics. *Oncogene* 22(42), 6479–6483 (2003).

35 Sharma S, Kelly TK, Jones PA. Epigenetics in cancer. *Carcinogenesis* 31(1), 27–36 (2010).

36 Issa JP. CpG island methylator phenotype in cancer. *Nat. Rev. Cancer* 4(12), 988–993 (2004).

37 Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB, Issa JP. CpG island methylator phenotype in colorectal cancer. *Proc. Natl Acad. Sci. USA* 96(15), 8681–8686 (1999).

38 Tanaka N, Huttenhower C, Nosho K *et al.* Novel application of structural equation modeling to correlation structure analysis of CpG island methylation in colorectal cancer. *Am. J. Pathol.* 177(6), 2731–2740 (2010).

39 Weisenberger DJ, Siegmund KD, Campan M *et al.* CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. *Nat. Genet.* 38(7), 787–793 (2006).

40 Park SJ, Rashid A, Lee JH, Kim SG, Hamilton SR, Wu TT. Frequent CpG island methylation in serrated adenomas of the colorectum. *Am. J. Pathol.* 162(3), 815–822 (2003).

41 Ogino S, Cantor M, Kawasaki T *et al.* CpG island methylator phenotype (CIMP) of colorectal cancer is best characterised by quantitative DNA methylation analysis and prospective cohort studies. *Gut* 55(7), 1000–1006 (2006).

42 Shen L, Toyota M, Kondo Y *et al.* Integrated genetic and epigenetic analysis identifies three different subclasses of colon cancer. *Proc. Natl Acad. Sci. USA* 104(47), 18654–18659 (2007).

43 Samowitz WS, Albertsen H, Herrick J *et al.* Evaluation of a large, population-based sample supports a CpG island methylator phenotype in colon cancer. *Gastroenterology* 129(3), 837–845 (2005).

44 Toyota M, Ohe-Toyota M, Ahuja N, Issa JP. Distinct genetic profiles in colorectal tumors with or without the CpG island methylator phenotype. *Proc. Natl Acad. Sci. USA* 97(2), 710–715 (2000).

45 Tainsky MA. Cancer biomarker discovery: speed-bumps and tire shredders. *Cancer Biomark.* 6(5–6), 225–227 (2010).

46 Issaq HJ, Waybright TJ, Veenstra TD. Cancer biomarker discovery: Opportunities and pitfalls in analytical methods. *Electrophoresis* 32(9), 967–975 (2011).

47 Crea F, Nobili S, Paolicchi E *et al.* Epigenetics and chemoresistance in colorectal cancer: an opportunity for treatment tailoring and novel therapeutic strategies. *Drug Resist. Updat.* 14(6), 280–296 (2011).

48 Naylor S. Biomarkers: current perspectives and future prospects. *Expert Rev. Mol. Diagn.* 3(5), 525–529 (2003).

49 Perera FP, Weinstein IB. Molecular epidemiology: recent advances and future directions. *Carcinogenesis* 21(3), 517–524 (2000).

50 Vineis P, Perera F. Molecular epidemiology and biomarkers in etiologic cancer research: the new in light of the old. *Cancer Epidemiol. Biomarkers Prev.* 16(10), 1954–1965 (2007).

51 Levin B, Lieberman DA, Mcfarland B *et al.* Screening and surveillance for the early detection of colorectal cancer and adenomatous polyps, 2008: a joint guideline from the American Cancer Society, the US Multi-Society Task Force on Colorectal Cancer, and the American College of Radiology. *Gastroenterology* 134(5), 1570–1595 (2008).

52 Gatto NM, Frucht H, Sundararajan V, Jacobson JS, Grann VR, Neugut AI. Risk of perforation after colonoscopy and sigmoidoscopy: a population-based study. *J. Natl Cancer Inst.* 95(3), 230–236 (2003).

53 Frazier a L, Colditz GA, Fuchs CS, Kuntz KM. Cost–effectiveness of screening for colorectal cancer in the general population. *JAMA* 284(15), 1954–1961 (2000).

