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DNA methylation in colorectal cancer – Impact on screening and therapy monitoring modalities?

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Abstract. Colorectal cancer (CRC) is a common malignancy. It arises from benign neoplasms and evolves into adenocarcinomas through a stepwise histological progression sequence, proceeding from either adenomas or hyperplastic polyps/serrated adenomas. Genetic alterations have been associated with specific steps in this adenoma-carcinoma sequence and are believed to drive the histological progression of CRC. Recently, epigenetic alterations (especially DNA methylation) have been shown to occur in colon polyps and CRC. The aberrant methylation of genes appears to act together with genetic alterations to drive the initiation and progression of colon polyps to CRC.
DNA methylation changes have been recognized as one of the most common molecular alterations in human tumors, including CRC. Because of the ubiquity of DNA methylation changes and the ability to detect methylated DNA in several body fluids (blood, stool), this specifically altered DNA may serve, on the one hand, as a possible new screening marker for CRC and, on the other hand, as a tool for therapy monitoring in patients having had neoplastic disease of the colorectum.
As many CRC patients present with advanced disease, early detection seems to be one of the most important approaches to reduce mortality. Therefore, an effective screening test would have substantial clinical benefits. Furthermore, early detection of progression of disease in patients having had CRC permits immediate commencement of specific treatment regimens (e.g. curative resection of liver and lung metastases) and probably longer survival and better quality of life.

Keywords: Colorectal cancer, DNA methylation, stool, screening, therapy monitoring, review

1. Colorectal cancer in general

Colorectal cancer (CRC) is the third most common malignant neoplasm worldwide [1]. The annual incidence of CRC in North America and Europe is approximately 30–50/100 000 [2]. In 2005, the American Cancer Society estimated 145290 new cases of and 56290 deaths from CRC in the United States. Thus, CRC is the third most common malignancy and the third-leading cause of cancer death in women and men in the United States. In women, it ranks third after lung and breast cancer; in men, it ranks third after lung and prostate cancer [3]. The lifetime incidence of CRC among women and men at average risk is sufficiently high at 6%, or 1 in 18 [4].

Despite advances in surgical techniques and adjuvant therapy, there has been only a modest improvement in survival for patients with advanced neoplasms [5]. Hence, effective primary and secondary preventive approaches must be developed to reduce mortality from CRC. Genetics, experimental and epidemiologic studies suggest that CRC results from complex interactions between inherited susceptibility and environmental factors [6–9].

Because of the frequency of the disease, demonstrated slow growth of primary lesions and better survival of patients with early-stage lesions, CRC is a suit-

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able disease for screening. Several studies have demonstrated benefits of CRC screening [10–12]. Most CRCs appear in the rectum (38%) or sigmoid colon (29%) and therefore around two-thirds of CRCs fall within the reach of sigmoidoscopy [13].

2. Early detection in general

Advances in cancer treatment and improvements in cancer outcome over the past few decades have been modest, despite significant investment in cancer research. A great deal of research is invested in improving treatment for advanced disease, because most people who develop cancer have advanced disease at the time of diagnosis. For example, of those with lung, colorectal or breast cancer in the United States, 72%, 57% and 34%, respectively, have regional or distant spread of their disease at the time of diagnosis. Despite huge effort, only modest gains in the survival of cancer patients with advanced disease at the time of diagnosis have been achieved over the past few decades. Comparably less effort has been put into strategies for the early detection of cancer although the promise of early detection is that it will identify cancer while still localized and curable, not only preventing mortality, but also reducing morbidity and costs [14].

Cervical cancer (CC) provides an excellent example of the power of early detection, and subsequent treatment, in reducing the burden of cancer. CC is also an excellent example of intensive research into molecular alterations during pathogenesis of a specific cancer type and subsequently of the establishment of evidence-based screening programs and even therapy or prevention strategies. That is why the history of CC research can serve as an interesting example of how to introduce an efficient screening program and develop new options for therapy or prevention. Therefore, it should be examined more closely:

At the beginning of the twentieth century, mortality due to invasive CC was among the highest for women. By the middle of the twentieth century, pathologists had shown that the natural history of CC progressed through stages of increasingly severe cervical intraepithelial neoplasia (CIN) and that these stages could be histologically identified using exfoliated cells. Subsequently, an exfoliated cytological staining procedure (Papanicolau (PAP) smear) that can detect premalignant and malignant changes in the cervical epithelium was developed. Furthermore, programes and policies were introduced in developed countries to implement widespread early detection of pre-neoplastic cervical lesions. Since 1950, there has been an approximately 70% decline in the incidence of, and mortality due to, invasive CC in the United States, whereas in developing countries where PAP smear screening is not widespread, CC remains a major public health problem [14]. The effectiveness of this screening approach is mostly due to its high acceptance in the population, to the fact that the "organ of interest" is easily accessible in a non-invasive procedure and the fact that changes can easily be identified by a very well established marker like cytology. Detailed knowledge of the alterations during pathogenesis (progressing from low-grade to intermediate- to high-grade CIN and eventually to invasive cancer) has also contributed to the cytological screening of CC.

New technologies – including DNA methylation analyses – offer a variety of new opportunities for developing biomarker-based tests that are less expensive and more accurate than currently used screening tests. Additionally, CC also illustrates the potential power of using molecular tests to enhance the accuracy of early detection. PAP smears are performed on millions of women each year. Thus a large number of both false-negatives and false-positives occur. The development of molecular methods to augment, or possibly replace, PAP smears has been spurred by the recognition that cervical neoplasia is caused by persistent infection with oncogenic human papillomaviruses (HPVs). Since the late 1990s, studies have shown that relatively inexpensive, easy-to-use, molecular tests for the presence of HPV can be performed on cervical swabs collected either by a practitioner or by a woman herself and will detect pre-invasive CC with greater sensitivity and no (or slight) loss of specificity in comparison to PAP smears (for review see [14]).

The challenges faced by early-detection researchers can be classified in terms of the steps needed to produce a useful population screening test: discovery, development and evaluation. Sullivan Pepe et al. [15] reported five phases of biomarker development for early detection of cancer: *Phase 1* is represented by preclinical exploratory studies aiming to evaluate the expression and regulation of thousands of genes and proteins in tumor and comparable healthy organ tissue to identify candidates for early detection. *Phase 2* includes assay development and validation, which is realized with markers in specimens that can be obtained non-invasively, such as serum, plasma, urine, sputum or stool and that correlate with disease. The goal of phase 2 is to evaluate ability of these markers to discrimi-

nate between patients with clinically established disease and healthy controls. *Phase 3* uses retrospective, longitudinal studies. This phase relies on the existence of repositories of clinical specimens, typically serum, that have been routinely collected and stored. Samples obtained from individuals before they were diagnosed with the cancer of interest are compared with samples from healthy age-matched controls. Phase 3 is vitally important because it provides a window on the natural history of the disease and how it relates to levels of the biomarker under study. If more samples are available during the prediagnostic period of the cancer patients, phase 3 studies can determine how long before normal clinical diagnosis a tumor marker might be able to detect disease. *Phase 4* uses prospective screening studies to evaluate whether the potential screening marker is, in fact, able to detect the disease while it is still localized, and to estimate expected screening costs. *Phase 5* includes cancer control studies which should directly evaluate the impact of screening on population disease morbidity and mortality. Although the five phases are not necessarily sequential, they are ordered according to strength of evidence from weakest to strongest, and results from earlier phases will typically be required to justify conducting later-phase studies [14,15].

Furthermore, it must also be mentioned that the power of a screening test is not only dependent on its specificity and sensitivity, but also on people's willingness to participate in a given screening program. This willingness is strongly influenced by whether the screening is easy to perform, safe and practicable in clinical routine. For example, it was recently reported by the American Cancer Society [12] that 88% of American women aged between 18 and 44 undergo PAP smear testing, which is an easy-to-perform and safe screening tool. In comparison, 60.5% of American women aged 40 to 64 undergo mammogram examination, which needs technical equipment and can be painful. Additionally, about 50% of American men were screened by digital rectal examination and PSA testing for prostate cancer. This very low number may reflect the reduced willingness of men to consult a doctor for screening purposes. With regard to CRC screening, only 40% of American women and men aged 50 yrs and older (without any sex-specific differences in the prevalence of screening) reported recent screening with an endoscopic procedure (either sigmoidoscopy or colonoscopy) and only 20% of either sex reported having undergone screening with a fecal occult blood test (FOBT). The reasons for such a low number of participants may be uncomfortable and unpleasant preparation procedures for endoscopy, sometimes painful examination procedures, complications during endoscopy and low sensitivity or specificity of FOBT. Because the prevalence of CRC screening is only approximately 50% (FOBT or lower endoscopy, or both) the substantial problem of too many average risk adults not being screened with any of the recommended tests persists [12].

