The interplay between genetics and epigenetics in colorectal cancer

Citation for published version (APA):

Derks, S. (2009). *The interplay between genetics and epigenetics in colorectal cancer*. [Doctoral Thesis, Maastricht University]. Datawyse / Universitaire Pers Maastricht. <https://doi.org/10.26481/dis.20091002sd>

Document status and date:

Published: 01/01/2009

DOI:

[10.26481/dis.20091002sd](https://doi.org/10.26481/dis.20091002sd)

Document Version:

Publisher's PDF, also known as Version of record

Please check the document version of this publication:

- A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
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Sarah Derks

ISBN 978 90 5278 858 6

Cover	Abel Derks
Lay-out	Yvonne Leenders
Printed by	Datawyse Universitaire Pers Maastricht

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The interplay between genetics and epigenetics in colorectal cancer

PROEFSCHRIFT

Ter verkrijging van de graad van doctor
aan de Universiteit Maastricht,
op gezag van de Rector Magnificus,
Prof. mr. G.P.M.F. Mols,
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen
op vrijdag 2 oktober 2009 om 12:00 uur

door

Sarah Derks

geboren op 20 juni 1981 te 's-Gravenhage

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The studies described in this thesis were funded by the Kootstra Talent Fellowship, the Transnational University Limburg and the Dutch Cancer Society (KWFVU02-2618).

Financial support for printing of this thesis was provided by Oncomethylome Sciences, Roche Diagnostics, GC Biotech BV and the Jurriaanse Stichting.

"A map of many colors, with street signs so we can navigate, routes that we can choose, destinations that we can change. Maybe the gene isn't selfish. Maybe it's actually sensitive. [...] and the epigenome may prove to be one of the more beautiful, delicate, subtle maps of all time".

J Neimark

List of Abbreviations

ACF	aberrant crypt foci
Aza	5-Aza-2'deoxyctidine
CAE	cancer associated events
cDNA	complementary DNA
CGH	comparative genomic hybridization
ChIP	chromatin immunoprecipitation
CIMP	CpG island methylator phenotype
CIN	chromosomal instability
CRA	colorectal adenoma
CRC	colorectal cancer
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
EPM	extensive promoter methylation
FAP	Familial Adenomatous Polyposis
HAT	histone acetyltransferases
HDAC	histone deacetylases
HNPCC	hereditary nonpolyposis colorectal cancer
HPLC	high performance liquid chromatography
ISH	in situ hybridization
LOH	loss of heterozygosity
LRES	long-range epigenetic silencing
MAP	MUTYH-associated polyposis
MBD	methyl binding domain
MI	methylation index
MINT	methyalted in tumor
miRNA	microRNA
MLPA	multiplex ligation probe amplification
MMR	mismatch repair
MSI	microsatellite instability
MSP	methylation-specific PCR
MSS	microsatellite stability
PcG	polycomb group
PCR	polymerase chain reaction
PRC	polycomb repressive complex
RNA	ribonucleic acid
SCLC	small cell lung carcinoma
SNP	single nuclear polymorphism
TNM	tumor node metastasis
TSA	Trichostatin A
TSG	tumor suppressor gene
TSS	transcription start site

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CHAPTER 1|

General introduction

Introduction

Colorectal cancer

Colorectal cancer (CRC) is a significant health problem in the western world. It is the second cause of cancer deaths for both men and women, leading to an annual mortality rate of 207,400 in Europe¹. CRC is most frequently diagnosed in patients age 50 years and older, and rarely occurs before age 40. While approximately 5 percent of CRCs develop due to inherited colon cancer syndromes as the Familial Adenomatous Polyposis (FAP)², Lynch syndrome³ also referred to as hereditary non-polyposis colon cancer (HNPCC), and MUTYH-associated polyposis (MAP)⁴, the majority of CRCs are sporadic carcinomas.

Optimal management and treatment of CRCs requires accurate staging. Currently, TNM staging, based on depth of tumor (T) invasion, lymph node (N) status and the presence or absence of distant metastasis (M), is the gold standard in routine medical practice⁵. When CRC is detected at an early stage and confined to the bowel wall (stage I) the five-year survival is over 90%. However, in case of lymph node involvement (stage III) or metastatic disease (stage IV) five-year survival decreases to around 68% and 10% respectively⁶. In early stage disease surgical resection is sufficient for curative treatment while later stage disease requires adjuvant chemotherapy. Patients within the same TNM stage, however, can demonstrate considerable variation in survival. Therefore, other factors to provide additional prognostic and predictive information are needed.

Furthermore, it is expected that a great reduction in CRC incidence and mortality can be achieved by detection and removal of premalignant lesions. It is generally accepted that CRCs develop from benign precursor lesions, often colorectal adenomatous polyps (CRAs). CRAs are common in adults over 50 years, but only a low percentage of these lesions will eventually develop into a malignant tumor⁷. Studying the normal colon-, adenoma- to carcinoma progression in CRCs will lead to a better understanding of the disease and might provide molecular markers for early detection of CRC in screening programs.

Colorectal cancer (epi)genetics

During the last two decades it has become increasingly apparent that multiple genetic and epigenetic abnormalities drive CRC development (Figure 1).

Figure 1: History of colorectal cancer genetics and epigenetics. TSG; tumor suppressor gene

Pioneering research of the Vogelstein laboratory in 1988 has led to a model for the development of colorectal neoplasia, which has become revolutionary in the understanding of the multistep pathogenesis of human cancer in general^{8,9}. In this model CRC is a genetic disease, driven by accumulating abnormalities such as *KRAS*¹⁰ mutations and allelic deletions of chromosomes 5, 17, and 18. In 1989, *TP53*, located on the frequently deleted chromosomal arm 17p, was identified as tumor suppressor gene (TSG) and shown to be mutated in a high percentage of CRCs¹¹. This was followed by the identification of mutations in the adenomatous polyposis coli (*APC*) gene as cause of the inherited colon cancer syndrome FAP as well as being one of the earliest abnormalities in sporadic colorectal cancer¹². Another hereditary form of CRC, the Lynch syndrome, was shown to be caused by the disruption of mismatch repair genes, leading to microsatellite instability (MSI), a feature also present in 15% of sporadic CRCs¹³. Later, chromosomal instability (CIN), was identified as underlying feature of most human tumors, accounting for genetic instability in the remaining 85% of CRCs¹⁴. CIN describes a condition of aneuploidy and an increased rate of loss of heterozygosity (LOH) which occurs in a nonrandom pattern of associated genomic changes^{15,16}. Seven specific chromosomal changes, losses in 8p21-pter, 15q11-q21, 17p12-13 and 18q12-21 and gains in 8q23-qter, 13q14-31, and 20q13 have been demonstrated to be strongly associated with the progression of colorectal adenomas towards carcinomas and can be considered as cancer associated events (CAE)¹⁵.

Parallel to the identification of genetic alterations in CRCs, epigenetic abnormalities (heritable changes in gene expression by processes other than altered DNA sequence¹⁷) have been recognized to play a role in CRC develop-

ment¹⁸. Since its discovery in 1983, however, epigenetics has remained out-shadowed by a stronger interest in the genetics of CRC. The first epigenetic alteration linked to CRC development was a reduction of 5-methylcytosine at CpG dinucleotides in CRCs and CRAs, which was described in 1985¹⁹. DNA hypomethylation is linked to loss of genomic imprinting²⁰, oncogene activation²¹ and induction of genetic instability²². In 1989 DNA hypermethylation was identified as "third hit" in the Knudson model²³ for inactivation of tumor suppressor genes²⁴. Since the introduction of sodium bisulfite sequencing by Frommer and Clark^{25,26} and methylation specific PCR in 1996 by Herman *et al.*²⁷, DNA hypermethylation is the most extensively studied epigenetic modification in CRC. In the last decade a revolution in epigenetic cancer research has marked the place for epigenetics in CRC biology. It has become clear that promoter CpG island methylation occurs in a complex chromatin network and is influenced by histone-tail modifications that occur commonly in CRCs as well^{28,29}. In recent years microRNAs³⁰, Polycomb group proteins³¹ as well as chromatin looping³² and nucleosome positioning³³ have been added to the model of colorectal carcinogenesis. Numerous epigenetically modified genes have been identified, of which the clinical applications become increasingly clear.

Although extensive research has identified multiple genetic and epigenetic abnormalities in CRC, their interplay remains to be elucidated. In order to understand CRC, both abnormalities must be taken into account. We hypothesize that studying the timing and interplay of genetics and epigenetics in colorectal cancer will lead to a better understanding of the disease which will help to predict tumor behavior and disease progression and might provide markers for early detection and prognosis as well as new insights for treatment strategies.

Aim and outline of the thesis

The aim of this thesis is to study the interplay between genetics and epigenetics in colorectal cancer. We first review genetic and epigenetic abnormalities in colorectal carcinogenesis (chapter 2) and discuss the evidence for the (epi)genetic interplay and clinical application. In chapter 3 we present a methodological overview of promoter CpG island methylation analyses.

In order to understand the timing of genetic and epigenetic abnormalities in CRC development, frequently occurring genetic abnormalities as CIN and *TP53*-, *APC*- and *KRAS* mutation as well as promoter CpG island methylation of pivotal DNA repair- and tumor suppressor genes are studied in a well characterized series of normal colon, adenoma and carcinoma tissues in chapter 4. In this study CIN is analyzed by comparative genomic hybridization (CGH) which allows a detailed analysis of chromosomal gains and losses. In chapter 5 we investigate (epi)genetic interrelationships at the levels of individual (epi)genetic events and at the levels of CRC phenotypes. We classify CRCs based on the presence of CIN, MSI and a high number of promoter CpG island methylated genes and investigate whether genetic and epigenetic abnormalities collaborate or form different subgroups in colorectal carcinogenesis.

In order to investigate whether these subgroups of carcinomas are biologically distinct and differ in tumor behavior, we determine the prognostic sig-

nificance of epigenetic instability, defined as a high number of methylated genes, in a retrospective study in chapter 6.