54 Imperiale TF, Ransohoff DF, Itzkowitz SH, Turnbull BA, Ross ME. Fecal DNA versus fecal occult blood for colorectal-cancer screening in an average-risk population. *N. Engl. J. Med.* 351(26), 2704–2714 (2004).

55 Bosch LJ, Carvalho B, Fijneman RJ *et al.* Molecular tests for colorectal cancer screening. *Clin. Colorectal Cancer* 10(1), 8–23 (2011).

56 Chen WD, Han ZJ, Skoletsky J *et al.* Detection in fecal DNA of colon cancer-specific methylation of the nonexpressed vimentin gene. *J. Natl Cancer Inst.* 97(15), 1124–1132 (2005).

57 Itzkowitz S, Brand R, Jandorf L *et al.* A simplified, noninvasive stool DNA test for colorectal cancer detection. *Am. J. Gastroenterol.* 103(11), 2862–2870 (2008).

58 Itzkowitz SH, Jandorf L, Brand R *et al.* Improved fecal DNA test for colorectal cancer screening. *Clin. Gastroenterol. Hepatol.* 5(1), 111–117 (2007).

59 Li M, Chen WD, Papadopoulos N *et al.* Sensitive digital quantification of DNA methylation in clinical samples. *Nat. Biotechnol.* 27(9), 858–863 (2009).

60 Ahlquist DA, Skoletsky JE, Boynton KA *et al.* Colorectal cancer screening by detection of altered human DNA in stool: feasibility of a multitarget assay panel. *Gastroenterology* 119(5), 1219–1227 (2000).

61 Ned RM, Melillo S, Marrone M. Fecal DNA testing for colorectal cancer screening: the ColoSure™ test. *PLoS Curr.* 3, RRN1220 (2011).

62 Huang Z, Li L, Wang J. Hypermethylation of SFRP2 as a potential marker for stool-based detection of colorectal cancer and precancerous lesions. *Dig. Dis. Sci.* 52(9), 2287–2291 (2007).

63 Wang DR, Tang D. Hypermethylated *SFRP2* gene in fecal DNA is a high potential biomarker for colorectal cancer noninvasive screening. *World J. Gastroenterol.* 14(4), 524–531 (2008).

64 Oberwalder M, Zitt M, Wontner C *et al.* SFRP2 methylation in fecal DNA – a marker for colorectal polyps. *Int. J. Colorectal Dis.* 23(1), 15–19 (2008).

65 Nagasaka T, Tanaka N, Cullings HM *et al.* Analysis of fecal DNA methylation to detect gastrointestinal neoplasia. *J. Natl Cancer Inst.* 101(18), 1244–1258 (2009).

66 Zhang W, Bauer M, Croner RS *et al.* DNA stool test for colorectal cancer: hypermethylation of the secreted frizzled-related protein-1 gene. *Dis. Colon Rectum* 50(10), 1618–1626; discussion 1626–1617 (2007).

67 Lenhard K, Bommer GT, Asutay S *et al.* Analysis of promoter methylation in stool: a novel method for the detection of colorectal cancer. *Clin. Gastroenterol. Hepatol.* 3(2), 142–149 (2005).

68 Petko Z, Ghiassi M, Shuber A *et al.* Aberrantly methylated *CDKN2A*, *MGMT*, and *MLH1* in colon polyps and in fecal DNA from patients with colorectal polyps. *Clin. Cancer Res.* 11(3), 1203–1209 (2005).

69 Leung WK, To KF, Man EP *et al.* Detection of hypermethylated DNA or cyclooxygenase-2 messenger RNA in fecal samples of patients with colorectal cancer or polyps. *Am. J. Gastroenterol.* 102(5), 1070–1076 (2007).

70 Melotte V, Lentjes MH, Van Den Bosch SM *et al.* N-Myc downstream-regulated gene 4 (*NDRG4*): a candidate tumor suppressor gene and potential biomarker for colorectal cancer. *J. Natl Cancer Inst.* 101(13), 916–927 (2009).