3. Established screening modalities for colorectal cancer

Few of the advantages of CC screening are true for CRC. Despite its negative aspects, a huge effort to improve CRC screening has been made. CRC is a suitable disease for screening as it has a recognizable early stage and a defined natural history (with a long asymptomatic preclinical phase), surgical treatment is effective, and benefit is greater in early-stage disease.

In an interesting study, Hamilton et al. [16] evaluated the prediagnostic features of CRC using a population-based case-control study. In total, ten features were statistically significantly associated with CRC before diagnosis: *rectal bleeding*, *weight loss*, *abdominal pain*, *diarrhea*, *constipation*, *abnormal rectal examination*, *abdominal tenderness*, *hemoglobin* < 10.0 *g/dl*, *positive FOBT*, *blood glucose* > 10 *mM/l*. Furthermore, five of these features (*abdominal pain*, *rectal bleeding*, *anemia*, *positive FOBT*, *raised blood glucose*) remained statistically significantly associated with CRC for 180 days before diagnosis [16]. As a consequence, it should be kept in mind that people with symptoms or signs that suggest the presence of CRC or polyps fall outside the domain of screening and should be offered an appropriate diagnostic evaluation for earlier diagnosis of CRC.

Screening programs should begin by classifying the individual patient's level of risk based on personal (*CRC or an adenomatous polyp in the patient*), family (*CRC or an adenomatous polyp in patient's family*) and medical history (*e.g.*, *inflammatory bowel disease*), which will determine the appropriate approach to screening in that person. Clinicians should determine an individual patient's risk status well before the earliest potential initiation of screening (typically around age 20 years, but earlier if there is a family history of familial adenomatous polyposis (FAP)), and any predisposition to CRC should prompt further efforts to identify and define the specific condition associated with increased risk (for review see [10]). Men and women at

Table 1
Comparison of screening tests for colorectal cancer

Test	Sensitivity/ Specificity	Recommended screening frequency	Represents whole colon?	Advantages	Disadvantages
Fecal Occult Blood Testing (FOBT)*	50% sensitivity for CRC low (≤20%) sensitivity for adenomas [18]	Annually [19-22]	yes	Non-invasive; low cost; requires no bowel preparation; transportable specimens	Dietary restrictions recommended [10]; high rate of false-po itive findings [10,17]; no possibility to remove adenomas
Flexible Sigmoidoscopy (FS)*	97% sensitivity for CRC [10] 94% specificity for CRC [10] overall sensitivity and specificity for adenomas are not known [23]	Every five years [10]	no	Requires less bowel preparation; precancerous lesions can be removed	High costs; invasive; patient discomfort; missing of proximal neoplasias; risk of bowel perforation; requires trained examiners (for review see [10])
Colonoscopy*	97% sensitivity for CRC [10] 98% specificity for CRC [10] 90% sensitivity for adenomas ≥ 1 cm [24]	Every ten years [10]	yes	Precancerous lesions can be removed; reduced cancer incidence after polyp removal [25-27] or colonoscopy following FOBT screening [28]	High costs; invasive; patient discomfort; bowel preparation required; risk of bowel perforation; mortality rates of 1-3 per 10,000 [10]; requires trained examiners [10]
Double-Contrast Barium Enema (DBCE)*	Moderate sensitivity for CRC [10] Moderate to high specificity for CRC [10]	Every five years [10]	yes	Non-invasive; alternative for patients with incomplete colonoscopy or medical contraindication [17]	Patient discomfort; bowel preparation required; no possibility to remove adenomas
Virtual Colonoscopy+	High sensitivity and specificity for CRC 90% sensitivity and 72% specificity for adenomas [29,30]		yes	Non-invasive, no sedation needed; concurrent examination of extracolonic organs	Patient discomfort; bowel preparation required; high cost; high radiation dose; no possibility to remove adenomas; requires trained examiners
DNA quantity and quality in stool+	100% sensitivity and 81% specificity for CRC [31] Long (intact) DNA: 56% sensitivity for CRC [32]		yes	Non-invasive; does not require bowel preparation; transportable specimens; high patient acceptability	Research stage of development; time consuming assay; technology for large-scale screenings lacking at present
DNA mutation markers+	Sensitivity for CRC: KRAS: 60% [33,34] TP53: 28% [35] APC: 60% [36] BAT26: 40% [37]		yes	Non-invasive; does not require bowel preparation; transportable specimens; high patient acceptability	Research stage of development; time consuming assay; technology for large-scale screenings lacking at present
DNA methylation marker+	77%-90% sensitivity and 77% specificity for CRC [38]		yes	Non-invasive; does not require bowel preparation; transportable specimens; high patient acceptability	Research stage of development; time consuming assay; technology for large-scale screenings lacking at present

*Established Screening Tests. +Emerging Screening Tests

average risk should be offered screening for CRC and adenomatous polyps beginning at the age of 50 years. If the result of a screening test is abnormal, physicians should recommend a complete structural examination of the colon and rectum by colonoscopy (or flexible sigmoidoscopy and double-contrast barium enema if colonoscopy is not available). Screening strategies are not equal with regard to evidence of effectiveness, magnitude of effectiveness, risk, or up-front costs (for review see [10,17]). A comparison of potential screening tests for colorectal cancer is presented in Table 1.

4. Follow-up after curative resection for CRC

At diagnosis most patients undergo curative resection if CRC is limited to the bowel and the regional lymph nodes. Nevertheless, 30%–50% of patients will have recurrent disease and die of metastatic CRC despite initial radical resection [39,40]. In asymptomatic

patients, detection of a recurrent or metachronous tumor at an early stage improves the chance of another curative resection and may thereby increase overall patient survival. In particular, local recurrences as well as liver and lung metastases may not be clinically symptomatic when diagnosed during follow-up. Since CRC is one of the few solid tumors that may still be surgically treated in curative intention when recurrent disease has been diagnosed [41–43], different strategies to follow up patients after surgery have been developed ([44] and see CRC surveillance guidelines [45]). Additionally, it was seen that the practice of including patients with CRC in surveillance protocols for intensive follow-up significantly improved survival [46].

CRC recurrent disease mainly occurs in the regional abdominal lymph nodes, the liver or the lung and less frequently in the resected segment of the colorectum. Nevertheless, the life-threatening event in CRC is not lymph node metastasis *per se*, but hematogenous metastases which mainly affect the liver or the lung. Therefore, a screening test that is sensitive for hematogenous metastases and can be performed in patients' serum or plasma may have an impact on early detection of disease progression in patients having had CRC (Fig. 1). Such a test subsequently permits immediate start of specific treatment regimens and probably longer survival and better quality of life.

An increasing number of studies have reported the presence of methylated DNA in serum/plasma of patients with various types of malignancies and the absence of methylated DNA in normal control patients (for review see [47]). For the past five years our research group was mainly interested in evaluating DNA methylation changes in serum of cancer patients [47–52]. The potential for using epigenetic markers for prognostication and even for therapy monitoring in CRC appears to be great. A summary of reported methylated genes in serum/plasma of CRC patients is presented in Table 2c.

5. Adenoma-carcinoma sequence – genetic and epigenetic alterations

CRC develops as a result of the progressive accumulation of genetic and epigenetic alterations that lead up to the transformation of normal colonic epithelium to colon adenocarcinoma. The fact that CRC develops over about 5–15 years and progresses through parallel histological and molecular changes has permitted a detailed analysis of the events involved in its initiation and progression: firstly, cancer emerges via a multistep progression at both the molecular and the morphological level [7]; secondly, genetic and epigenetic alterations are pathogenic key events in cancer formation driving the initiation and progression of the adenoma-carcinoma sequence [53]; thirdly, it has been seen that hereditary cancer syndromes frequently correspond to germline forms of genetic and epigenetic key defects, whose somatic occurrences drive the emergence of sporadic CRC [54]. CRC is most commonly initiated by aberrant accumulation of *beta-catenin* in the Wingless/Wnt signaling pathway leading to transcription of WNT-target genes. Furthermore, other alterations that have been shown to play a central role in colorectal carcinogenesis affect *KRAS2*, *TP53* and elements of the TGF (transforming growth factor)-b signaling pathway, such as *TGFBR2* and *MADH4*/*SMAD4*. Epigenetic alterations – particularly aberrant DNA methylation – appear to affect genes whose inactivation can promote tumor formation by creating genomic instability (e.g. *MLH1* (mutL homologue 1)) or by causing primary inactivation of the methylated gene itself (e.g. *CDKN2A*) (for review see [55]).

5.1. Adenoma-carcinoma sequence – genetic alterations

Most sporadic CRCs are thought to develop from benign adenomas. Identification of the genetic abnormalities that seem to accumulate in a stepwise manner has led to the well-known model of the adenoma-carcinoma sequence [7,54,56]. The earliest identifiable lesion in CRC formation appears to be the ACF (aberrant crypt focus). The true neoplastic potential of this lesion is still undetermined, but it has been shown that some of these lesions harbor mutations in *KRAS2* or *APC* (adenomatous polyposis coli) and can progress to CRC (for review see [55]).