In chapter 7 we study the relationship between genetic and epigenetic phenotypes in CRC by focusing on the mechanistic interplay between genetics and epigenetics in TSG silencing. Therefore, we analyze promoter CpG island methylation as well as histone-tail modifications of genes on a chromosomal region frequently lost in CRC, chromosome 18q21 (chapter 7), and study their relationship. Finally, we will investigate the relationship between amplification of the oncogene *c-MYC* and promoter methylation of c-Myc target genes (chapter 8). c-Myc induced promoter methylation has been hypothesized³⁴, but the contribution to colorectal carcinogenesis remains to be elucidated.

The general discussion in Chapter 9 summarizes and discusses the major findings of the studies performed and ends with suggestions for clinical applications and future directions of the (epi)genetic interplay in CRC.

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CHAPTER 2|

Colorectal cancer epigenetics: complex simplicity

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Submitted for publication

Abstract

Colorectal cancer has long been considered a genetic disease, characterized by sequential accumulation of genetic alterations. Growing evidence indicates that epigenetic alterations add an additional layer of complexity to the pathogenesis of colorectal cancer. It is now becoming clear that colorectal cancer epigenetics will be the paradigm for multistep carcinogenesis, as it has been for cancer genetics.

Epigenetics involves the interplay between DNA methylation, histone modifications, nucleosomal occupancy and remodeling, chromatin looping and non-coding RNAs. In addition, evidence is emerging for epigenetic silencing of large chromosomal regions and for heritable methylation. Here we summarize recent evidence regarding the complexity of epigenetic regulation of gene expression in CRC and discuss the clinical use of these new insights.

Colorectal cancer pathogenesis

Colorectal cancer (CRC) is one of the most prevalent and well studied malignancies in the western world. The disease is thought to originate in multipotential stem cells which are located in the intestinal crypts in a niche of surrounding mesenchymal cells. These stem cells generate descendants which differentiate into goblet-, endocrine-, paneth- and absorptive cells. Eventually, the majority of colonic epithelial cells will migrate to the top of the crypt where they will undergo apoptosis or will be shed into the lumen of the gut¹⁻³. In healthy crypts, a balance between cell proliferation and cell death exists. This balance is regulated by multiple signaling pathways, including the WNT-, NOTCH-, EPHRINB- and TGF β /SMAD/BMP, RTK- and PI3K pathways. Mutations, either hereditary or acquired through lifestyle and environmental factors, affecting these pathways, can disturb the balance between cell proliferation and apoptosis and initiate CRC formation.

Two alternative models regarding the location of the intestinal cancer stem cell and CRC morphogenesis have been proposed⁴. The top-down model proposes that dysplastic, genetically altered cells are first observed at the luminal side of the crypt which then spread laterally and downward to replace the normal epithelium of the adjacent crypt. This implies that the dysplastic process proceeds from the top down and that the intercryptal zone appears to be the repository for the colonic stem cells⁵. The bottom-up model proposes that malignant transformation occurs at the stem cell population at the base of the crypt, giving rise to a clone that migrates up and colonizes the crypt. Fission of crypts eventually leads to the formation of uni- or monocryptal adenomas⁶, which are considered one of the earliest visible precursor lesions of CRC. Clusters of small numbers of aberrant crypts, called aberrant crypt foci (ACF), are thought to be an early step in the formation of colorectal adenomas (CRAs) and can be divided into two groups: dysplastic ACF and serrated epithelium. Serrated epithelium is associated with the formation of hyperplastic polyps and serrated adenomas, of which the malignant potential has yet to be determined. Dysplastic ACFs are considered the precursors of adenomas² (Figure 1).

Adenomas are often referred to as polyps, but they can also be present as non-polypoid lesions. These non-polypoid lesions are slightly elevated, completely flat or slightly compressed when compared to the surrounding normal mucosa and more difficult to detect by colonoscopy or computed tomography colonography. These lesions were thought to exist primarily in the Japanese population; however recent studies have demonstrated their prevalence throughout the world⁷⁻¹⁰. Whether non-polypoid adenomas represent an aggressive precursor entity or whether they result from necrosis and/or ulceration in polypoid adenomas remains to be established¹¹ (Figure 1).

Colorectal cancer genetics

CRC carcinogenesis is characterized by accumulating genetic alterations¹²⁻¹⁵. A large number of mutations have been identified in the CRC genome. The majority of these are thought to be harmless 'passenger' mutations, providing no positive or negative selection advantage. Approximately 15 'driver' mutations are thought to be positively selected for during initiation, progression and maintenance of CRC^{16,17}.

Figure 1: Complex pathogenesis of colorectal cancer

Three CRC developmental pathways have been identified, the polypoid-, serrated- and depressed pathway. The 'classical' polypoid route starts with the development of polypoid adenomas, which grow above the surface of the intestinal mucosa, and which stepwise progress to CRC. Polypoid adenomas and carcinomas are found sporadically as well as in the hereditary familial adenomatous polyposis (FAP) syndrome. The polypoid route is characterized by accumulating genetic alterations, such as mutations in *APC* and *TP53*, CIN and LOH. A subset of CRCs, including the hereditary Lynch syndrome CRCs, are predominantly observed in females and located proximally. These lesions are thought to arise via flat or slightly elevated precursors, the serrated polyps. Serrated adenomas/carcinomas are characterized by MSI, CIMP, *KRAS* and *BRAF* mutations. A third subgroup of CRCs is thought to arise from depressed precursor lesions which progress in depth rather than above the mucosa surface. These precursors are hard to detect by standard colonoscopy and colonography and are thought to rapidly progress to CRC, however underlying biology of these lesions remains to be elucidated. Not every precursor lesion is thought to progress to CRC, although evidence on the (epi)genetic background of the high-risk precursor lesions is limited. CIN, chromosomal instability; LOH, loss of heterozygosity; MSI, microsatellite instability; CIMP, CpG island methylator phenotype

These 'driver' mutations affect pathways regulating proper development and homeostasis of the intestinal epithelium such as proliferation, migration, differentiation, adhesion, cell death, DNA stability and repair. Pathways frequently affected in CRC include WNT-, TGF β /BMP/SMAD-, TP53-, RTK- and PI3K signaling pathways¹⁶.

WNT signaling is affected by highly prevalent mutations in the tumor suppressor gene *adenomatous polyposis coli* (*APC*) in sporadic CRC and in the inherited familial adenomatous polyposis (FAP) syndrome and less frequently occurring mutations in β -catenin, *AXIN1*, *AXIN2* and *TCF4*¹⁸⁻²⁵. The TGF β /BMP/SMAD pathway is frequently affected by mutations in *TGF β receptor 2* (*TGF β R2*)^{26,27}, *SMAD2*²⁸, *SMAD4*²⁹ and *activin receptor type II* (*ACVR2*)³⁰. The tumor suppressor gene *TP53*, encoding a transcription factor which is activated upon cellular stress and inhibits cell growth and induces cell death, is frequently mutated in CRC³¹ as is, to a lesser extent, its downstream target *BAX*³². Receptor tyrosine kinase (RTK) signaling is impaired at the level of *BRAF*^{33,34}, *KRAS*³⁵ and *FES*^{36,37}. Mutations in *PIK3CA*, the catalytic subunit of the lipid kinase phosphoinositide 3 kinase PI3K, which phosphorylates the inositol ring of phosphoinositides thereby acting as a signal transducer³⁸⁻⁴⁰ and in *phosphatase and tensin homologue deleted on chromosome 10* (*PTEN*)^{41,42} have been observed frequently in CRC.

Less prevalent are mutations in DNA stability genes such as DNA mismatch repair genes *MSH2*, *MSH6*, *hMLH1* and *PMS2*, which are associated with a 1000-fold increased spontaneous gene mutation rate in microsatellite repeat

sequences⁴³, causing microsatellite instability (MSI) in the hereditary non polyposis colon cancer (HNPCC)/Lynch syndrome^{44,45}. Alterations in components of the base excision repair machinery, such as the *MYH* glycosylase, impairing the repair of G to T transversions caused by oxidative stress, have been described in families with an autosomal recessive form of polyposis⁴⁶⁻⁴⁹. In addition, approximately 85% of CRCs are characterized by chromosomal instability (CIN), an increased rate of losses or gains of (parts of) chromosomes and increased rate of loss of heterozygosity (LOH). This involves gain of chromosomes 1q32, 7p, 7q, 8q, 13q, 20p, 20q and loss of chromosomes 8p21-pter, 15q11-q21, 17p12-13, 18q12-21⁵⁰⁻⁵³. The frequency of chromosomal alterations is low in CRAs and high in CRCs, even in early stage CRC. Adenoma to carcinoma progression is thus associated with a clear increase in the level of CIN. Whether CIN is an early event and driving force in CRC development or an epiphenomenon remains to be established, although the identification of alterations in 'CIN' genes involved in maintaining sister chromatid cohesion (*CDC4*, *SMC1L1*, *CSPG6*, *STAG3*, *NIPBL*), double strand break repair (*MRE11*), chromosome segregation (*hZw10*, *hZwilch* and *hRod*)⁵⁴⁻⁵⁷ in CRC and mathematical models⁵⁸ favor the first assumption.

Colorectal cancer epigenetics

As described above, the genetic background of CRC has been studied extensively and is considered the classical paradigm for multistep carcinogenesis. Recently, an understanding of the role of epigenetics in cancer development, specifically CRC, has been emerging. Chronic injury or inflammation is hypothesized to initiate permanent epigenetic silencing of tumor suppressor genes in the WNT-, NOTCH-, EPHRINB- and TGF β /SMAD/ BMP, RTK- and PI3K pathways. This pushes these cells into a 'stem-like' state allowing these cells to clonally expand and acquire additional tumor promoting genetic and epigenetic alterations resulting in abnormal survival and prevention of differentiation, and eventually in the formation of a cancer, a process called 'epigenetic cellular addiction'⁵⁹⁻⁶¹. It is currently understood that epigenetic regulation of gene expression is altered at multiple levels.