71 Hellebrekers DM, Lentjes MH, Van Den Bosch SM *et al.* GATA4 and GATA5 are potential tumor suppressors and biomarkers in colorectal cancer. *Clin. Cancer Res.* 15(12), 3990–3997 (2009).

72 Glöckner SC, Dhir M, Yi JM *et al.* Methylation of *TFPI2* in stool DNA: a potential novel biomarker for the detection of colorectal cancer. *Cancer Res.* 69(11), 4691–4699 (2009).

73 Kim MS, Louwagie J, Carvalho B *et al.* Promoter DNA methylation of oncostatin m receptor-beta as a novel diagnostic and therapeutic marker in colon cancer. *PLoS ONE* 4(8), e6555 (2009).

74 Bosch LJ, Oort FA, Neerincx M *et al.* DNA methylation of phosphatase and actin regulator 3 detects colorectal cancer in stool and complements FIT. *Cancer Prev. Res. (Phila.)* 5(3), 464–472 (2012).

▪▪ **First study reporting a high sensitivity and specificity for the fecal immunochemical test in combination with a methylation marker in fecal DNA for early detection of CRC.**

75 Ahlquist DA, Zou H, Domanico M *et al.* Next-generation stool DNA test accurately detects colorectal cancer or large adenomas. *Gastroenterology* 142(2), 248–256 (2011).

▪ **Reports high sensitivity and specificity of a methylation marker panel in fecal DNA for the early detection of CRC.**

76 Leung WK, To KF, Man EP *et al.* Quantitative detection of promoter hypermethylation in multiple genes in the serum of patients with colorectal cancer. *Am. J. Gastroenterol.* 100(10), 2274–2279 (2005).

77 Ebert MP, Model F, Mooney S *et al.* Aristaless-like homeobox-4 gene methylation is a potential marker for colorectal adenocarcinomas. *Gastroenterology* 131(5), 1418–1430 (2006).

78 Lofton-Day C, Model F, Devos T *et al.* DNA methylation biomarkers for blood-based colorectal cancer screening. *Clin. Chem.* 54(2), 414–423 (2008).

79 Herbst A, Rahmig K, Stieber P *et al.* Methylation of *NEUROG1* in serum is a sensitive marker for the detection of early colorectal cancer. *Am. J. Gastroenterol.* 106(6), 1110–1118 (2011).

80 Devos T, Tetzner R, Model F *et al.* Circulating methylated *SEPT9* DNA in plasma is a biomarker for colorectal cancer. *Clin. Chem.* 55(7), 1337–1346 (2009).

81 Payne SR. From discovery to the clinic: the novel DNA methylation biomarker (m)SEPT9 for the detection of colorectal cancer in blood. *Epigenomics* 2(4), 575–585 (2010).

82 Tanzer M, Balluff B, Distler J *et al.* Performance of epigenetic markers SEPT9 and ALX4 in plasma for detection of colorectal precancerous lesions. *PLoS ONE* 5(2), e9061 (2010).

83 Warren JD, Xiong W, Bunker a M *et al.* Septin 9 methylated DNA is a sensitive and specific blood test for colorectal cancer. *BMC Med.* 9(1), 133 (2011).

84 Tang D, Liu J, Wang DR, Yu HF, Li YK, Zhang JQ. Diagnostic and prognostic value of the methylation status of secreted frizzled-related protein 2 in colorectal cancer. *Clin. Invest. Med.* 34(2), E88–E95 (2011).

85 Ahlquist DA, Taylor WR, Mahoney DW *et al.* The stool DNA test is more accurate than the plasma septin 9 test in detecting colorectal neoplasia. *Clin. Gastroenterol. Hepatol.* 10(3), 272–277 (2012).

86 Deschoolmeester V, Baay M, Specenier P, Lardon F, Vermorken JB. A review of the most promising biomarkers in colorectal cancer: one step closer to targeted therapy. *Oncologist* 15(7), 699–731 (2010).

87 Graziano F, Cascinu S. Prognostic molecular markers for planning adjuvant chemotherapy trials in Dukes' B colorectal cancer patients: how much evidence is enough? *Ann. Oncol.* 14(7), 1026–1038 (2003).