Accumulating evidence demonstrates that some CRC arise from hyperplastic polyps via a serrated adenoma intermediate [57]. Interestingly, this hyperplastic polyp-serrated adenoma–adenocarcinoma sequence is more common in the proximal colon, and these tumors more often show increased DNA methylation and mutations in *BRAF* [58,59].

Adenomas are generally masses that protrude into the gut lumen (polyps). They can either be pedunculated (with a stalk) or sessile (without a stalk). More rarely, adenomas can be flat or depressed. The epithelium of adenomas can form glands (tubular adenoma), finger-like projections (villous adenoma) or a combi-

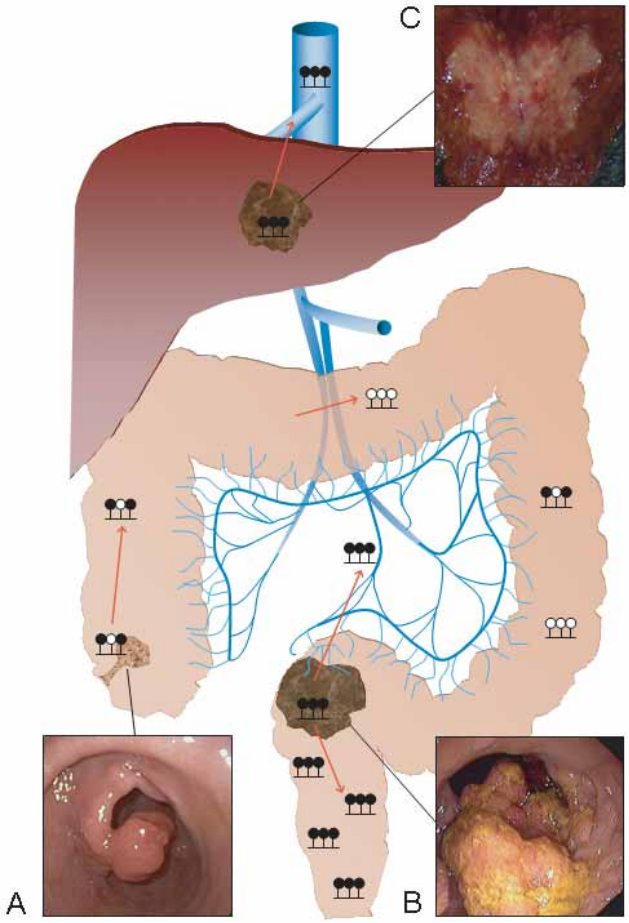

Fig. 1. Methylated DNA can be detected in tumor-derived DNA found either in the bloodstream of CRC patients or in samples drained to the outside of the body (e.g. stool). Three different situations are demonstrated: (**A**) colorectal polyp; (**B**) CRC and (**C**) metastasized CRC (e.g. after primary curative resection). Fully methylated (tumor-specific) DNA is represented by black lollipops, hemi-methylated (tumor-specific) DNA is represented by black mixed with white lollipops and normal DNA is represented be solely white ones. In situations (A) and (B) it seems that methylated DNA can be more sufficiently detected in stool samples, because tumor-specific methylated DNA is shed into the gut lumen and is then be transported to the outside of the body by the physiological bowel movement. It seems that less tumor-derived DNA will be transported into the bloodstream, especially in terms of pre-neoplastic lesions (which are not invasive by definition). Situation (C) represents metastasized CRC (e.g. after primary curative resection). The life-threatening event in CRC is not lymph node metastasis *per se*, but hematogenous metastases which mainly affect the liver or the lung. Therefore, it seems plausible to use methylated tumor-derived DNA, especially in patients bloodstream for early detection of recurrent diesease. DNA methylation changes in CRC patients may serve, on the one hand, as a possible new screening marker for CRC (fecal DNA; maybe in combination with bloodstream testing) and, on the other hand, as a tool for therapy monitoring and early detection of recurrent disease (bloodstream testing) in patients having had CRC.

nation of both (tubulovillous adenomas). The time required for the development of malignancy from adenoma is lengthy, with even conservative estimates indicating an interval of 5–10 years [60]. Moreover, only a small percentage of adenomas progress to carcinoma. Although there is presently no clear way to identify which adenomas will become malignant, subsequent progression is associated with severe dysplasia, patient age, size of adenoma and histological type [61]. Adenomas that are $>$ 1 cm, show severe dysplasia and/or a villous architecture are described as advanced [23]. The average time for an asymptomatic early CRC to become an advanced symptomatic lesion is thought to be around 2–3 years [62]. Additionally, survival from CRC is intimately related to its stage, with early CRC having an excellent outcome [62].

De novo CRCs [63] are typically superficial and flat (non-polyploid), with no detectable adenomatous remnants. Interestingly, these tumors might evolve through a distinct genetic pathway, in which the frequency of *KRAS* mutations is lower than in the adenoma-carcinoma sequence [64–66]. Approximately 15%

Table 2a
DNA methylation in CRC cell lines

Gene	Name	CRC cell line	Promotor methylation status	Gene expression	Gene expression after treatment	Technique used	Reference
APC	adenomatosis polyposis coli	DLD-1, SW480, Colo320, HT29, Colo201	HM	No		MSP	**Sakamoto** *et al.* (2001) [116]
APC	adenomatosis polyposis coli	11 MSS CRC-CL 9 MSI CRC-CL	HM 2/11 HM 2/9			MSP	**Lind *et al.*** (2004) [105]
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3	RKO, DLD-1, SW48, SW480, HT29, CoLo320	HM	No	Yes/5AdC	COBRA	**Murai** *et al.* (2005) [117]
CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	11 MSS CRC-CL 9 MSI CRC-CL	1/11 2/9			MSP	**Lind** *et al.* (2004) [105]
CDH13	Cadherin 13, H-cadherin	13 CRC-CL	HM 7/13 (54%)	No in 6/7	4/4 Yes/5AdC	MSP	**Toyooka** *et al.* (2002) [118]
CDH4	Cadherin 4, R-cadherin	RKO	HM	no	Yes/5AdC	MSP	**Miotto** *et al.* (2004) [119]
CDNK2A ($P14^{ARF}$)	cyclin-dependent kinase inhibitor 2A	DLD-1, HCT-15, SW48	HM	No	Yes/5dAC	MSP	**Zheng** *et al.* (2000) [120]
CDNK2A ($P14^{ARF}$)	cyclin-dependent kinase inhibitor 2A	11 MSS CRC-CL 9 MSI CRC-CL	5/11 8/9			MSP	**Lind *et al.*** (2004) [105]
CDNK2A (P16)	cyclin-dependent kinase inhibitor 2A	Six CRC-CL	5/6			MSP	**Wagner** *et al.* (2002) [121]
CDNK2A (p16INK4A)	cyclin-dependent kinase inhibitor 2A	11 MSS CRC-CL 9 MSI CRC-CL	8/11 7/9			MSP	**Lind *et al.*** (2004) [105]
CDX1	caudal type homeo box transcription factor 1	37 CRC-CL	HM 7/37 (19%)	No	5/5 Yes/5AdC	MSP	**Wong** *et al.* (2004) [122]
CDX1	caudal type homeo box transcription factor 1	CaCo2, SW480, HCT116, ColoDM, DLD-1, RKO	HM	No	Yes/5AdC	MSP	**Suh** *et al.* (2002) [123]
CHFR	heckpoint with forkhead and ring finger domains	HCT116, DLD1, HT29	HM	No/reduced	Yes/5AdC	MSP	**Corn** *et al.* (2003) [124]
COL1A2	Collagen, typeI, alpha2(I)	HCT116, SW480, SW620	HM	No	Yes/5AdC		**Sengupta** *et al.* (2003) [125]
DAPK1	Death associated protein kinase1	Nine CRC-CL	3/9	No in 5/9		COBRA	**Satoh** *et al.* (2002) [126]
EphA7	EPH receptor A7	DLD-1, HCT116, SW620	HM	No	Yes/5AdC	MSP	**Wang** *et al.* (2005) [127]
GATA-4	GATA binding protein 4	RKO, DLD1, HCT116, HT29, LoVo, SW480	HM	No in 4/6	Yes/5AdC	MSP	**Akiyama** *et al.* (2003) [128]
GATA-5	GATA binding protein 5	RKO, DLD1, HCT116, HT29, LoVo, SW480	HM	No in 5/6	Yes/5AdC	MSP	**Akiyama** *et al.* (2003) [128]
ID4	inhibitor of DNA binding 4	SW480, DLD1	HM	No	Yes/5Aza	MSP	**Umetani** *et al.* (2004) [129]
MGMT	O-6-methylguanine-DNA methyltransferase	11 MSS CRC-CL 9 MSI CRC-CL	5/11 5/9			MSP	**Lind *et al.*** (2004) [105]
MLH1	MutL homolog 1, colon cancer, nonpolyposis type 2	11 MSS CRC-CL 9 MSI CRC-CL	0/11 3/9			MSP	**Lind *et al.*** (2004) [105]
PTGIS	Prostaglandin I2 (prostacyclin) synthase	HCT116	HM	No	Yes/5dAC+HDAC	BS	**Frigola** *et al.* (2005) [130]
PTGS2 (Cox-2)	prostaglandin-endoperoxide synthase 2 (Cyclooxygenase)	Eight CRC-CL	HM 1/8 (RKO)	No	Yes /5AdC	COBRA	**Toyota** *et al.* (2000) [131]
RASSF1	Ras association (RalGDS/AF-6) domain family 1	Five CRC-CL	HM 4/5			MSP	**Wagner** *et al.* (2002) [121]
RASSF2	Ras association (RalGDS/AF-6) domain family 2	LOVO, DLD-1, RKO	HM	No	Yes/5AdC	COBRA	**Akino** *et al.* (2005) [132]
RUNX3	runt-related transcription factor 3	32 CRC-CL	HM 12/32	No	Yes /5AdC	MSP	**Ku** *et al.* (2004) [133]
SFRP1	Secreted frizzled-related protein 1	RKO, DLD-1, HCT116, LOVO, SW480	HM	No	Yes/5AdC	MSP	**Suzuki** *et al.* (2002) [108]
SLC5A8	solute carrier family 5 (iodide transporter), member 8	31 CRC-CL	HM 16/31	No in 23/31	6/8 Yes/5Aza	MSP	**Li** *et al.* (2003) [101]
SMARCA3 (HLTF)	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 3	34 CRC-CL	HM 9/34	No in 9/9	Yes/5Aza	MSP	**Moinova** *et al.* (2002) [134]
TMEFF2 (TPEF/HPP1)	transmembrane protein with EGF-like and two follistatin-like domains 2	LOVO, DLD-1	HM	No	Yes/5AdC	ML	**Ebert** *et al.* (2005) [135]
TMEFF2 (TPEF/HPP1)	transmembrane protein with EGF-like and two follistatin-like domains 2	HT29, LOVO	HM	No	Yes/5AdC	COBRA	**Young** *et al.* (2001) [136]