DNA methylation

One of the most studied epigenetic alterations in CRC is DNA methylation. DNA methylation is a key mechanism of epigenetic regulation of gene expression in vertebrates occurring predominantly at CpG dinucleotides, although CpA, CpT and CpC methylation has been reported as well⁶². DNA methylation is catalyzed by DNA methyltransferases (DNMTs). Mammalian DNMTs include DNMT1, DNMT2, DNMT3a, DNMT3b and DNMT3L. "De novo" methyltransferases DNMT3a and -3b, together with their catalytically inactive cofactor DNMT3L, establish DNA methylation patterns during embryonic development, while DNMT1 is involved in maintaining DNA methylation patterns from parental to daughter genomes. DNMT2 has been shown to have tRNA methyltransferase activity⁶³. A role for DNMT1 and -3b in mediating aberrant DNA methylation in CRC has been established in CRC cell lines deficient for these enzymes, where disruption of only one of these DNMTs resulted in a modest decrease in global methylation, while disruption of both enzymes eliminated

the majority of methyltransferase activity and reduced more than 95% of genomic DNA methylation⁶⁴⁻⁶⁷.

DNA hypomethylation

One of the first recognized epigenetic alterations in CRAs and CRCs is a depletion of the overall 5-methylcytosine content (global DNA hypomethylation)⁶⁸, a phenomenon which occurs gradually, age-dependently and early in the process of CRC carcinogenesis⁶⁹. Global DNA hypomethylation occurs at CpG dinucleotides in high- and moderate repetitive sequences (satellite- and LINE- repeats, dispersed retrotransposons and endogenous retroviral elements), unique sequences such as oncogenes and imprinted loci, CpG islands and, as recently identified, in highly conserved sequences up to 2 kb distant of CpG islands, termed CpG island shores⁷⁰⁻⁷².

Originally, global hypomethylation in CRC was hypothesized to be associated with widespread oncogene activation such as for example *S100A4*, a gene involved in metastasis⁷³ and *P-cadherin (CDH3)*⁷⁴. However, more attention has been addressed to an alternative mechanism, namely induction of genomic instability. This is supported by observations that hypomethylation precedes loss of diploidy, probably due to errors of methylation replication in pericentromeric sites which are sensitive for recombination and chromosome replication⁶⁹ and that hypomethylation is predominantly observed in microsatellite stable (MSS), CIN CRCs⁷⁵⁻⁷⁷. This is underscribed by the observation that the hypomethylated DNMT1/DNMT3b deficient CRC cell line HCT116 is characterized by aneuploidy and structural chromosomal rearrangements^{78,79}. Experimental evidence linking DNA hypomethylation to chromosomal instability comes from mouse models showing that *DNMT* hypomorphic *Apc*^{MIN/+} mice develop microadenomas associated with LOH of *APC*, probably through increased genetic instability, although the incidence and growth of macroscopic CRCs was strongly suppressed. This suggests that global hypomethylation induces early stage CRC, while inhibiting later stages of CRC⁸⁰⁻⁸³.

Loss of imprinting (LOI)

Among the sequences that are subject to DNA hypomethylation are imprinted loci, loci with specific expression of one parental allele, and silencing, often established by methylation of a regulatory sequence, of the other allele. Abnormal activation of the normally silent maternally inherited allele of the *insulin-like growth factor II (IGF2)* gene by hypomethylation of the proximal promoter of *IGF2* has been observed in CRC and CRC cell lines. As a consequence, overexpression of *IGF2*, an autocrine and paracrine growth factor, activates the IGF1 receptor (IGFR), which upon activation autophosphorylates and activates the PI3K/AKT and GRB2/RAS/ERK pathway⁸⁴.

Different mouse models support a causal role for LOI in CRC carcinogenesis. Holm *et al.*⁸⁵ showed that imprinting-free mouse ES cells acquired characteristics of transformed cells such as increased proliferation and immortality. When transplanted in SCID mice, imprinting-free ES cells developed CRAs. Moreover, chimeric mice derived from these ES cells developed tumors in multiple tissues. Further evidence comes from a H19^{+/-}/APC^{+ /MIN} LOI mouse model which demonstrates that LOI mice develop intestinal tumors and a less differentiated normal intestinal epithelium, a phenomenon which is also observed in patients with *IGF2* LOI⁸⁶. To further investigate the mechanism of

IGF2 LOI induced CRC carcinogenesis, Feinberg and coworkers used the same $H19^{+/-}/APC^{+/MIN}$ model to show that LOI leads to increased expression of proliferation-associated genes in the mouse intestinal crypt and enhanced sensitivity to IGF-II signaling, blockage of which resulted in a reduced number of aberrant crypt foci after treatment with azoxymethane⁸⁴. Whether hypo-⁸⁷ or hypermethylation⁸⁸ of the differentially methylated region (DMR) of *H19* is involved in the LOI of *IGF2* and whether *H19*, which is over-expressed in CRC⁸⁹, functions as an oncogene or tumor suppressor gene in CRC⁹⁰ remains to be established.

DNA hypermethylation

The best characterized epigenetic alteration in CRC is promoter hypermethylation, which occurs at CpG dinucleotide-dense regions, called CpG islands, present at the 5' region of approximately 60% of all genes^{91,92}. The majority of CpG islands lack methylation in normal colon mucosa, independent of the transcriptional status of the gene. Hypermethylation of promoter CpG islands has been observed for an increasing number of tumor suppressor- and DNA repair genes and can have the same effect as coding-region mutations or loss of the locus and is therefore considered the 'third hit'⁹¹ in the Knudson model for inactivation of tumor suppressor genes.

Aberrant promoter CpG island methylation is related to aging, but CRC-specific hypermethylation events have been identified. Promoter CpG island hypermethylation in CRC affects WNT-, RTK-, NOTCH-, TP53-, PI3K-, retinoic acid-, and IGF signaling as well as genes involved in cell cycle regulation, transcription regulation, DNA repair/stability, apoptosis, adhesion angiogenesis, invasion and metastasis, axon guidance, transmembrane glycoproteins, peptide hormones and chromatin organization (Table 1).

The recognition that a distinct subset of CRAs and CRCs display significantly more promoter methylation than others has led to the introduction of the concept of CpG island methylator phenotype (CIMP)⁹³⁻⁹⁵. CIMP CRCs have been shown to be associated with older age, female sex, family history of CRC, proximal location in the colon, mucinous cell differentiation, specific precursor lesions (serrated adenomas), smoking, MSI, *BRAF* and *KRAS* mutations¹⁵⁷⁻¹⁶⁴. The existence of two CIMP subgroups, each with specific molecular characteristics, has recently been postulated^{165,166}. Whether the detection of CIMP depends on the markers analyzed, experimental tools and modes of data analysis and whether CIMP is just an epiphenomenon associated with aging has been discussed extensively^{48,95,161,167,168}. This dispute is in part the result of a lack of uniform definition of CIMP and differences in interpretation of the data.

Recently, it was reported that not only CpG islands in promoter regions, but also in highly conserved sequences up to 2 kb distant, termed CpG island shores, are affected by hypermethylation and downregulation⁷². The effect of this on gene regulation remains to be explored.

Hypermethylated DNA becomes silenced via recruitment and binding of methyl-CpG binding proteins and associated corepressors such as HDAC2, which create a repressive chromatin structure. Three families of methyl-CpG binding proteins have been identified thus far: the methyl-binding domain (MBD) family of proteins containing MBD1, MBD2, MBD4 and MeCP2, the zinc finger proteins Kaiso, ZBTB4 and ZBTB38, and the SET-and-RING-Finger-Associated (SRA) proteins UHRF1 and UHRF2. Loss of methyl CpG-binding

proteins such as MBD2 and Kaiso has been shown to suppress intestinal carcinogenesis in *APC*^{MIN/+} mice^{169,170}, and induces tumor suppressor gene expression sensitizing CRC cell lines to cell cycle arrest and cell death¹⁷¹.

Table 1: Promoter CpG island hypermethylated genes and miRNAs in colorectal cancer

Signaling pathways/ cancer hallmarks	Promoter CpG island hypermethylated genes / miRNAs	Reference
WNT	APC *, SFRP1 *, SFRP2 *, SFRP4 *, SFRP5 *, SOX17 *, WNT5a , DKK1 *, DKK3 , WIF1 *, AXIN2 *	12,23,96-104
RTK	RASSF1A , RASSF2A , EPHB2 , RAB32 , NORE1 *	105-109
NOTCH	NEURL	110
TP53	P14 ^{ARF} *, HIC1 * DFNA5 *	111-113
PI3K	PIK3CG	114
ER	ER *	115
Retinoid acid	RARβ , CRABP1 *	116,117
IGF	IGFBP3 *, IGFBP7 *	118,119
Cell cycle regulation	P16 ^{INK4A} *, KLF4 *	111,120
Transcription regulation	GATA4 *, GATA5 *, RUNX3 *, CDX1 , HLTF , FOXL2 *, ALX4 *	110,121-125
DNA repair/stability	hMLH1 , O⁶MGMT , WRN *, CHFR	126-129
Apoptosis	BNIP3 , IRF8 , DAPK1 *, HRK *	130-133
Adhesion	CDH4 , CDH13	134,135
Angiogenesis	THBS1 *	136
Invasion & metastasis	TIMP3 , RECK , CXCL12 *	137-139
Axon guidance	SLIT2 *	140
Transmembrane (glyco)proteins	MUC1 , NMDAR2A , HPP1 , SPARC , DCC *, CD133	141-145
Peptide hormones	SST , TAC1 *	146
Chromatin organization	PRDM2	147,148
Unknown function/pathway	VIM , NDRG4 *	149
'Classical' CIMP-panel	MINT1 , MINT2 , MINT31 , CDKN2A *, hMLH1	93
'New' CIMP-panel	CACNA1G *, IGF2 *, NEUROG1 *, RUNX3 *, SOCs1	150
miRNAs	miRNA-124a, miRNA-34b, miRNA-34c, miRNA- 148 ^a , miRNA-9, hsa-miR-342	151-154

This table, which is not exhaustive, represents genes and miRNAs of major signaling pathways/cancer hallmarks which are epigenetically silenced by promoter CpG island hypermethylation. * PcG targets^{155,156}, **bold** mutation target, underscored deletion target (event occurring in >40% of CRCs⁵⁰).