88 Klump B, Nehls O, Okech T *et al.* Molecular lesions in colorectal cancer: impact on prognosis? Original data and review of the literature. *Int. J. Colorectal Dis.* 19(1), 23–42 (2004).

89 Belt EJ, Van Stijn MF, Bril H *et al.* Lymph node negative colorectal cancers with isolated tumor deposits should be classified and treated as stage III. *Ann. Surg. Oncol.* 17(12), 3203–3211 (2010).

90 George S, Primrose J, Talbot R *et al.* Will Rogers revisited: prospective observational study of survival of 3592 patients with colorectal cancer according to number of nodes examined by pathologists. *Br. J. Cancer* 95(7), 841–847 (2006).

91 Nosho K, Baba Y, Tanaka N *et al.* Tumour-infiltrating T-cell subsets, molecular changes in colorectal cancer, and prognosis: cohort study and literature review. *J. Pathol.* 222(4), 350–366 (2010).

92 Prall F, Nizze H, Barten M. Tumour budding as prognostic factor in stage I/II colorectal carcinoma. *Histopathology* 47(1), 17–24 (2005).

93 Pritchard CC, Grady WM. Colorectal cancer molecular biology moves into clinical practice. *Gut* 60(1), 116–129 (2011).

94 Fretwell VL, Ang CW, Tweedle EM, Rooney PS. The impact of lymph node yield on Duke's B and C colorectal cancer survival. *Colorectal Dis.* 12(10), 995–1000 (2010).

95 Chau I, Allen MJ, Cunningham D *et al.* The value of routine serum carcino-embryonic antigen measurement and computed tomography in the surveillance of patients after adjuvant chemotherapy for colorectal cancer. *J. Clin. Oncol.* 22(8), 1420–1429 (2004).

96 Oussoultzoglou E, Rosso E, Fuchshuber P *et al.* Perioperative carcinoembryonic antigen measurements to predict curability after liver resection for colorectal metastases: a prospective study. *Arch. Surg.* 143(12), 1150–1158; discussion 1158–1159 (2008).

97 Tan E, Gouvas N, Nicholls RJ, Ziprin P, Xynos E, Tekkis PP. Diagnostic precision of carcinoembryonic antigen in the detection of recurrence of colorectal cancer. *Surg. Oncol.* 18(1), 15–24 (2009).

98 Kelley RK, Venook a P. Prognostic and predictive markers in stage II colon cancer: is there a role for gene expression profiling? *Clin. Colorectal Cancer* 10(2), 73–80 (2011).

99 Umetani N, Takeuchi H, Fujimoto A, Shinozaki M, Bilchik a J, Hoon DS. Epigenetic inactivation of ID4 in colorectal carcinomas correlates with poor differentiation and unfavorable prognosis. *Clin. Cancer Res.* 10(22), 7475–7483 (2004).

100 Tanaka M, Chang P, Li Y *et al.* Association of *CHFR* promoter methylation with disease recurrence in locally advanced colon cancer. *Clin. Cancer Res.* 17(13), 4531–4540 (2011).

101 Krtolica K, Krajnovic M, Usaj-Knezevic S, Babic D, Jovanovic D, Dimitrijevic B. Comethylation of *p16* and *MGMT* genes in colorectal carcinoma: correlation with clinicopathological features and prognostic value. *World J. Gastroenterol.* 13(8), 1187–1194 (2007).

102 Whitehall VL, Walsh MD, Young J, Leggett BA, Jass JR. Methylation of *O*-6-methylguanine DNA methyltransferase characterizes a subset of colorectal cancer with low-level DNA microsatellite instability. *Cancer Res.* 61(3), 827–830 (2001).

103 Xu XL, Yu J, Zhang HY *et al.* Methylation profile of the promoter CpG islands of 31 genes that may contribute to colorectal carcinogenesis. *World J. Gastroenterol.* 10(23), 3441–3454 (2004).

104 Ogino S, Nosho K, Kirkner GJ *et al.* CpG island methylator phenotype, microsatellite instability, *BRAF* mutation and clinical outcome in colon cancer. *Gut* 58(1), 90–96 (2009).