of CRCs are familial, with autosomal-dominant and autosomal-recessive modes of inheritance. The most common inherited conditions are familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC). It arises because of mutations in mismatch-repair genes, including *MLH1*, *MSH2*, *MSH3*, *MSH6*, *PMS1* and *PMS2* [67]. The loss of reparative mechanisms leads to an increased mutation rate especially in repetitive DNA sequences (microsatellite sequences), resulting in microsatellite instability (MSI) [68,69]. For example, *BAT26* (big adenine tract 26) is a microsatellite region that is altered in almost all mismatch-repair-deficient CRCs.

Additional mutated genes that can be detected in

Table 2b
DNA methylation in CRC tissue

Gene	Name	Tissue specimen	Histological type	Fraction methylated	Percentage methylated	Technique used	Reference
ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1	Formalin-fixed, paraffin-embedded or fresh frozen	CRC	67/275	24	MSP	**Van Rijnsoever** *et al.* (2002) [137]
APBA1 (MINT1)	amyloid beta (A4) precursor protein-binding, family A, member 1 (Methylated in Tumor 1)	Endoscopic biopsy specimen	Serrated Adenomas Tubular Adenomas Normal Tissue	11/27 3/34 0/16	40 9 0	MSP	**Park** *et al.* (2003) [138]
APBA2 (MINT2)	amyloid beta (A4) precursor protein-binding, family A, member 2 (Methylated in Tumor 2)	Endoscopic biopsy specimen	Serrated Adenomas Tubular Adenomas Normal Tissue	17/27 6/34 1/17	63 18 6	MSP	**Park** *et al.* (2003) [138]
APBA2 (MINT2)	amyloid beta (A4) precursor protein-binding, family A, member 2 (Methylated in Tumor 2)	Formalin-fixed, paraffin-embedded or fresh frozen	CRC	102/ 275	37	MSP	**Van Rijnsoever** *et al.* (2002) [137]
APC	adenomatosis polyposis coli		25 MSS CRC 28 MSI CRC	7/25 10/28	28 36	MSP	**Lind *et al.*** (2004) [105]
APC	adenomatosis polyposis coli		Normal tissue CRC Liver metastasis	0/21 10/47 10/24	0 21 42	ML	**Ebert** *et al.* (2005) [135]
APC	adenomatosis polyposis coli	Fresh frozen	CRC Adenoma	3/31 3/40	9 8	MSP	**Kim** *et al.* (2003) [139]
APC	adenomatosis polyposis coli	Formalin-fixed, paraffin-embedded	CRC Normal Tissue		7,4 3,3	MSP	**Xiong** *et al.* (2001) [140]
APC	adenomatosis polyposis coli	Fresh frozen	CRC Normal tissue Adenomas	20/108 0/28 9/48	18 0 18	MSP	**Esteller** *et al.* (2000) [141]
AXIN-2	AXIN 2 (CONDUCTIN, AXIL)	Fresh tissue	MSI CRC MSS CRC	5/10 0/10	50 0	HDO-MA	**Koinuma** *et al.* (2006) [106]
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3		CRC	40/61	66	COBRA	**Murai** *et al.* (2005) [117]
CDH1	Cadherin 1, type 1, E-cadherin (epithelial)		24 MSS CRC 28 MSI CRC	10/24 11/28	42 39	MSP	**Lind *et al.*** (2004) [105]
CDH1	cadherin 1, type 1, E-cadherin (epithelial)	Formalin-fixed, paraffin-embedded or fresh frozen	CRC	12/22	54,5	MSP	**Kanazawa** et al. (2002) [142]
CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	Fresh frozen	CRC	34/61	56	MSP	**Garinis** *et al.* (2002) [143]
CDH13	H- Cadherin	Fresh frozen	CRC	23/61	38	MSP	**Hibi** *et al.* (2005) [144]
CDH13	H- cadherin	Fresh frozen	CRC	27/84	32	MSP	**Hibi** *et al.* (2004) [145]
CDH13	H- cadherin	Fresh tissue	CRC Adenoma Non-malignant mucosa CRC-CL	17/35 8/19 2/33 7/13	49 42 6 54	MSP	**Toyooka** *et al.* (2002) [118]
CDH4	Cadherin 4, R-cadherin		CRC Normal tissue f. CRC-Pat Adenoma Normal tissue	38/49 5/17 10/10 0/10	78 29 100 0	MSP	**Miotto** *et al.* (2004) [119]
CDKN2A	cyclin-dependent kinase inhibitor 2A	Formalin-fixed, paraffin-embedded	Adenomas	14/41	34	MSP	**Petko** *et al.* (2005) [96]
CDNK2A (P16)	cyclin-dependent kinase inhibitor 2A	Fresh frozen	CRC Duke's A, B CRC Duke's C,C Total	4/33 22/29 26/62	12 76 42	MSP	**Yi** *et al.* (2001) [146]
CDNK2A (P16)	cyclin-dependent kinase inhibitor 2A	Formalin-fixed, paraffin-embedded or fresh frozen	CRC	100/275	36	MSP	**Van Rijnsoever** *et al.* (2002) [137]
CDNK2A (P16)	cyclin-dependent kinase inhibitor 2A	Formalin-fixed, paraffin-embedded	CRC Normal Tissue	62 62	3,8 2,3	MSP	**Xiong** *et al.* (2001) [140]
CDNK2A (P16)	cyclin-dependent kinase inhibitor 2A		CRC	6/29	21	MSP	**Wagner** *et al.* (2002) [121]
CDNK2A (P16)	cyclin-dependent kinase inhibitor 2A	Endoscopic biopsy specimen	Serrated Adenomas Tubular Adenomas Normal Tissue	11/27 10/34 0/16	40 29 0	MSP	**Park** *et al.* (2003) [138]
CDNK2A (P16)	cyclin-dependent kinase inhibitor 2A		CRC (88) CIMP+ CIMP- Adenoma (45) CIMP+ CIMP-	 21/41 0/47 12/22 0/23	 51 0 55 0	MSP BS	**Toyota** *et al.* (2000) [147]
CDNK2A (P16)	cyclin-dependent kinase inhibitor 2A	Fresh frozen	Liver metastasis	8/11	73	MSP	**Nakayama** *et al.* (2003) [148]
CDNK2A (P16)	cyclin-dependent kinase inhibitor 2A	Fresh frozen	CRC	20/52	38	MSP	**Zou** *et al.* (2002) [114]
CDNK2A (P16)	cyclin-dependent kinase inhibitor 2A	Fresh frozen	CRC	44/94	47	MSP	**Nakayama** *et al.* (2002) [113]
CDNK2A (P16)	cyclin-dependent kinase inhibitor 2A	Fresh frozen	CRC	31/58	53	MSP	**Lecomte** *et al.* (2002) [115]
CDNK2A ($P14^{ARF}$)	cyclin-dependent kinase inhibitor 2A	Fresh frozen	Adenoma from: FAP-patient Multiple Adenoma patient MSI-H CRC-Pat. MSS/MSI-L CRC-Pat.	 13/32 20/29 12/14 14/16	 41 69 86 88	MSP	**Wynter** *et al.* (2006) [149]
CDNK2A ($P14^{ARF}$)	cyclin-dependent kinase inhibitor 2A		24 MSS CRC 28 MSI CRC	3/24 17/28	12 39	MSP	**Lind *et al.*** (2004) [105]
CDNK2A ($P14^{ARF}$)	cyclin-dependent kinase inhibitor 2A		CRC Dysplasia UC mucosa Normal Tissue	19/38 4/12 3/5 3/40	50 33 60 3,7	MSP BS	**Sato** *et al.* (2002) [86]