Heritability of DNA methylation

With the recognition of altered CpG island hypermethylation in CRC, the question emerged whether aberrant methylation could be transmitted through the germline and influence CRC risk. Although CIMP was shown to be strongly associated with a family history of CRC in one study¹⁵⁸, Joensuu *et al.*¹⁷² found no evidence for a heritable basis of promoter CpG island methylation. Occasionally, somawide monoallelic germline epimutations (defined as epigenetic silencing of a gene that is normally active or epigenetic activation of a gene that is normally silent) of *hMLH1* have been observed in *hMLH1* wild-type HNPCC/Lynch patients¹⁷³⁻¹⁷⁵. These epimutations arise either de novo in the germline during oogenesis or in the zygote¹⁷⁶ or are transmitted from mother to son¹⁷⁷. Affected individuals developed early-onset cancers with loss of hMLH1 protein, and in this case a positive family history of CRC or other HNPCC/Lynch-related cancers leads to epigenetic alterations affecting CRC susceptibility.

A Mendelian inherited, allele specific mosaic methylation of *MSH2* in a *MSH2* wild-type family in which several members developed CRC and other HNPCC/Lynch-related cancers was recently reported by Chan *et al.*¹⁷⁸ and Ligtenberg *et al.*¹⁷⁹. Whether these epimutations are truly transmitted through the germline, a phenomenon which has been documented in plants and mice, or are influenced by cis- or transacting factors that influence epigenetic patterning, remains to be determined¹⁸⁰⁻¹⁸³.

Post-translational histone modifications

As described above, CRC is thought to initiate in pluripotent intestinal stem- or progenitor cells. These cells are characterized by tight control of transcriptional activity of genes regulating proliferation and differentiation ensuring that these genes are properly regulated in stem cells and upon external differentiation signals to differentiate into lineage-specific progeny. Transcriptional control of these genes is regulated by a specific pattern of posttranslational histone modifications, such as phosphorylation, ubiquitylation, sumoylation, ADP ribosylation, deimination and proline isomerization¹⁸⁴. These modifications, which are dynamic and rapidly changing, function either by disrupting contacts within and between nucleosomes or by the recruitment of non-histone proteins to chromatin thereby dictating the higher-order chromatin structure¹⁸⁵. The effects of posttranslational modifications depend on the amino acid type, its position in the histone tail and the type of modification. The effects of histone (de)acetylation and (de)methylation in CRC are beginning to be understood. While transcriptionally active genes are enriched for di- and trimethylation of histone H3 at lysine 4 (H3K4me2/me3) and acetylation of key H3 and H4 amino acids around the transcription start site, transcriptionally inactive genes show lower levels of these active marks and are characterized by trimethylation of histone H3 at lysines 9 and 27 (H3K9me3 and H3K27me3). This combination of the repressive chromatin marks (H3K9me3 and H3K27me3) in combination with the active H3K4me2 mark is referred to as 'bivalent' or 'poised' chromatin.

This repressive chromatin configuration is mediated by the Polycomb group (PcG) of proteins, transcriptional repressors that silence specific groups of genes¹⁸⁶. PcG proteins are subdivided into two groups based on their association with multimeric complexes called Polycomb repressive complexes (PRCs). The PRC2 complex is involved in initiating of silencing by trimethylation of H3K27 and to a lesser extent H3K9 and includes the CBX and HPH family of proteins, YY1, BMI1, RING1/1a and RING2/1b. The PRC1 complex is involved in maintenance of silencing by recognizing the H3K27me3 mark and includes HKMT, EED1,-3,-4, EZH1, EZH2 and SUZ12. PRC-mediated transcriptional silencing is thought to involve ATP-dependent nucleosome-remodeling by the SWI/SNF complex as well as by direct blocking of the transcription initiation machinery¹⁸⁶. PRC-mediated transcriptional silencing has been hypothesized to predispose PcG-target genes to promoter CpG island hypermethylation¹⁸⁷⁻¹⁸⁹. Many of the genes reported to be hypermethylated in CRC are PcG target genes¹⁵⁵ (Table 1). Indeed, EZH2 has recently been linked to DNMTs establishing a role for this protein in the induction and targeting of DNA methylation¹⁹⁰. These data suggest that tumor-specific targeting of the novo methylation is pre-programmed by epigenetic marks that normally regulate the expression of embryonic genes, by locking a stem cell phenotype initiating abnormal clonal expansion^{191,192}.

Long range epigenetic silencing

For most genes studied, it has been presumed that aberrant epigenetic silencing in cancer is a local phenomenon silencing individual CpG islands. However, recent studies provide evidence that epigenetic silencing can in some cases span large chromosomal regions containing multiple genes. Frigola *et al.* demonstrated epigenetic silencing of a 4Mb chromosomal region at chromosome 2q14.2¹⁹³ and introduced the term 'long range epigenetic silencing' (LRES), a phenomenon related to X-inactivation and imprinting¹⁹⁴. Promoter CpG island methylation was observed in three distinct regions of up to 12 successive CpG islands (spanning 1 MB) harboring several candidate tumor suppressor genes. Although not promoter CpG island methylated, the neighboring genes in this 2q14.2 4 MB region were also transcriptionally suppressed and marked with the inactive chromatin mark H3K9me2¹⁹³.

Subsequently, LRES has been reported for a 4 Mb region at chromosome 3p22, harboring the *hMLH1*, in MSI CRCs¹⁹⁵. The majority of these genes, including genes reported to exhibit tumor suppressor functions (*CTDSPL/HYA22*, *DLEC1*), WNT signaling activators (*LRRFIP2*) and genes frequently deleted and/or hypermethylated in cancer (*ITGA9*, *CTDSPL*, *PLCD1*), showed promoter CpG island hypermethylation, transcriptional inactivation, presence of the inactive mark H3K9me2 and absence of H3K9 acetylation, associated with active chromatin. As was observed for the 2q14.2 region, genes in the 3p22 region that are resistant to promoter CpG island methylation are also transcriptionally repressed, as indicated by the H3K9me2 mark in combination with the active mark H3K9 acetylation. Another example of LRES, characterized by frequent and dense promoter CpG island methylation, downregulation and bivalent (H3K4me3 and H3K27me3) chromatin marking of neighboring genes located at a 1.25 Mb region at 5q35.2 has recently been reported¹⁹⁶.

We have recently shown that H3K27me3 and H3K9me3-mediated LRES does not occur at a 4 MB region at the frequently lost 18q21 locus harboring the TSGs *MBD1*, *CXXC1*, *SMAD4*, *DCC* and *MBD2*. We demonstrated that *DCC* is characterized by promoter CpG island methylation, repressive histone-tail marks H3K27me3 and H3K9me3 but not the active chromatin mark H3K4me3, while its neighboring genes *MBD1*, *CXXC1*, *SMAD4* and *MBD2*, although downregulated in CRC, show the opposite, concluding that H3K27me3 and H3K9me3-mediated epigenetic silencing of *DCC* in CRC is a focal process not affecting neighboring genes on chromosomal region 18q21¹⁹⁷ and that LRES is not a universal phenotype.

It is at this time unclear as to what may initiate LRES, whether being initiated by one critical gene target that recruits the histone methylation machinery thereby spreading the heterochromatin state to innocent bystander genes, or by coordinated silencing of neighboring genes.

Nucleosomal positioning

In addition to promoter CpG island methylation and covalent histone tail modifications, nucleosomal occupancy has been implicated in epigenetic silencing in CRC. Epigenetic regulation of gene expression requires active eviction of nucleosomes from the transcription start sites. The positioning of these nucleosomes occurs during replication and is guided by DNA sequence, chromatin assembly proteins such as chaperone- and escort proteins, ATP-

dependent nucleosome remodellers, histone variants and post-translational histone modifications¹⁹⁸.

Lin *et al.*¹⁹⁹ showed that epigenetic silencing of the bidirectional *hMLH1/EPM2AIP1* promoter in CRC cell lines is associated with the positioning of three nucleosomes just upstream of the transcription start site of the active *hMLH1* promoter, inducing a mitotically heritable, permanently repressed, non permissive chromatin state. Upon gene reactivation and DNA demethylation by 5-aza-2'-deoxycytidine, nucleosomal depletion at the *hMLH1* locus occurs, converting the nonpermissive chromatin state into permissive chromatin state including active histone marks. Depletion of nucleosomes just upstream of transcription start sites suggests that lack of nucleosomes is needed to allow access of the transcriptional machinery to the permissive promoter.

Although the bidirectional *hMLH1/EPM2AIP1* promoter has, so far, been the only gene for which nucleosomal occupancy has been demonstrated in CRC, nucleosome depletion upstream of transcription start sites of active genes has been demonstrated in a variety of model systems including yeast and flies²⁰⁰⁻²⁰³ and evidence for the involvement of this mechanism in the silencing of other CRC TSGs can be expected soon.

Chromatin looping

Transcriptional regulation has been associated with higher-order chromatin structure. However, the exact relation between chromatin folding and gene expression remains unclear. Recently, a direct link between PcG mediated silencing, promoter CpG island hypermethylation and high order chromatin folding has been reported in CRC²⁰⁴. Tiwari *et al.* demonstrate the presence of PcG/H3K27me3-enriched chromatin elements at the base of chromatin loop intersection points over a 100 kb region at the *GATA4* locus in *GATA4* transcriptionally inactive cells, whereas this hub-like structure is absent in *GATA4* expressing cells. These data indicate that the PcG machinery might be a major factor in determining a three-dimensional repressive chromatin superstructure. Whether this silencing model will be applicable to other tumor suppressor genes in CRC remains to be determined.