105 Shima K, Morikawa T, Baba Y *et al.* MGMT promoter methylation, loss of expression and prognosis in 855 colorectal cancers. *Cancer Causes Control* 22(2), 301–309 (2011).

106 Kohonen-Corish MR, Daniel JJ, Chan C *et al.* Low microsatellite instability is associated with poor prognosis in stage C colon cancer. *J. Clin. Oncol.* 23(10), 2318–2324 (2005).

107 Mokarram P, Kumar K, Brim H *et al.* Distinct high-profile methylated genes in colorectal cancer. *PLoS ONE* 4(9), e7012 (2009).

108 Yi JM, Dhir M, Van Neste L *et al.* Genomic and epigenomic integration identifies a prognostic signature in colon cancer. *Clin. Cancer Res.* 17(6), 1535–1545 (2011).

109 Ahn JB, Chung WB, Maeda O *et al.* DNA methylation predicts recurrence from resected stage III proximal colon cancer. *Cancer* 117(9), 1847–1854 (2011).

110 Ward RL, Cheong K, Ku SL, Meagher A, O'connor T, Hawkins NJ. Adverse prognostic effect of methylation in colorectal cancer is reversed by microsatellite instability. *J. Clin. Oncol.* 21(20), 3729–3736 (2003).

▪ **First study reporting a prognostic significance for CpG island methylation phenotype in microsatellite-stable CRC patients.**

111 Barault L, Charon-Barra C, Jooste V *et al.* Hypermethylator phenotype in sporadic colon cancer: study on a population-based series of 582 cases. *Cancer Res.* 68(20), 8541–8546 (2008).

▪ **First population-based study investigating the prognostic relevance of CpG island methylator phenotype in three subgroups.**

112 Saltz LB, Niedzwiecki D, Hollis D *et al.* Irinotecan fluorouracil plus leucovorin is not superior to fluorouracil plus leucovorin alone as adjuvant treatment for stage III colon cancer: results of CALGB 89803. *J. Clin. Oncol.* 25(23), 3456–3461 (2007).

113 Edwards MS, Chadda SD, Zhao Z, Barber BL, Sykes DP. A systematic review of treatment guidelines for metastatic colorectal cancer. *Colorectal Dis.* 14(2), e31–e47 (2012).

114 Goldberg RM. Therapy for metastatic colorectal cancer. *Oncologist* 11(9), 981–987 (2006).

115 Tejpar S, Bertagnolli M, Bosman F *et al.* Prognostic and predictive biomarkers in resected colon cancer: current status and future perspectives for integrating genomics into biomarker discovery. *Oncologist* 15(4), 390–404 (2010).

116 Simon R. Development and validation of biomarker classifiers for treatment selection. *J. Stat. Plan. Inference* 138(2), 308–320 (2008).

117 Amado RG, Wolf M, Peeters M *et al.* Wild-type *KRAS* is required for panitumumab efficacy in patients with metastatic colorectal cancer. *J. Clin. Oncol.* 26(10), 1626–1634 (2008).

118 Di Fiore F, Blanchard F, Charbonnier F *et al.* Clinical relevance of *KRAS* mutation detection in metastatic colorectal cancer treated by Cetuximab plus chemotherapy. *Br. J. Cancer* 96(8), 1166–1169 (2007).

119 Lievre A, Bachet JB, Le Corre D *et al. KRAS* mutation status is predictive of response to cetuximab therapy in colorectal cancer. *Cancer Res.* 66(8), 3992–3995 (2006).

120 Boland CR, Goel A. Microsatellite instability in colorectal cancer. *Gastroenterology* 138(6), 2073–2087.e3 (2010).

121 Des Guetz G, Schischmanoff O, Nicolas P, Perret GY, Morere JF, Uzzan B. Does microsatellite instability predict the efficacy of adjuvant chemotherapy in colorectal cancer? A systematic review with meta-analysis. *Eur. J. Cancer* 45(10), 1890–1896 (2009).