Table 2b, continued

CDNK2A (p16INK4A)	cyclin-dependent kinase inhibitor 2A	Fresh frozen	Adenoma from: FAP-patient Multiple Adenoma patient MSI-H CRC-Pat. MSS/MSI-L CRC-Pat.	 17/33 13/29 9/14 10/16	 52 45 64 63	MSP	**Wynter** *et al.* (2006) [149]
CDNK2A (p16INK4A)	cyclin-dependent kinase inhibitor 2A		25 MSS CRC 28 MSI CRC	7/25 10/28	28 36	MSP	**Lind *et al.*** (2004) [105]
CHFR	checkpoint with forkhead and ring finger domains	Fresh frozen and paraffin-embedded	CRC Normal tissue	11/30 2/9	37 22	MSP	**Corn** *et al.* (2003) [124]
COL1A2	Collagen, typeI, alpha2(I)	Fresh frozen	CRC	5/6	83	MSP	**Sengupta** *et al.* (2003) [125]
DAPK1	Death associated protein kinase 1	CRC		4/28	14	COBRA	**Satoh** *et al.* (2002) [126]
DAPK1	Death associated protein kinase 1	Fresh frozen	CRC patients Normal tissue	67/122 0/10	55 0	MSP	**Yamaguchi** *et al.* (2003) [150]
EphA7	EPH receptor A7	Formalin-fixed, paraffin-embedded	CRC	37/75	49	MSP	**Wang** *et al.* (2005) [127]
ESR1	Estrogen Receptor 1	Fresh frozen	UC with neoplasia UC without neoplasia		25.4 – 17.8 4.0 – 6.4	COBRA	**Tominaga** *et al.* (2005) [151]
ESR1	Estrogen Receptor 1	Formalin-fixed, paraffin-embedded	CRC Normal Tissue		28,1 7,0	MSP	**Xiong** *et al.* (2001) [140]
GATA-4	GATA binding protein 4		CRC	30/45	67	MSP	**Akiyama** *et al.* (2003) [128]
GATA-5	GATA binding protein 5		CRC	28/44	64	MSP	**Akiyama** *et al.* (2003) [128]
ID4	inhibitor of DNA binding 4	Formalin-fixed, paraffin-embedded	Normal tissue Adenoma CRC Liver-metastasis	0/9 0/13 49/92 19/26	0 0 53 73	MSP	**Umetani** *et al.* (2004) [129]
MGMT	O-6-methylguanine-DNA methyltransferase	Formalin-fixed, paraffin-embedded	Adenomas 19/39		49	MSP	**Petko** *et al.* (2005) [96]
MGMT	O-6-methylguanine-DANN methyltransferase		25 MSS CRC 28 MSI CRC	10/25 11/28	40 39	MSP	**Lind *et al.*** (2004) [105]
MGMT	O-6-methylguanine-DANN methyltransferase	Fresh frozen	Adenoma from: FAP-patient Multiple Adenoma patient MSI-H CRC-Pat. MSS/MSI-L CRC-Pat.	 22/33 12/29 6/14 8/15	 66 41 43 53	MSP	**Wynter** *et al.* (2006) [149]
MINT31	Homo sapiens clone MINT31 colon cancer differentially methylated CpG island genomic sequence.	Endoscopic biopsy specimen	Serrated Adenomas Tubular Adenomas Normal Tissue	8/28 8/34 1/16	29 24 6	MSP	**Park** *et al.* (2003) [138]
MLH1	MutL homolog 1, colon cancer, nonpolyposis type 2		25 MSS CRC 28 MSI CRC	0/25 11/28	0 39	MSP	**Lind *et al.*** (2004) [105]
MLH1	MutL homolog 1, colon cancer, nonpolyposis type 2	paraffin-embedded and fresh frozen	HNPCC	1/14		MSP	**Gazzoli** *et al.* (2002) [152]
MLH1	MutL homolog 1, colon cancer, nonpolyposis type 2	Formalin-fixed, paraffin-embedded	MSS MSI	18/33 18/90	55 20	COBRA	**Nakagawa** *et al.* (2001) [93]
MLH1	MutL homolog 1, colon cancer, nonpolyposis type 2	Endoscopic biopsy specimen	Serrated Adenomas Tubular Adenomas	4/26 0/34	15 0	MSP	**Park** *et al.* (2003) [138]
MLH1	MutL homolog 1, colon cancer, nonpolyposis type 2	Fresh frozen	IBD neoplasia MSI-H MSI-L MSS	 6/13 1/16 4/27	 46 6 15	MSP	**Fleisher** *et al.* (2000) [153]
MYOD (MYF-3)	myogenic differentiation 1	Fresh frozen	Adenomas CRC	15/17 104/105	88 99	SB	**Shannon** *et al.* (1999) [154]
PTGIS	Prostaglandin I2 (prostacyclin) synthase	Fresh frozen	Adenoma CRC	3/10 43/100	30 43	BS	**Frigola** *et al.* (2005) [130]
PTGS (Cox-2)	prostaglandin-endoperoxide synthase 2 (Cyclooxygenase)		CRC Adenoma	12/92 7/50	13 14	COBRA	**Toyota** *et al.* (2000) [131]
RASSF1	Ras association (RalGDS/AF-6) domain family 1	Formalin-fixed, paraffin-embedded	CRC Normal tissue HD Normal tissue CRC-Pat	45/222 0/6 1/7	20 0	MSP	**Van Engeland** *et al.* (2002) [155]
RASSF1	Ras association (RalGDS/AF-6) domain family 1		MS1+ CRC HNPCC	16/31 6/20	52 30	MSP	**Oliveira** *et al.* (2005) [156]
RASSF2	Ras association (RalGDS/AF-6) domain family 2		Adenoma CRC	21/49 51/122	43 42	COBRA	**Akino** *et al.* (2005) [132]
RUNX3	runt-related transcription factor 3		CRC	31/92	34	MSP	**Imamura** *et al.* (2005) [157]
SFRP1 SFRP2 SFRP4 SFRP5	Secreted frizzled-related protein 1, 2, 4, 5		CRC	118/124 111/124 36/124 73/124	95 90 29 59	MSP	**Suzuki** *et al.* (2002) [108]
SLC5A8	solute carrier family 5 (iodide transporter), member 8		CRC Normal tissue CRC-Pat Normal tissue HD	38/64 0/26 0/12	59	MSP	**Li** *et al.* (2003) [101]
SMARCA3 (HLTF)	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 3		CRC	27/63	43	MSP	**Moinova** *et al.* (2002) [134]
TMEFF2 (TPEF/HPP1)	transmembrane protein with EGF-like and two follistatin-like domains 2	Fresh tissue	Normal tissue CRC Liver metastasis	1/21 36/47 19/24	5 77 79	ML	**Ebert** *et al.* (2005) [135]
TMEFF2 (TPEF/HPP1)	transmembrane protein with EGF-like and two follistatin-like domains 2	Fresh frozen	Adenoma from: FAP-patient Multiple Adenoma patient MSI-H CRC-Pat. MSS/MSI-L CRC-Pat.	 10/32 17/26 9/14 7/13	 31 65 64 54	COBRA	**Wynter** *et al.* (2006) [149]
TMEFF2 (TPEF/HPP1)	transmembrane protein with EGF-like and two follistatin-like domains 2	Fresh tissue	Adenoma HP AC	6/9 17/27 46/55	66 63 84	COBRA	**Young** *et al.* (2001) [136]
TMEFF2 (TPEF/HPP1)	transmembrane protein with EGF-like and two follistatin-like domains 2	Formalin-fixed, paraffin-embedded	UC mucosa Dysplasia CRC	0/5 4/10 24/48	0 40 50	MSP	**Sato** *et al.* (2002) [87]
VIM	Vimentin	Fresh tissue	CRC patients Healthy Donor	95/153 1/46	62 2	MSP	**Chen** *et al.* **(2005)** [158]