Small non-coding RNAs

Epigenetic regulation of gene expression can also occur at the RNA level in that RNA-mediated downregulation of gene expression is conferred by small (~21-23 nucleotides) non-coding microRNAs (miRNAs) that are processed from long double stranded (ds) RNA or hairpin RNA precursors by the RNase III family enzymes DROSHA and DICER. Gene silencing occurs by annealing of miRNAs to mRNA- and core promoter sequences that are fully or partially complementary and involves translational repression, accelerated mRNA turnover and heterochromatin formation²⁰⁵. Although the total number of miRNAs and their role in epigenetic silencing via heterochromatin formation, has yet to be unraveled, the role of miRNAs in targeting multiple mRNAs and core promoters in multiple biological pathways including development, proliferation, differentiation, and apoptosis, has been studied more clearly²⁰⁶⁻²¹¹. miRNAs aberrantly expressed in CRC include miR-21²⁰⁶, a negative regulator of the tumor suppressor gene *Pdcd4*, miRNA-141 and miRNA-200c which inhibit epithelial differentiation²¹², miRNA-34a, a negative regulator of TP53-

induced apoptosis²¹³, miRNA-145²¹⁴, miRNA-135a and miRNA-135b constitutively activating the WNT and miRNA-373 and miRNA-520c which stimulate migration and invasion *in vitro* and *in vivo* by suppressing CD44 *in vitro*²¹⁵. The observation of repressed miRNAs in CRC initiated the search for mechanisms of transcriptional silencing. Recently miRNA-124a, a miRNA regulating the expression of cyclin D kinase 6 (CDK6) and subsequent phosphorylation of the CDK6 target Retinoma blastoma (Rb) protein, was reported to be associated with CpG island hypermethylation and absence of histone H3 and H4 acetylation, H4K4me3 and occupancy by MeCP2 and MBD2¹⁵¹. miRNA-34b and miRNA-34c, both targeting the TP53 pathway are frequently promoter hypermethylated and H3K4 trimethylated in CRC¹⁵². Promoter CpG island methylation of miRNA-148a, miRNA-34b, miRNA-34c and miRNA-9 has been associated with metastasis formation in CRC¹⁵³. Expression of hsa-miR-342, a miRNA encoded in an intron of the *Ena/Vasp-like (EVL)* gene and inducing apoptosis, is regulated by hypermethylation of the *EVL* CpG island¹⁵⁴.

Complex interplay between epigenetic alterations in CRC

Since alterations in DNA methylation, histone tail modifications, chromatin looping, nucleosomal positioning and RNA-mediated epigenetic silencing are observed simultaneously in CRC, the question emerges how these phenomena are related mechanistically (Figure 2).

Several studies have investigated the relationship between hypo- and hypermethylation: Bariol *et al.* and Frigola *et al.*^{216,217} concluded that DNA hypo- and hypermethylation are independent processes, although Matsuzaki *et al.* and Deng *et al.*^{75,218}, reported the opposite.

The evidence that DNA methylation occurs in the context of histone tail modifications¹⁸⁴ is compelling. Already in 1999, Cameron *et al.*²¹⁹ showed that reversal of silencing of *hMLH1*, *TIMP3* and *p16^{INK4A}* in CRC cell lines requires demethylation prior to histone acetylation, indicating that, after establishment of transcriptional silencing by histone acetylation, DNA methylation becomes the dominant component for maintenance of silencing. This observation was later confirmed by Bachman *et al.*²²⁰ who reported that promoter CpG island methylation maintains silencing of *p16^{INK4A}*, a locus initially silenced by the repressive mark H3K9me2. Erasure of the DNA methylation signal leads to histone H4 acetylation, while resilencing of this locus occurs independently of *p16^{INK4A}* promoter CpG island methylation and is associated with the repressive mark H3K9me2. Promoter hypermethylation and H4 deacetylation were shown to finally lock the *p16^{INK4A}* gene in a repressed state. These data provide insight in the order of events underlying transcriptional silencing of tumor suppressor genes and suggest that promoter CpG island hypermethylation is superimposed on the bivalent chromatin state of genes important in embryonic stem cell proliferation and differentiation.

Recently, DICER-mediated RNA-dependent transcriptional silencing (RdTS) and DNA methylation, a phenomenon described in plants in which RNA species direct DNA methylation and repressive histone modifications to homologous DNA sequences²²¹, has been reported for a select group of CpG islands (*SFRP4* and *ICAM-1*) in the CRC cell line HCT116²²² which is associated with specific mark H3K9me2.

Figure 2: Colorectal cancer epigenetics

Epigenetic regulation of gene expression in colorectal cancer involves promoter CpG island and CpG island shore hypo- and hypermethylation, histone tail modifications, nucleosomal remodeling and -positioning, non-coding RNAs and chromatin looping. Although the exact interplay between these processes has not yet been elucidated and the initiating event has not been identified, evidence is emerging that causal relationships exists. Silencing of tumor suppressive miRNAs by promoter CpG island hypermethylation (black arrow) has been reported. In addition, concurrent promoter CpG island methylation, PcG mediated-histone tail modifications and chromatin looping have been observed for *GATA4* (grey lines), underscoring the role of epigenetic alterations in the organization of high order chromatin structure. CGI, CpG island

Interplay between genetics and epigenetics

In theory, genetic and epigenetic alterations can occur in every possible combination (Figure 3). Integrating data from genome-wide genetic and epigenetic analysis in CRC reveals that some genes are specifically affected by epigenetic inactivation, others are targeted by genetic events and for a subgroup of genes a combination of genetic and epigenetic inactivation has been observed (Table 1). What determines the mechanism of inactivation has remained a mystery. Simultaneous gene mutation and promoter CpG island methylation has, for example, been described for *p16^{INK4A}*²²³. Concomitant loss and promoter CpG island methylation has been observed for regions 8p21pter (*GATA4*, *SFRP1*)²²⁴, 15q11-q21 (*THBS1*), 17p12-13 (*HIC1*)²²⁵ and 18q12-21 (*DCC*)²²⁶. In addition, a combination of copy number gain and promoter CpG island methylation (*GATA5* and *CDH4* on region 20q13) can occur concomitantly as well, although it is not clear whether these events occur simultaneously. In the case of copy number gains, epigenetic inactivation of tumor suppressor genes on this locus could serve to counteract the effect of copy number gains on gene expression. Genome wide mutation-¹⁶ and promoter CpG island methylation analyses¹¹⁰, revealed that the majority of mutations occur heterozygously and at a low frequency, in contrast to a much higher number of approximately 200-400 genes that are hypermethylated per CRC, which can occur homozygously^{110,227}. Only a small percentage

Figure 3: Interplay between genetic and epigenetic alterations in colorectal cancer.

Genetic and epigenetic alterations in CRC occur in multiple combinations and equivalently alter important signal transduction pathways. Black arrows indicate genetic events causing epigenetic alterations and vice versa. Grey lines indicate events that occur concomitantly in regulating gene expression of a specific locus. Grey, dotted lines indicate associations that have been hypothesized/observed but for which a causal relationship has not yet been established.

(approximately 20%) are targets of both mutation and promoter CpG island methylation^{110,228}. Genes affected by both mechanisms, are more frequently affected by hypermethylation than mutation. Within one tumor cell, mutation and hypermethylation most frequently show a mutually exclusive character¹¹⁰. This has also been observed for *APC* promoter methylation and mutations which occur most frequently in the absence of each other²²⁹.

The same inverse relationship has been observed for DNA promoter hypermethylation and DNA copy number changes. The association between these two events shows that only a small number (approximately 2%) of tumor suppressor genes inactivated by promoter CpG island methylation is located on chromosomal regions affected by deletion or copy number change^{226,227,230}.

The observation that promoter CpG island methylation and copy number changes infrequently occur in the same CRC is also reflected by the inverse relationship between CIMP and CIN, as reported by multiple groups^{160,162,166,231}. Therefore, CIMP and CIN CRCs are thought to develop from two distinct etiological mechanisms which rarely overlap¹⁶⁶.

Who's the boss?

As described above, genetics and epigenetics join forces in altering gene expression in CRC and it is debated which type of alteration is dominant¹⁶⁷. CIMP has been shown to be strongly associated with MSI through a similar association with promoter methylation of mismatch repair gene *hMLH1*¹²⁶. Another example of how epigenetic instability initiates genetic instability in CRC development is provided by promoter methylation of DNA repair gene

*O*⁶-methylguanine DNA methyltransferase (*O*⁶MGMT), a DNA repair protein which removes alkyl groups from the *O*⁶ position of guanine, an important site of DNA alkylation²³². Since *O*⁶MGMT is required for this repair process, *O*⁶MGMT loss of function results in failure to remove the alkyl groups and subsequent conversion of a guanine-cytosine pair to an adenine-thymine pair which leads to an oncogenic potential of *KRAS*^{233,234}, *TP53*²³⁵ and *PIK3CA*²³⁶. Although reports on these specific association are conflicting^{237,238} it provides an example of how epigenetic modifications can initiate genetic instability. An association between decreased activity of the checkpoint control gene *CHFR* and CIN has been shown in CRC cell lines^{239,240}. However, as *CHFR* promoter methylation has been associated with *hMLH1* promoter methylation¹²⁹ and MSI, a role for *CHFR* promoter methylation in inducing CIN is debated²⁴¹.

Genetic alterations initiating epigenetic modifications have also been reported. Multiple studies have shown that activation of oncogenic signaling pathways (*KRAS*, *c-MYC*) initiates and is associated with promoter CpG island methylation^{163,166,242-245}, although no direct evidence exists on how *KRAS* and *c-MYC* can induce widespread DNA methylation.

An additional mechanism by which genetic abnormalities can induce promoter CpG island methylation, are double strand DNA breaks²⁴⁶, caused by reactive oxygen species during ageing and chronic inflammation. When unrepaired, these breaks will introduce chromosomal translocations and cell death. When present in gene promoter regions, silencing proteins are recruited to break-sites along with silencing histone marks²⁴⁷, resulting in transient silencing of the gene, in order to assure breakage repair before transcription can be resumed. Recently, it has been shown that transient silencing marks occasionally become permanent when promoter CpG island methylation spreads, a process which is highly dependent on the presence of the deacetylase *SIRT1*²⁴⁷.

A third mechanism on how genetics affects epigenetics are germline single nucleotide polymorphisms (SNPs) and mutations in genes encoding enzymes regulating epigenetic regulation of gene expression. For example, SNPs in the histone methyltransferase *SMYD3*²⁴⁸, and in folate metabolizing enzymes such as methylenetetrahydrofolate (*MTHFR*), and methionine synthase (*MTR*) have been associated with CRA and CRC risk^{249,250}. Whether these SNPs are also associated with DNA- and histone methylation remains to be elucidated. The recent identification of non-coding RNAs as epigenetic regulators of gene expression leads to the search for germline SNPs in 3' UTRs targeted by miRNA, which will alter the strength of miRNA binding and regulation of the expression of target genes. Recently, statistically significant associations between variant alleles of the *CD86* and *INRS* gene UTRs miRNA binding sites, affecting binding of miR-337, miR-582, miR-200^a, miR-184, miR-212, and CRC risk have been observed²⁵¹.