122 Bertagnolli MM, Niedzwiecki D, Compton CC *et al.* Microsatellite instability predicts improved response to adjuvant therapy with irinotecan, fluorouracil, and leucovorin in stage III colon cancer: Cancer and Leukemia Group B Protocol 89803. *J. Clin. Oncol.* 27(11), 1814–1821 (2009).

123 Fallik D, Borrini F, Boige V *et al.* Microsatellite instability is a predictive factor of the tumor response to irinotecan in patients with advanced colorectal cancer. *Cancer Res.* 63(18), 5738–5744 (2003).

124 Popat S, Hubner R, Houlston RS. Systematic review of microsatellite instability and colorectal cancer prognosis. *J. Clin. Oncol.* 23(3), 609–618 (2005).

125 Ogino S, Meyerhardt JA, Kawasaki T *et al.* CpG island methylation, response to combination chemotherapy, and patient survival in advanced microsatellite stable colorectal carcinoma. *Virchows Arch.* 450(5), 529–537 (2007).

126 Shen L, Catalano PJ, Benson AB 3rd, O'Dwyer P, Hamilton SR, Issa JP. Association between DNA methylation and shortened survival in patients with advanced colorectal cancer treated with 5-fluorouracil based chemotherapy. *Clin. Cancer Res.* 13(20), 6093–6098 (2007).

127 Jover R, Nguyen TP, Perez-Carbonell L *et al.* 5-fluorouracil adjuvant chemotherapy does not increase survival in patients with CpG island methylator phenotype colorectal cancer. *Gastroenterology* 140(4), 1174–1181 (2011).

128 Esteller M, Herman JG. Generating mutations but providing chemosensitivity: the role of *O6*-methylguanine DNA methyltransferase in human cancer. *Oncogene* 23(1), 1–8 (2004).

129 Jacinto FV, Esteller M. *MGMT* hypermethylation: a prognostic foe, a predictive friend. *DNA Repair (Amst.)* 6(8), 1155–1160 (2007).

130 Agrelo R, Cheng WH, Setien F *et al.* Epigenetic inactivation of the premature aging Werner syndrome gene in human cancer. *Proc. Natl Acad. Sci. USA* 103(23), 8822–8827 (2006).

131 Kawasaki T, Ohnishi M, Suemoto Y *et al. WRN* promoter methylation possibly connects mucinous differentiation, microsatellite instability and CpG island methylator phenotype in colorectal cancer. *Mod. Pathol.* 21(2), 150–158 (2008).

132 Laird PW. The power and the promise of DNA methylation markers. *Nat. Rev. Cancer* 3(4), 253–266 (2003).

133 Shivapurkar N, Gazdar a F. DNA methylation based biomarkers in non-invasive cancer screening. *Curr. Mol. Med.* 10(2), 123–132 (2010).

134 Esteller M, Sanchez-Cespedes M, Rosell R, Sidransky D, Baylin SB, Herman JG. Detection of aberrant promoter hypermethylation of tumor suppressor genes in serum DNA from non-small cell lung cancer patients. *Cancer Res.* 59(1), 67–70 (1999).

135 Bailey VJ, Easwaran H, Zhang Y *et al.* MS-qFRET: a quantum dot-based method for analysis of DNA methylation. *Genome Res.* 19(8), 1455–1461 (2009).

136 Rifai N, Gillette MA, Carr SA. Protein biomarker discovery and validation: the long and uncertain path to clinical utility. *Nat. Biotechnol.* 24(8), 971–983 (2006).

137 Jeddeloh JA, Greally JM, Rando OJ. Reduced-representation methylation mapping. *Genome Biol.* 9(8), 231 (2008).

138 Oda M, Glass JL, Thompson RF *et al.* High-resolution genome-wide cytosine methylation profiling with simultaneous copy number analysis and optimization for limited cell numbers. *Nucleic Acids Res.* 37(12), 3829–3839 (2009).

139 Kibriya MG, Raza M, Jasmine F *et al.* A genome-wide DNA methylation study in colorectal carcinoma. *BMC Med. Genomics* 4, 50 (2011).