Table 2c
DNA methylation in serum/plasma and stool of CRC patients

Gene	Name	Specimen	Donor	Fraction methylated	Percentage methylated	Technique used	Reference
CDH4	Cadherin 4, R-cadherin	Peripheral blood	CRC patient Blood lymphocytes	32/46 0/17	70 0	MSP	**Miotto** *et al.* (2004) [119]
CDKN2A (P16)	cyclin-dependent kinase inhibitor 2A	Stool	Patients with adenomas Healthy Donor	9/29 3/19	31 16	MSP	**Petko** *et al.* (2005) [96]
CDNK2A (P16)	cyclin-dependent kinase inhibitor 2A	Serum	CRC patients	13/44	30	MSP	**Nakayama** *et al.* (2002) [113]
CDNK2A (P16)	cyclin-dependent kinase inhibitor 2A	Serum	CRC patients with recurrence	31/45	69	MSP	**Nakayama** *et al*. (2003) [148]
CDNK2A (P16)	cyclin-dependent kinase inhibitor 2A	Serum	CRC patients Patients with Adenomas Healthy Individuals	14/20 0/34 0/10	70	MSP	**Zou** *et al.* (2002) [114]
CDNK2A (P16)	cyclin-dependent kinase inhibitor 2A	Plasma	CRC patients	21/31	68	MSP	**Lecomte** *et al.* (2002) [115]
DAPK1	Death associated protein kinase 1	Serum	CRC patients	3/14	21	MSP	**Yamaguchi** *et al.* (2003) [150]
HIC1	Hypermethylated in cancer 1	Stool	CRC patients Patients with Adenomas Patients with HP Healthy Individuals		42 31 0 0	MSP	**Lenhard** *et al.* (2005) [159]
MGMT	O-6-methylguanine-DNA methyltransferase	Stool	Patients with adenomas Healthy Donor	14/29 5/18	48 27	MSP	**Petko** *et al.* (2005) [96]
MLH1	MutL homolog 1, colon cancer, nonpolyposis type 2	Serum	Matched serum samples to MLH1 positive CRCs	3/9	33		**Grady** *et al*. (2001) [112]
MLH1	MutL homolog 1, colon cancer, nonpolyposis type 2	Serum	CRC patients Healthy Donor	19/49 1/41	39 2	MSP	**Leung** *et al.* (2005) [160]
PGR	progesterone receptor	Stool	CRC patients Healthy Donor	18/23 8/26	78 31	ML	**Müller** *et al.* (2004) [38]
SFRP2	Secreted frizzled-related protein 2	Stool	CRC patients Healthy Donor	19/23 6/26	83 26	ML	**Müller** *et al.* (2004) [38]
SFRP5	Secreted frizzled-related protein 5	Stool	CRC patients Healthy Donor	18/23 9/26	78 35	ML	**Müller** *et al.* (2004) [38]
SMARCA3 (HLTF)	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 3	Serum	CRC patients Healthy Donor	14/49 3/41	29 7	MSP	**Leung** *et al.* (2005) [160]
VIM	Vimentin	Stool	CRC patients Healthy Donor	43/94 20/198	46 10	MSP	**Chen** *et al.* **(2005)** [158]

CRC-CL Colorectal cancer cell lines
CRC-Pat Colorectal cancer patient
HD Healthy donor
HP Hyperplastic polyp

HM Hypermethylation

FAP Familial Adenomatous Polyposis
UC Ulcerative Colitis
IBD Inflammatory Bowel disease neoplasia
HNPCC hereditary nonpolyposis colon cancer
MSI-H Microsatellite Instability high-frequency
MSI-L Microsatellite Instability low-frequency
MSS Microsatellite stable
CIMP+ CpG island methylator phenotype positive
CIMP- CpG island methylator phenotype negative

HDO-MA high-density oligonucleotide microarrays
MSP Methylation specific PCR
COBRA Combined bisulfite restriction analysis
ML MethyLight
SB Southern Blot
BS Bisulfite sequencing

early colorectal carcinogenesis are e.g. *SMAD2/4*, *TP53* (for review see [23]).

As outlined above, the Wingless/Wnt signaling pathway plays a vital role in malignant transformation during the adenoma-carcinoma sequence [55]. Aberrant WNT signaling is an early event in the process of carcinogenesis in approximately 90% of CRCs [70]. It occurs mainly through inactivating mutations of the tumor suppressor gene *APC* [71–73] and less often through mutations of *β-catenin* or *AXIN2* [74,75]. These alterations result in cellular accumulation of *β-catenin*, which subsequently serves as an activator of T-cell factor/lymphoid-enhancing factor (Tcf/LEF)-dependent transcription. Several β-catenin/TCF target genes are presumed to contribute to tumor initiation and progression in mice and humans [76].

5.2. DNA methylation in general

Changes in the status of DNA methylation, known as epigenetic alterations, are one of the most common molecular alterations in human neoplasia [77]. Epigenetic changes differ from genetic changes mainly in that they occur at a higher frequency than do genetic changes, are reversible upon treatment with pharmacological agents and occur at defined regions in a gene. Epigenetics describes a trait that is heritable, yet not based on a change in primary DNA sequence [77–79]. In recent years it has become clear that there is a synergy between genetic and epigenetic changes and that Knudson's two-hit hypothesis needs to be revised: instead of only two possibilities (loss of heterozygosity or homozygous deletion), there is also a third possibility – transcriptional silencing by DNA methylation

of promoters – that can disable tumor-suppressor gene transcription [80].

Cytosine methylation occurs after DNA synthesis, by enzymatic transfer of a methyl group from the methyl donor S-adenosylmethionine to the carbon-5 position of cytosine. Cytosines are methylated in the human genome mostly when located 5' to a guanosine. In human somatic cells, 5mC accounts for $\sim$ 1% of total DNA bases and therefore affects 70%–80% of all CpG dinucleotides in the genome. These CpG dinucleotides are severely under-represented in the human genome, because they are affected by a high rate of methylcytosine-to-thymine transition mutations. Remaining CpG dinucleotides are unequally distributed across the human genome, which means there exist stretches of sequences without CpG dinucleotides interspersed by so-called CpG islands. CpG islands are defined as a 500-base-pair window with a G:C content of at least 55% and an observed overexpected frequency of at least 0.65. Computational analysis of the human genome sequence predicts 29000 CpG islands. It has been increasingly recognized over the past years that the CpG islands of a large number of genes, which are mostly unmethylated in normal tissue, are methylated to varying degrees in human cancers. Methylation of some CpG islands in non-malignant tissue also increases with age, whereas the total genomic content of 5mC declines. The same is true during carcinogenesis of several tumors (e.g. adenoma-carcinoma sequence), where methylation takes place at specific promoter regions, followed by general hypomethylation of the whole genome, and this is thought to induce a higher rate of chromosomal instability (for review see [77–79,81]). Post-synthetic covalent addition of a methyl group to cytosine is mediated by the three known active DNA cytosine methyltransferases (DNMT1, 3a, and 3b). When DNA containing a symmetrically methylated CpG dinucleotide is replicated, the result is two double-stranded DNA molecules, each containing a methylated CpG dinucleotide on the parental strand, but also containing an unmethylated CpG dinucleotide on the newly synthesized strand. The methylated state of the site in the parent molecule is maintained in the daughter molecules when a maintenance methyltransferase recognizes the hemimethylated site and methylates the unmethylated cytosine, restoring the symmetrically methylated CpG dinucleotide pair. DNMT1 is mainly responsible for maintenance of DNA methylation, whereas DNMT3a and DNMT3b have been shown to methylate hemimethylated and unmethylated DNA with equal efficiency. Overexpression of both DNMT1 and DNMT3 mRNAs has been reported in human tumors.

The reciprocal relationship between the density of methylated cytosine residues and the transcriptional activity of a gene has been widely documented. It should be emphasized, however, that this inverse correlation has been demonstrated conclusively only for methylation in the promoter regions and not in the transcribed parts of a gene. Several tumor-suppressor genes contain CpG islands in their promoters, and many of them show evidence of methylation silencing. After changes associated with histone deacetylation have occurred and these CpG islands have become methylated, the relevant genes become irrevocably silent (for review see [77–79,81]).