Somatic alterations in epigenetic enzymes in CRC include mutations in DNA methyltransferase *DNMT1*²⁵², the histone acetyltransferases *EP300*²⁵³⁻²⁵⁵, *CREBBP*^{254,255} and *pCAF*²⁵⁵, the histone deacetylase *HDAC2*²⁵⁶⁻²⁵⁹, the histone methyltransferases *MLL3*¹⁶, the methyl-CpG-binding protein *MBD4*^{260,261} and the histone methyltransferase *PRDM2/RIZ1*^{147,262,263}, of which the latter is also affected by promoter CpG island hypermethylation²⁶⁴, as well as amplification of *MLL4*²⁶⁵. In addition, overexpression of the DNA methyltransferases *DNMT1*, *-3A* and *-3B*^{266,267}, histone deacetylases *HDAC7A*²⁶⁸ and *SIRT1*²⁶⁹, histone methyltransferases *EZH2*²⁷⁰ and *SMYD3*²⁷¹ and downregulation of histone deacetylases *HDAC1*, *HDAC5* and *SIRT1*²⁶⁸ has been observed in

CRC. Whether the underlying mechanism involves genetic alterations, still has to be unraveled.

Although cause-and-consequence relations have not been elucidated for the majority of the associations discussed above, specific associations indicate that genetic and epigenetic events collaborate in the disruption of important signalling pathways. Aberrant WNT signaling provides an excellent example of how genetic (*APC*, *AXIN1*²², *AXIN2*²³, *β-catenin*²⁷²) and epigenetic (*APC*, *SFRPs*²⁷³, *WIF-1*¹⁰¹, *DKK1*, *DKK3*⁹⁹, *SOX17*⁹⁷ and *DACT3*²⁷⁴) events collaborate in accumulating β-catenin in the nucleus to transcriptionally drive WNT target genes. The same has been observed for the RAS-RAF-MEK-ERK pathway in which *KRAS*³⁵, *BRAF*²⁷⁵ and *NF1*²⁷⁶ are mutation targets, while negative regulators of *KRAS*, *RASSF1A*¹⁰⁵, *RASSF2A*²⁷⁷ and *NORE1A* (*RASSF5*)¹⁰⁹ are affected by promoter CpG island methylation.

Translational CRC epigenetics

The observation that epigenetic changes are highly prevalent and play an important role in CRC carcinogenesis make them excellent markers for early detection, prediction of prognosis and response to therapy.

Early detection

CRC screening by colonoscopy²⁷⁸ or fecal occult blood testing (FOBT)^{279,280} has been reported to reduce mortality in randomized clinical trials, providing a rationale for CRC screening. However, the burdening colonoscopy procedure and its associated risks, and the low sensitivity of FOBT enforce the need for more sensitive non-invasive tests.

Epigenetic diagnostic tests for non-invasive early detection of CRC need to be easy to conduct, sensitive, specific and cost-effective when compared to other CRC screening modalities and would preferably be positioned as a pre-selection screening test for colonoscopy. Several studies have shown proof-of-principle for the detection of promoter CpG island hypermethylation of CRA or CRC-derived DNA in stool^{149,281-285} or blood^{125,286} using sensitive methylation-specific PCR (MSP)-based assays. In order to increase the sensitivity of detection of methylation markers in stool, Zou *et al.*²⁸⁷ developed a capture-assay using MBD protein columns to capture methylated DNA, which markedly increased the sensitivity without decreasing specificity.

The observation that *IGF2* LOI is present in normal colonic mucosa and blood of CRA and CRC patients and a subset of healthy people^{87,288-290} initiated the development of a blood-based test analyzing *IGF2* LOI in peripheral blood lymphocytes²⁹¹ showing that abnormal imprinting of *IGF2* is strongly and independently associated with CRA and CRC. Recently, Ito *et al.*²⁸⁹ showed that hypomethylation of the proximal promoter of *IGF2* occurs as an acquired tissue-specific event in 80% of CRCs, which is higher than the prevalence of *IGF2* LOI in a population-based study. However, the numbers of cases and controls analyzed in the majority of these studies are low, data have not been confirmed in independent prospective screening studies and sensitivity, specificity and cost-effectiveness have not been compared directly to the gold standard for detecting CRA and CRC (colonoscopy) and the currently widely used screening modality (i)FOBT.

Prognostic markers

The current gold standard for determining prognosis in CRC is the TNM staging system which is highly predictive of outcome at the extremes (e.g. stage I versus stage IV), but less informative for intermediate groups. Identifying molecular markers that identify patients at risk for recurrence and with a poor prognosis could enhance current CRC treatment, especially in the intermediate cancer stages. Although an increasing number of publications report on the prognostic significance of epigenetic markers implicated in CRC, a clear distinction should be made between epigenetic markers that act as independent prognostic markers (that add additional information on prognosis to clinical characteristics such as TNM-stage and do not act as intermediate factor between clinical prognostic factors and outcome) and markers that can predict treatment outcome²⁹².

Several publications report a prognostic role for single promoter methylation markers in CRC²⁹². For example, *p16^{INK4A}* methylation alone, or in combination with *KRAS* mutations, was found to be associated with shortened survival in several studies²⁹³⁻²⁹⁶, but not in other¹¹¹, in tumor tissue, but also as free-circulating tumor-associated DNA in plasma²⁹⁷. In a subgroup of stage II patients, *HPP1* and/or *HLTF* methylation in serum was also found to be associated with a worse prognosis²⁹⁸. *O⁶MGMT* and *p14^{ARF}* promoter methylation have also been mentioned as prognostic factors in CRC²⁹⁹⁻³⁰¹, but their effects seem to be dependent of the presence of other methylated markers or adjuvant treatment^{301,302}. In addition, methylation of *ID4*, a gene controlling cell differentiation, was suggested as a prognostic marker in stage I-IV CRC. However, it is not yet clear whether this is independent of other important prognostic factors such as TNM stage and tumor grade as these were not included in the analyses³⁰³.

A prognostic role was also suggested for CIMP, showing a worse prognosis for patients with CIMP CRCs in most studies, although conflicting results have been reported as well^{157,304-311}, possibly reflecting differences in patient populations, confounding effects of other molecular events and different definitions of CIMP³¹⁰. While most studies focus on the influence of DNA hypermethylation on CRC prognosis, hypomethylation of *LINE-1*, has also been implicated for poor prognosis^{217,312}, independent of other clinical and molecular characteristics³¹³. In addition, high *miRNA-21* expression has been shown to be associated with poor survival, independently of clinical covariates as well as poor therapeutic outcome in two independent training and test cohorts of CRC patients²¹¹.

Predictive markers

Methylation markers have been implicated as predictive markers in treatment response. Evidence on the predictive role of CIMP is scarce and inconsistent. Previously, Ogino *et al.*³⁰⁷ showed a poor outcome after 5-FU treatment which was also observed in another study among advanced CRC cases³⁰⁸. Other studies however, show contrasting results and report a survival benefit for CRCs from 5-FU^{305,314}. These controversial results could be due to different marker panels and inconsistent definitions of CIMP, however Ward *et al.* previously showed that the observed adverse prognostic effect of CIMP was lost within the methylated CRC showing MSI³⁰⁴ suggesting a possible role for MSI in treatment prediction. However, although MSI seems to have a major

effect on the expression of CIMP¹⁵⁹, more recent results imply that MSI in sporadic CRC can be explained by CIMP-associated *hMLH1* methylation, pointing out that an accurate CIMP definition could be essential for understanding the role of MSI as well¹⁶¹. Single epigenetic markers that are suggested as predictive for treatment response include promoter CpG island methylation of *Werner syndrome gene (WRN)*¹²⁸, *O⁶MGMT*³⁰² and the *UDP-glucuronosyl-transferase UGT1A1*, which are thought to influence to the effects of and response to chemotherapy^{128,315}.

Truncating mutations in the human histone deacetylase *HDAC2* gene, associated with resistance to the antiproliferative and proapoptotic effects of the HDAC inhibitor trichostatin A (TSA), were recently described in MSI CRC^{257,259}. This suggests that HDAC2-deficiency confers resistance to TSA-induced histone acetylation, although this could not be confirmed by others²⁵⁸, and that HDAC2 mutational status could predict the response of treatment with HDAC inhibitors in CRC.

Despite numerous studies on the relevance of epigenetic markers in CRC, the number of clinically useful markers is still limited. Research on molecular markers is susceptible to publication bias and false-positive results due to small population sizes and small effect sizes and lacks confirmation studies analyzing the reproducibility of the data³¹⁶⁻³¹⁸. Moreover, the majority of studies describe single epigenetic markers, even though it is suggested that using an approach of combining several markers is more likely to identify relevant prognostic factors³¹⁹.

Conclusions

The role of epigenetics in normal development and carcinogenesis of the gut is beginning to emerge. It is becoming clear that epigenetic alterations not only complement genetic alterations in the pathogenesis of CRC, but that complex interactions between different chromatin modifications such as DNA methylation, non-coding RNAs, positioning of nucleosomes and chromatin binding proteins, posttranslational modifications of histone tails and chromatin looping exist. Novel high-throughput technologies will 1) enable detailed mapping of the CRC chromatin landscape thereby providing novel insights in CRC etiology and biology, 2) identify additional genetic variations in epigenetic regulators associated with CRC and 3) will identify potential epigenetic markers for early detection, prediction of prognosis and response to therapy. To maximize the potential of these novel technologies and to thoroughly evaluate the prognostic and predictive potential of epigenetic disease markers, the American Association for Cancer Research Human Epigenome Task Force and the Scientific Advisory Board of the Epigenome Network of Excellence from the European Union recently urged the scientific community to collaborate³²⁰. In addition, in order to be able to prevent and treat CRC by interfering with altered epigenetic regulation of gene expression, the mystery of what causes and determines altered epigenetic regulation of gene expression in CRC epithelium and its associated stroma needs to be addressed. Although a causal role for genetic susceptibility and environmental factors has been proposed, independent population-based studies assessing the influence of genetic variation and environmental factors such as diet and lifestyle on epigenetic regulation of gene expression should be conducted to test

these hypotheses. Only by considering the complex interactions of CRC genetics and epigenetics we will be able to obtain insight in CRC carcinogenesis and translate these findings to clinical practice.