140 Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc. Natl Acad. Sci. USA* 93(18), 9821–9826 (1996).

141 Eads CA, Danenberg KD, Kawakami K *et al.* MethyLight: a high-throughput assay to measure DNA methylation. *Nucleic Acids Res.* 28(8), E32 (2000).

142 Tost J, Gut IG. DNA methylation analysis by pyrosequencing. *Nat. Protoc.* 2(9), 2265–2275 (2007).

143 Gonzalgo ML, Liang G. Methylation-sensitive single-nucleotide primer extension (Ms-SNuPE) for quantitative measurement of DNA methylation. *Nat. Protoc.* 2(8), 1931–1936 (2007).

144 Brena RM, Auer H, Kornacker K, Plass C. Quantification of DNA methylation in electrofluidics chips (Bio-COBRA). *Nat. Protoc.* 1(1), 52–58 (2006).

145 Ehrich M, Nelson MR, Stanssens P *et al.* Quantitative high-throughput analysis of DNA methylation patterns by base-specific cleavage and mass spectrometry. *Proc. Natl Acad. Sci. USA* 102(44), 15785–15790 (2005).

146 Van Vlodrop IJ, Niessen HE, Derks S *et al.* Analysis of promoter CpG island hypermethylation in cancer: location, location! *Clin. Cancer Res.* 17(13), 4225–4231 (2011).

147 Mischak H, Allmaier G, Apweiler R *et al.* Recommendations for biomarker identification and qualification in clinical proteomics. *Sci. Transl. Med.* 2(46), 46ps42 (2010).

148 Dancey JE, Dobbin KK, Groshen S *et al.* Guidelines for the development and incorporation of biomarker studies in early clinical trials of novel agents. *Clin. Cancer Res.* 16(6), 1745–1755 (2010).

149 Williams SA, Slavin DE, Wagner JA, Webster CJ. A cost–effectiveness approach to the qualification and acceptance of biomarkers. *Nat. Rev. Drug Discov.* 5(11), 897–902 (2006).

150 Sawyers CL. The cancer biomarker problem. *Nature* 452(7187), 548–552 (2008).

151 Diamandis EP. Cancer biomarkers: can we turn recent failures into success? *J. Natl Cancer Inst.* 102(19), 1462–1467 (2010).

152 Luo YX, Chen DK, Song SX, Wang L, Wang JP. Aberrant methylation of genes in stool samples as diagnostic biomarkers for colorectal cancer or adenomas: a meta-analysis. *Int. J. Clin. Pract.* 65(12), 1313–1320 (2011).

153 Hopewell S, Loudon K, Clarke MJ, Oxman a D, Dickersin K. Publication bias in clinical trials due to statistical significance or direction of trial results. *Cochrane Database Syst. Rev.* 1, MR000006 (2009).

154 Ioannidis JP, Trikalinos TA. An exploratory test for an excess of significant findings. *Clin. Trials* 4(3), 245–253 (2007).

155 Kyzas PA, Loizou KT, Ioannidis JP. Selective reporting biases in cancer prognostic factor studies. *J. Natl Cancer Inst.* 97(14), 1043–1055 (2005).

156 Debauve G, Capouillez A, Belayew A, Saussez S. The helicase-like transcription factor and its implication in cancer progression. *Cell. Mol. Life Sci.* 65(4), 591–604 (2008).

157 Mccart a E, Vickaryous NK, Silver A. Apc mice: models, modifiers and mutants. *Pathol. Res. Pract.* 204(7), 479–490 (2008).

158 Mohammad HP, Zhang W, Prevas HS *et al.* Loss of a single Hic1 allele accelerates polyp formation in Apc(Delta716) mice. *Oncogene* 30(23), 2659–2669 (2011).

159 Wales MM, Biel MA, El Deiry W *et al.* p53 activates expression of HIC-1, a new candidate tumour suppressor gene on 17p13.3. *Nat. Med.* 1(6), 570–577 (1995).

160 McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM. REporting recommendations for tumor MARKer prognostic studies (REMARK). *Nat. Clin. Pract. Oncol.* 2(8), 416–422 (2005).