Advances in the technology of DNA methylation analysis have spurred the discovery of numerous cases of hypermethylation of tumor-suppressor gene promoters in human tumors. Furthermore, it has become clear that methylated DNA can be detected in tumor-derived DNA found in the serum of cancer patients [47–52]. Additionally, methylated DNA can also be found in samples obtained from cancer patients by draining to the outside of the body either physiologically (stool, vaginal secretion [38,82]) or artificially (peritoneal fluids [83]).

5.3. Adenoma-carcinoma sequence – epigenetic alterations

The past decade saw a large number of studies dealing with DNA methylation changes in tumorigenesis of CRC. The data concerning hypermethylation were predated by studies of global hypomethylation at an early stage in colorectal neoplasia (for review see [84]). Age is the principal function of CRC incidence, and age-related methylation changes are well documented for CRC [84]. Another risk factor for CRC is ulcerative colitis (UC). Interestingly, it was found that both the dysplastic and nondysplastic mucosa of UC patients with neoplasia have significantly elevated levels of age-related methylation, indicating that chronic inflammation is associated with high levels of methylation, perhaps as a result of increased cell turnover, and that UC can be viewed as resulting in premature aging of colorectal epithelial cells [85]. Furthermore, specific hypermethylation was also seen to be a very early event in UC-associated carcinogenesis, thus indicating the possibility that hypermethylation can serve as a biomarker for early detection of cancer or dysplasia in UC [86, 87].

Deficiencies in the mismatch repair (MMR) system result in mutation rates 100-fold greater than for normal cells as a direct consequence of an inability to faithfully replicate the genome. In particular, these mutations are evident as frameshifts in microsatellite sequences. They are normally stable, but slippage during DNA replication generates insertions/deletions and, if perpetuated, engenders microsatellite instability (MSI), the hallmark of the replication error phenotype. MSI is also present in 10%–15% of cases of sporadic colorectal cancer, but is rarely caused by mutation. It has been reported that a strong correlation exists between genetic instability and methylation capacity, indicating that methylation abnormalities may play a role in chromosome segregation in cancer cells. A central MMR gene, called *MLH1*, was reported to be methylated in sporadic CRCs and strongly associated with MSI [84,88–90]. The finding of aberrant methylation of *MLH1* in sporadic MSI colon cancers, and the restoration of MLH1 expression by demethylating the *MLH1* promoter in cell lines derived from such cancers, strongly suggests that such aberrant methylation could be a cause rather than a consequence of colon carcinogenesis [84,88–90]. Fine-structure analysis of the methylation status of specific CpGs in the *MLH1* promoter has shown that the methylation status of small clusters of CpGs in the 5' region of the *MLH1* promoter appears to dictate the transcriptional status of the gene [91]. Hawkins and Ward [92] also reported such a *MLH1* hypermethylation in hyperplastic polyps of patients with sporadic CRC with MSI, suggesting that hypermethylation of the *MLH1* gene is a critical step in progression to carcinoma. It was recently shown that methylation of *MLH1* promoter in the normal colonic mucosa is closely associated with age and the development of sporadic MSI+ colon cancers [93].

As mentioned above, several findings gave rise to the hypothesis that epigenetic and genetic changes act together to promote cancer formation [94]. Although mutation of *CDKN2A*/$p16$ has not been described in CRC, methylation of *CDKN2A*/$p16$ is detected in 40% of CRCs [95]. Furthermore, it has been reported that methylation plays an important role in colon adenomas [96,97]. For example, *CDKN2A*/$p16$ methylation is more common in adenomas with tubulovillous/villous histology, a characteristic associated with more frequent predisposition to invasive carcinoma [97]. This observation demonstrates that aberrant promoter methylation occurrs early in the adenoma carcinoma sequence, although it does not confirm that the aberrant *CDKN2A*/$p16$ methylation is a primary, rather than a secondary, event in the tumorigenesis process. Additionally, DNA methylation status of *MGMT*, *CDKN2A*, and *MLH1* in colon adenomas and hyperplastic polyps has been determined to evaluate the timing and frequency of these events in the adenoma-carcinoma progression sequence and subsequently to analyze the potential for these methylated genes to be molecular markers for adenomas and hyperplastic polyps [96]. It has been revealed that methylated *MGMT*, *CDKN2A* and *MLH1* occur in 49%, 34% and 7% of adenomas and in 5%, 10% and 7% of hyperplastic polyps, respectively, and that they are more common in histologically advanced adenomas. Furthermore, methylated *CDKN2A*, *MGMT* and *MLH1* were detected in fecal DNA from 31%, 48% and 0% of individuals with adenomas, indicating the potential of fecal DNA-based assays as a useful diagnostic test for polyps [96].

In addition to the interest in the role of epigenetic alterations in established cancers, the evidence showing increased methylation in CpG islands in non-neoplastic tissues has prompted considerable interest in the role aberrant DNA methylation may have as a pre-neoplastic event. Indeed, there is evidence that aberrant CpG island methylation may occur as the result of a genetic predisposition or a field effect. Ahuja et al. [98] showed that aberrant CpG island methylation occurs in histologically normal colon epithelium in an age-dependent fashion and that half of the genes involved in this age-related methylation are the same as those involved in colon carcinogenesis. The cause of this age-related DNA methylation is unknown, but current models suggest that the methylation occurs as the consequence of local predisposing factors in DNA (e.g. methylation control centers, such as Sp1 (specificity protein 1) sites or tandem B1 elements), environmental exposures, and/or a genetic predisposition to aberrant DNA methylation (for review see [55]). Furthermore, it is likely detection of colon adenomas with methylation may identify colonic epithelium that is at significant risk for genetic alterations that will lead to colon tumor formation [99].

As mentioned above, the adenoma–carcinoma progression is believed to be an evolutionary process in which neoplastic cells acquire heritable genetic and epigenetic alterations that drive the carcinogenesis process. Each major step in this evolutionary process is usually accompanied by a recognizable histological change that proceeds from a benign tubular adenoma to an advanced adenoma (e.g. tubulovillous or villous adenoma) and finally to invasive adenocarcinoma. It

is supposed for CRC that specific gene mutations (e.g. *APC* mutations) initiate the formation of tubular adenomas and that others (e.g. *TP53* mutations) drive the malignant transformation of the adenomas (for review see [55]).

Aberrant DNA methylation of specific loci has been identified in the earliest precursor lesions for colon adenocarcinomas, aberrant crypt foci (ACF). *MINT1*, *MINT31*, *SLC5A8* and *MGMT* methylation has been found in ACFs and in adenomas [97,100,101]. Additionally, early work has reported that CRCs with hypermethylated *MLH1* and/or *CDKN2A*/$p16$ may belong to a distinct subclass of CRCs, termed the CIMP (CpG island methylator phenotype), that demonstrate genome-wide aberrant methylation of gene promoters and that may be caused by a distinct and unique mechanism. Interestingly, the specific genes commonly found to be methylated in CRC differ from those commonly found to be methylated in other tumor types, suggesting that there is a selective process driving the occurrence of methylated genes [95,102].

Bai et al. [103] found that the methylation status of genes is established in the adenoma phase of the adenoma–carcinoma sequence, suggesting that these events occur during initiation of the colon neoplasms and do not have a functional role in the progression of colon cancer. In contrast, it has been observed that a subset of genes (*MLH1*, *RASSF1*, *CDKN2A*, *GSTP1*, *THBS1* and *TIMP3*) was more commonly methylated in CRCs than in adenomas, suggesting that at least some genes may affect the transformation step in CRC formation [104]. The same study group found no difference in the proportion of genes methylated in progressively more advanced stages of adenocarcinoma, but they did not assess the frequencies of specific methylated genes in various stages of CRC, which would be more informative with regard to understanding the role of epigenetic events in the clonal evolution of CRC [104].

Furthermore, recent studies have shown that epigenetic silencing of genes involved in Wingless/Wnt signaling is an alternative mechanism in colorectal carcinogenesis. Compared to inactivating *APC* mutations, epigenetic silencing of *APC* seems to play a minor role [105]. Koinuma et al. [106] documented epigenetic silencing of *AXIN2* in MSI+ colorectal cancer. Nevertheless, two groups have documented frequent promotor hypermethylation and epigenetic silencing of genes encoding secreted frizzled-related proteins (SFRPs) which are thought to contribute to constitutive WNT signaling [107,108]. While *SFRP* and *WIF-1* [109] methylation-associated silencing occurs across the whole spectrum of colorectal tumorigenesis, Aguilera et al. [110] demonstrated that Dickkopf-1 (*DKK-1*) promotor hypermethylation was present only in advanced colorectal neoplasms.

At bottom, significant evidence is provided that the aberrant methylation of genes contributes to the initiation of adenomas and their progression to CRC. A summary of reported methylated genes in CRC cell lines or CRC tissue is presented in Table 2a and 2b.