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CHAPTER 3|

Methylation-specific PCR unraveled

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Cellular Oncology 26(5-6), 291-9 (2004)

Summary

Methylation-specific PCR (MSP) is a simple, quick and cost-effective method to analyze the DNA methylation status of virtually any group of CpG sites within a CpG island. The technique comprises two parts: 1) sodium bisulfite conversion of unmethylated cytosines to uracil under conditions whereby methylated cytosine remains unchanged and 2) detection of the bisulfite induced sequence differences by PCR using specific primer sets for both unmethylated and methylated DNA.

This review discusses the critical parameters of MSP and presents an overview of the available MSP variants and the (clinical) applications.

DNA methylation

DNA methylation is the post-replicative addition of a methyl group to the 5-carbon position of the cytosine ring, resulting in 5-methylcytosine. The transfer of a methyl group from S-adenosylmethionine (methyl donor) to cytosine (methyl acceptor) is catalyzed by DNA methyltransferases (DNMTs) and predominantly occurs on cytosines located 5' to guanine, the so-called CpG dinucleotides¹. CpG dinucleotides are underrepresented in the genome as a result of evolution-induced depletion related to the propensity of methylcytosine to deaminate spontaneously²⁻⁵. Only small regions of DNA named CpG islands contain CpG dinucleotides at its mathematically predicted frequency^{6,7}. A CpG island is defined as a 0.5-4 kb long stretch of DNA with a G:C content > 55% and an observed over expected frequency > 0.65^{1,6}. These CpG islands contain approximately 20% of all CpG dinucleotides and are associated with the promoter regions of approximately half of all genes². CpG island containing promoters are normally protected from methylation, while CpG dinucleotides which are not associated with CpG islands are heavily methylated⁸. Methylated cytosines are associated with DNMTs, methyl binding proteins, histone deacetylases and transcription repressor proteins which collectively organize a transcriptional repressive, late-replicating chromatin structure^{2,9,10}. DNA methylation is essential for normal mammalian development and is associated with genomic imprinting, transcriptional inactivation of the X-chromosome and ageing^{2,8}. Recently it has been shown that aberrant promoter methylation plays a role in cancer by transcriptional silencing of tumor suppressor genes and inactivation of DNA repair genes². Analyzing promoter hypermethylation can contribute to the understanding of cancer and has been proposed as molecular marker for diagnostic purposes¹¹.

Detection of DNA methylation

Various methods have been developed to analyze DNA methylation. Amongst these methods are restriction enzyme- and sodium bisulfite based approaches which directly detect methylation at the level of a single gene or the whole genome (for extensive reviews see references^{11,12}).

Restriction-enzyme based methods are based on the inability of methylation sensitive restriction enzymes to cleave methylated cytosines in their recognition site. The identification of the methylation status relies on Southern hybridization techniques or PCR and is based on the length of the digested DNA fragment^{12,13}. The inability to digest methylated sequences results in longer

fragments, indicating a methylated CpG dinucleotide. Although restriction-enzyme based methods are simple, rapid and highly sensitive, the technique is limited to specific restriction sites and requires a substantial amount of high quality DNA. In addition, incomplete digestion can lead to false positive results. The technique is suitable for genome-wide methylation analyses and marker discovery techniques but is less convenient for analyzing the methylation status of specific CpG sites¹².

The introduction of sodium bisulfite genomic sequencing by Frommer *et al.* in 1992¹⁴ has made methylation studies accessible to a wide range of laboratories. Sodium bisulfite (NaHSO_3) based detection of DNA methylation can circumvent the problems associated with restriction enzyme analysis and is now extensively used. Treatment of single-stranded DNA with sodium bisulfite results in sequence differences due to deamination of unmethylated cytosines to uracil under conditions whereby methylated cytosines remain unchanged. The difference in methylation status marked by bisulfite reactivity can accurately be determined and quantified by PCR-based technology. Bisulfite sequencing techniques provide qualitative data on the methylation status of 5-methylcytosines in the amplicon between the sequence primers and thus requires primers specific for bisulfite converted, but not specific for unmethylated or methylated DNA. Although this approach provides detailed information on the methylation status of all CpG sites, the method is laborious and time-consuming^{11,12}.

Methylation-specific PCR

In 1996, Herman and colleagues¹⁵ introduced methylation-specific PCR (MSP). MSP is based on the use of two distinct methylation specific primers sets for the sequence of interest (Figure 1). The unmethylated (U) primer will only amplify sodium bisulfite converted DNA in unmethylated condition, while the methylated (M) primer is specific for sodium bisulfite converted methylated DNA. MSP provides a positive, sensitive (detection of 1 methylated allele in a background of 1000 unmethylated alleles), quick and cost-effective test to analyze the methylation status of CpG dinucleotides in a CpG islands making the technique suitable for high-throughput analysis of clinical samples^{12,15}. Here we describe the most critical parameters determining the success and specificity of MSP, i.e. bisulfite conversion, primer design and PCR and discuss several post PCR validation approaches. For a detailed MSP-protocol, see reference¹⁶.

Sodium bisulfite treatment

- Chemical reaction conditions to obtain optimal conversion of cytosine to uracil and limited degradation and fragmentation of the DNA have to be achieved. This can be ensured by treating (up to 1 µg) single stranded DNA (protein- and RNA free) with sodium bisulfite (pH 5.0, final concentration 2.5-3 M) for 16 hours at 50 °C. Increasing the amount of input DNA carries the risk of incomplete conversion and leads to false-positive results. Shorter incubation times in combination with higher incubation temperatures are also possible.

- When using low quantities or paraffin-derived DNA, it is recommended to add a carrier (e.g. glycogen) to facilitate DNA precipitation after bisulfite treatment.
- Commercial kits, containing all reagents necessary for sodium bisulfite modification of DNA, are available.
- After sodium bisulfite conversion, DNA is single stranded, thus making the DNA sensitive for degradation. Therefore, sodium bisulfite treated DNA should be treated as RNA and stored at -20°C to prevent further degradation. To avoid repeated freezing and thawing, aliquoting is recommended.

Primer design

- The primers should contain one to three CpG dinucleotides in the 3' region of the primers to accomplish optimal discriminative power between methylated and unmethylated DNA and to increase the specificity of the primer annealing.
- Since amplification of unconverted unmethylated CpG dinucleotide will lead to an overrepresentation of methylation levels, the primers must be specific for sodium bisulfite converted DNA and therefore have to contain a substantial number of non-CpG cytosines in the original template. To assure sodium bisulfite conversion-specific primer annealing, the MSP primers should be tested on non-converted genomic DNA.
- The primer should be at least 23-24 bp in length to achieve gene specific primer annealing.
- It is preferable to design both U and M primer pairs with similar melting temperatures. Since the sodium bisulfite conversion decreases the GC content of the U primer, the sequence of the U-primer needs to be extended into the 5' region. This constraint will inevitably lead to a variation in start position and length but allows paired analyses in a single thermocycler and provides a convenient way to recognize each amplicon after gel electrophoresis.
- To achieve good sensitivity and specificity, the U and M primer should be comparable in their sequence and amplify the same region.
- To facilitate MSP on fragmented DNA obtained from formalin fixed, paraffin-embedded archival clinical tissue, the MSP product to be amplified should not exceed 150 bp¹⁷.
- Software tools are available to support MSP primer design^{18,19}.

Figure 1: Methylation-specific PCR (MSP)

PCR conditions

- To facilitate amplification of GC-rich DNA sequences it is recommended to use β -mercaptoethanol in the PCR buffer.
- The annealing temperature for specific determination of methylation patterns is critical. MSP relies on the specific annealing of primers based on matches or mismatches of the primer sequence to bisulfite treated DNA. Predicted annealing temperatures should be used to examine amplification of unmethylated or methylated sequences, but ideally the most specific amplification will be determined empirically through examination of multiple annealing temperatures. This is most efficiently accomplished by using a thermocycler with a gradient block. Published annealing temperatures should be confirmed with the thermocycler in use in an investigators laboratory prior to embarking on additional methylation analysis.
- Cycles of amplification must be determined for each MSP reaction condition. If high numbers of amplification cycles are used, even specific M-primers can rarely accept mismatches when annealing to DNA, which will result in the appearance of amplification in the methylated reaction. Typical amplification should not exceed 35 cycles, but must be determined empirically.
- To control PCR efficiency, a dilution series of methylated DNA in a background of unmethylated DNA can be included.

To interpret the MSP results properly, it is important to include positive, negative and H₂O controls. Positive controls are specific for U and M sequences and give an impression of the specificity of the respective reactions. DNA known to be U for the gene of interest can be used as positive control for the U condition. Methylated PCR product in the M reaction of the U control implicates lack of specificity of the M reaction and disturbs proper interpretation of the results. As positive control for the M reaction, *in vitro* methylated DNA can be used.

After amplification, the products of the U and M reactions can be visualized by 6-8% nondenaturing polyacrylamide gel electrophoresis, allowing clear separation of the small products. High percentage horizontal agarose gels can be used as alternative. Fully unmethylated samples will reveal a PCR product only when U-primers are used, as fully methylated samples will give a distinct band in the M reaction. Since clinical tissue samples should be considered heterogeneous, both U and M amplicons can be expected.

Post-PCR validation

- Following PCR amplification, specificity of amplification can be confirmed by product analysis. MSP amplification results in loss of information of the CpG sites contained within the primer, but all CpG sites between primers in the PCR product remain informative. False priming, for reasons described above, can be examined by post-PCR analysis of the PCR product. Three principle ways can be employed. The use of fluorescent dyes (Syber Green) can facilitate the examination of differences in melting temperature based on CG content, much the same way as DGGE (described below). This however, does not provide much additional information on location of the methylation changes in the amplified product. Post-PCR restriction analysis can provide information for specific sequences present

in restriction sites¹⁵. In addition, sequencing of the PCR product can be performed and provides complete CpG methylation information on all CpG sites within the PCR product.

MSP has been widely used for promoter methylation profiling of cancer²⁰, diagnosing Prader-Willi- and Angelman Syndrome by making use of abnormal methylation at the *small nuclear ribonucleoprotein-associated polypeptide N (SNRPN)* gene²¹ and to evaluate X chromosome inactivation²².