5.4. Epigenetic alterations in the bloodstream of CRC patients

An increasing number of studies have reported the presence of methylated DNA in serum/plasma of patients with various types of malignancies and the absence of methylated DNA in normal control patients (for review see [47]). For the past five years our research group was mainly interested in evaluating DNA methylation changes in serum of cancer patients [47–52]. We came to the conclusion that there is great potential for the use of these epigenetic markers as early detection markers, markers for prognostication and even for therapy monitoring. In terms of CRC, several interesting studies have been reported: Two decades ago, Shapiro et al. [111] for the first time reported markedly elevated circulating DNA levels in patients with malignant gastrointestinal disease as compared to moderately elevated levels in benign disease and minimal values in normal controls. Grady *et al.* [112] found methylated *MLH1* promoter DNA in the serum of patients with microsatellite unstable CRCs. Other studies reported aberrant $p16$ methylation in the serum of CRC patients, indicating its potential role as a tumor marker [113,114]. Methylated $p16$ tumor DNA in the serum or plasma of CRC patients seems to be associated with later Dukes' stages and with poorer prognosis [114,115]. A summary of reported methylated genes in serum/plasma of CRC patients is presented in Table 2c. The potential use of circulating methylated DNA in serum/plasma of CRC patients for therapy monitoring is presented in Fig. 1.

5.5. Epigenetic alterations in stool of CRC patients

Detection of hypermethylated DNA markers might help identify patients with CRC and precursors using stool. An initial feasibility study using a panel of three markers (*CDKN2A*, *MGMT* and *MLH1*) detected hypermethylation in DNA extracted from stools of seven of 12 CRC patients (giving a sensitivity of 58%), but

also in three of ten normal controls (giving a specificity of 70%) (for review see [23]).

Very recently, our study group [38] was able to demonstrate secreted frizzled-related protein 2 (*SFRP2*) methylation – an antagonist of the WNT signaling pathway that is commonly methylated in CRC tissue specimens [108] – as the most sensitive single DNA-based marker in stool for identification of CRC (sensitivity 77%–90%, specificity 77%). For this study we used MethyLight analysis of fecal DNA from three independent sets of patients. Additionally, DNA methylation was detected in three of five proximal (right-sided) cancers.

Furthermore, Petko et al. [96] demonstrated that detection of methylated genes in fecal DNA from individuals with colon polyps carrying methylated genes is possible. Detection of DNA methylation in fecal DNA holds promise as a key component of screening modalities for CRC, not least of all in view of its potential contribution to detecting proximal (right-sided) cancers (Fig. 1). It remains to be seen whether a combination of genetic and epigenetic markers will identify CRC at an early stage. A summary of reported methylated genes in stool of CRC patients is presented in Table 2c.

Consequently, detection of genetic or epigenetic alteration or both in several specimens (stool, blood) from patients with CRC may have the potential for early detection of CRC. Effective early detection of adenomas would offer several benefits, such as lowered CRC incidence and reduced need for surgical intervention. As a lengthy period is required for CRC to develop from an adenoma, an effective adenoma screening test would need to be performed less frequently than a test for early CRC. However, because only a small minority of adenomas is destined to progress to malignancy, detection of adenomas would involve gross overtreatment of patients, which would be costly and harmful, both physically and psychologically. It could be argued that the optimal test would be one that accurately detected advanced adenomas with a high chance of malignant progression, but this requires better understanding of the natural history of such lesions [23]. Genetic and epigenetic markers may also serve as tools for therapy monitoring in cancer patients in order to detect early progression of the disease and offer immediate and specific treatment regimens (e.g. curative resection of liver and lung metastases) as a means of ultimately ensuring longer survival and better quality of life.

6. Conclusions

CRC is a common malignancy. Advances in cancer treatment and improvements in cancer outcome over the past few decades have been modest. In the United States, 57% of CRC patients have regional or distant spread of their disease at the time of diagnosis [14]. Only modest gains in the survival of CRC patients with advanced disease at time of diagnosis have been achieved over the past few decades. Early detection seems to be one of the most important approaches to reducing mortality by identifying cancer while still localized and curable, as well as to reducing morbidity and costs. Furthermore, longer survival and better quality of life can be achieved with earlier detection of progressive CRC.

Cervical cancer (CC) provides an excellent example of the power of early detection. Its effectiveness is mostly due to: its high acceptance in the population; the fact that the "organ of interest" is easily accessible in a non-invasive procedure; detailed knowledge of the alterations during pathogenesis (progressing from low-grade to intermediate- to high-grade CIN and eventually to invasive cancer); and that changes can easily be identified by a very well-established marker like cytology.

With regard to CRC, the natural history of the disease also seems to show progression from low-grade to intermediate- to high-grade lesions and eventually to invasive cancer (adenoma-carcinoma model of CRC carcinogenesis [7,54,56]). Especially alterations in the WNT pathway occur in malignant transformation during the adenoma-carcinoma sequence. Aberrant WNT signaling is an early event in the process of carcinogenesis in approximately 90% of CRCs [70]. Consequently, many of the genetically or epigenetically altered genes occurring during colorectal carcinogenesis (e.g. in the WNT pathway) may have some potential to serve as early detection markers or markers for therapy monitoring.

Early-detection researchers should therefore try to gain more insight into the molecular alterations occuring during progression from adenoma to carcinoma and should try to evaluate all possible target genes for early detection or therapy monitoring in CRC (as we attempted for DNA methylation markers in this review). Sullivan Pepe et al. [15] reported five phases of biomarker development for early detection of cancer. Up to now, research for early detection and therapy monitoring markers in CRC is still in *Phase 1* and *Phase 2* of the reported five phases: many preclinical ex-

ploratory studies aiming to evaluate the expression and regulation of several genes (by mutation or epigenetic alteration) and proteins in CRC tissues and cell lines and comparable healthy organ tissue to identify candidates for early detection. *Phase 2* of early-detection research still takes the shape of assay development and validation in specimens obtained non-invasively, such as serum, plasma or stool. The goal of these studies is to evaluate their ability to differentiate between patients with clinically established disease and healthy controls. To our knowledge, nothing has yet been published on the recommended *phases 3*, *4* and *5*, including multicenter prospective screening studies and estimation of expected screening costs.

Finally, it must also be mentioned that the power of a screening test is not only dependent on its specificity and sensitivity but also on people's willingness to participate in a given screening program. Looking at CRC screening, only 40% of American women and men aged 50 years and older reported recent screening with an endoscopic procedure and only 20% of both sexes reported having undergone screening with a fecal occult blood test [12]. Reasons for such a small number of participants may be uncomfortable and unpleasant preparation procedures for endoscopy, sometimes painful examination procedures, complications during endoscopy and low sensitivity or specificity of FOBT.

Changes in DNA methylation have been recognized as one of the most common molecular alterations in human tumors, including CRC (for review see [84]). It has become clear that methylated DNA can be detected in tumor-derived DNA found in the bloodstream of cancer patients [47–52] and in samples obtained from cancer patients by draining to the outside of the body (stool, vaginal secretion [38,82] or peritoneal fluids [83]). This specifically altered DNA may serve, on the one hand, as a possible new screening marker for CRC and, on the other hand, as a tool for therapy monitoring in patients having had CRC.

Very recently, our study group was able to identify secreted frizzled-related protein 2 (*SFRP2*) methylation in stool as the most sensitive single DNA-based marker for identification of CRC [38]. Additionally, detection of DNA methylation was successfully achieved in three of five proximal (right-sided) cancers. Therefore, testing for methylated DNA in stool samples may have great potential as an alternative screening tool for CRC (Fig. 1). As well as being of potential value in population screening, an effective molecular stool test might also be of use in reducing the frequency of follow-up surveillance colonoscopies required for patients with known disease (e.g. CRC in own history, long-standing inflammatory bowel disease with the known increased risk for CRC development; for review see [23]).

CRC recurrent disease mainly occurs in the regional abdominal lymph nodes, the liver or the lung and less frequently in the resected segment of the colorectum. Nevertheless, the life-threatening event in CRC is not lymph node metastasis *per se*, but hematogenous metastases which mainly affect the liver or the lung. Therefore, a screening test that is sensitive for hematogenous metastases and could be performed in patients' serum or plasma will have impact on early detection of patients with progressive CRC (Fig. 1). Such a test subsequently offers immediate start of specific treatment regimens (e.g. curative resection of liver and lung metastases).

Finally, we conclude that DNA methylation changes in CRC patients may serve, on the one hand, as a possible new screening marker for CRC and, on the other hand, as a tool for therapy monitoring in patients having had CRC.

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Abbreviations

ACF	aberrant crypt focus
CC	cervical cancer
CRC	colorectal cancer
DCBE	double-contrast barium enema
FOBT	fecal occult blood test
FS	flexible sigmoidoscopy
MMR	mismatch repair
MSI	microsatellite instability
UC	ulcerative colitis

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