Variants and applications of MSP

(Multiplex) nested MSP

Since the publication of the 'original' MSP protocol¹⁵, several variants have been developed. To increase the sensitivity of MSP for detection of methylated DNA in a background of unmethylated DNA, Palmisano *et al.*²³ developed a nested, two-stage MSP. The first round PCR is performed using primers which amplify sodium bisulfite-modified DNA, although these primers do not discriminate between methylated and unmethylated alleles. This means that the primers are located in regions flanking the MSP primers and harboring a substantial number of cytosines but no CpG dinucleotides (analogue to bisulfite sequence primers). The obtained PCR product is diluted 50-100 fold and subjected to second round PCR reactions using U and M specific MSP primers. Nested MSP allows a more sensitive (1 methylated allele in >50,000 unmethylated alleles) detection of methylation in clinical samples harboring small amounts of poor quality DNA.

False positives, caused by the lack of the single-stranded conformation, renaturation and uncompleted sodium bisulfite conversion caused by the protection of a methylated cytosine of the neighboring unmethylated outer cytosine, have been reported^{12,24,25}. To check the completion of the bisulfite treatment, an internal standard that consists of an *in vitro* methylated oligonucleotide can be included²⁵. If DNA obtained from formalin fixed, paraffin-embedded archival tissue is used, it is recommended to design first round primers to obtain amplicons smaller than 150 bp to further enhance the success rate¹⁷. Van Engeland *et al.*¹⁷ multiplexed the first round primers of different genes allowing simultaneous analysis of multiple colorectal cancer tumor suppressor gene promoters. This approach offers the additional advantage that the PCR-product obtained with the first round PCR is stable and can be stored for a long time. This strategy has now been used successfully for methylation profiling of gastrointestinal stromal tumors²⁶ and hepatocellular carcinomas²⁷.

Classical MSP and its nested variant are very suitable for detection of methylated tumor DNA in a variety of body fluids. Colorectal-²⁸⁻³², lung-³³, liver-^{34,35}, head and neck-³⁶ and brain³⁷ tumor derived DNA has been detected in serum and plasma. Methylated gene promoters in urine and semen have been used to diagnose prostate cancer³⁸ and methylation markers present in ductal lavage has reported to be a useful adjunct to mammography in the early diagnosis of breast cancer³⁹. Sputum^{23,40} and bronchial brushes^{40,41} have been analyzed for promoter hypermethylation in order to diagnose lung

cancer while Rosas and co-workers reported that aberrant promoter methylation in saliva can be used for detecting and monitoring head and neck cancer⁴².

Quantitative MSP

To exactly quantify the number of methylated alleles, several quantitative MSP (Q-MSP) protocols have been developed which take advantage of the development of quantitative real-time PCR. Amongst these assays are approaches using fluorescently labelled MSP primers and those based on Taqman® technology (MethyLight)^{43,44}. Two primers and a fluorogenic, dual-labelled probe (reporter dye (e.g. FAM) and quencher dye (e.g. TAMRA)) specific for unmethylated or methylated DNA are used in MethyLight⁴³. During the extension phase of the PCR, the 5' to 3' exonuclease activity of the TaqDNA Polymerase cleaves the reporter from the probe, releasing it from the quencher, resulting in an increase in fluorescence emission. Data are normalized to an internal reference gene, e.g. *myoblast determination protein 1* (*MYOD1*) or β *actin* (*ACTB*) which are designed in a region that does not contain any CpG nucleotides to allow an unbiased amplification. Rand *et al.* reported false positive results due to the amplification of unconverted DNA and recommend the inclusion of a sodium bisulfite conversion detection step (ConLight-MSP) to avoid overestimation of DNA methylation⁴⁵. Primer design improvements, as described above for MSP primers, can also eliminate this amplification of unmodified DNA. Lo and colleagues include primers as well as probes specific to methylated, unmethylated or unconverted wild-type DNA^{44,46}. Most commonly, these studies determine only methylated bisulfite converted DNA for the gene of interest and compare these results to an internal reference gene⁴⁷⁻⁵⁴. It should be noted that the quantitation provided is for the relative amounts of methylated DNA with a particular methylation patterns compared to some input DNA (reference control gene or unmethylated sequence). This quantitation may reflect actual changes in methylation density or differences in tumor DNA within a specific sample. Recently, Fackler *et al.* introduced a quantitative multiplex nested MSP (QM-MSP) assay to further increase the sensitivity of this technology⁵⁵. In the first PCR reaction external primers lacking CG nucleotides, thereby obtaining DNA amplification independent of methylation status, are used. In the second reaction, quantitative analysis of methylated and unmethylated DNA for each gene was performed separately with the primer pairs and probes.

An additional advantage of Q-MSP is that it does not involve additional PCR steps, gel electrophoresis or hybridization. These characteristics makes Q-MSP applicable for fast screening of DNA methylation in clinical samples⁴³. Several studies describe the applicability of Q-MSP for analyzing formalin-fixed, paraffin-embedded tissues. Jeronimo and colleagues successfully compared *GSTP1* promoter methylation in formalin-fixed, paraffin-embedded tissue of non-neoplastic prostatic tissue with organ-confined prostate adenocarcinoma⁴⁷. Also, formalin-fixed, paraffin-embedded specimens of the upper gastrointestinal tract⁵⁴, prostate biopsies⁵⁶, breast tumors⁵⁵ and even laser-assisted micro dissected samples of breast tumors⁵¹ have been analyzed for promoter hypermethylation quantitatively. In addition, Q-MSP has been used to detect aberrant methylation profiles of circulating tumor DNA in serum of patients with oesophageal-⁵⁷, prostate-⁵⁸, lung-⁵⁰, liver-⁴⁶ and cervical⁵⁹ can-

cer. Furthermore, promoter hypermethylation is detected by Q-MSP in cervical scrapings⁴⁹.

In situ MSP

MSP data represent only a small percentage of the analyzed cells in a population unless the PCR is done semi-quantitative, with the result that the extent of heterogeneity for loss of expression during tumorigenesis cannot be defined. Furthermore, the precise timing of DNA methylation changes in specific cell types during embryonic development and carcinogenesis cannot be analyzed. To overcome this issue, Nuovo and coworkers combined MSP with *in situ* hybridization (ISH) allowing *in situ* detection of methylated DNA on tissue slides⁶⁰. Three sequential tissue sections are placed on a slide, treated with sodium bisulfite and analyzed by MSP. One slide is analyzed with U-primers, one slide with M-primers, while no primers are applied to the control slide. After amplification, *in situ* hybridization (ISH) is performed using a U- or M-specific internally digoxin-labeled probe, corresponding to the U and M products generated by solution-phase MSP. Visualization can be performed by nuclear fast red staining which colors the negative cells pink and the positive cells blue. *In situ* MSP offers information at the cellular level since visualization of the methylation of specific alleles in individual cells is possible. The method can be used to examine the role of CpG island methylation during embryogenesis, developmental timing of -and aberrant imprinting in specific cell types, mammalian X chromosome inactivation and tumor progression in clinical samples with heterogeneity and the precise cellular consequences of the methylation for gene expression^{12,60}.

In addition to gel- and fluorescence based detection of MSP products, HPLC and DGGE based MSP-variants have been developed.

HPLC

Recently, denaturing high-performance liquid chromatography (DHPLC) in combination with MSP for the analysis of methylation profiles has been described⁶¹⁻⁶³. After sodium bisulfite conversion, PCR amplification with primers specific for sodium bisulfite converted DNA, but not for unmethylated and methylated alleles is achieved, followed by denaturation and renaturation of the PCR products, enabling the formation of heteroduplex DNA detectable by HPLC. The principle of the method is based on different denaturing temperatures between bisulfite converted methylated and unmethylated fragments caused by different G/C contents. DHPLC in combination with MSP allows for the detection of minimal CpG methylation as well as complete CpG methylation of a sample. Furthermore, the degree of CpG site methylation can be estimated from the degree of heteroduplex formation and the presence of additional and distinct heteroduplex signals can indicate the presence of a mutation in the fragment analyzed.

Matin *et al.* also used HPLC to differentiate between methylated and unmethylated amplicons using ion-pair reversed-phase high performance liquid chromatography (IP RP HPLC), which is based on distinction of polynucleotide chain length after bisulfite treatment and primer extension reactions carried out with deoxy- and dideoxynucleotides⁶⁴.

DGGE

Epigenetically modified genotypes can be characterized by combining MSP with denaturing gradient gel electrophoresis (DGGE)⁶⁵. The principle of this application is that after bisulfite conversion, the amplicon of methylated DNA has a higher GC content compared to unmethylated DNA, resulting in different melting temperatures, thus the basis for separation is based on thermal stability. In addition to detection of almost any sequence change in every DNA region, the technique allows also detection of differentially methylated sequences which is not managed by the usage of restriction enzymes. The first studies described the ability to make a distinction between fully, hemi and unmethylated sites⁶⁶. Later on, a more sensitive method was developed by using a GC-clamp and the distinction between methylated or unmethylated CpG sites could be made on single base sequence changes in a certain fragment⁶⁵. This approach is not technically MSP, but can be used to examine overall methylation differences.

In summary, MSP is a rapid method to detect aberrant promoter methylation in tissue and body fluids and provides a non-invasive method for molecular diagnosis of diseases associated with aberrant DNA methylation.

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CHAPTER 4|

Promoter methylation precedes chromosomal alterations in colorectal cancer development

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Cellular Oncology 28(5-6), 247-57 (2006)

Abstract

Colorectal cancers are characterized by genetic and epigenetic alterations. This study aimed to explore the timing of promoter methylation and relationship with mutations and chromosomal alterations in colorectal carcinogenesis. Therefore promoter methylation status of *hMLH1*, *O⁶MGMT*, *APC*, *p14^{ARF}*, *p16^{INK4A}*, *RASSF1A*, *GATA4*, *GATA5*, and *CHFR* was evaluated using methylation-specific PCR in a series of 47 nonprogressed adenomas, 41 progressed adenomas (malignant polyps), 38 colorectal carcinomas and 18 paired normal tissues. Mutation status of *TP53*, *APC* and *KRAS* were studied by p53 immunohistochemistry and sequencing of the *APC* and *KRAS* mutation cluster regions. Chromosomal alterations were evaluated by comparative genomic hybridization. These analyses showed that nonprogressed adenomas, progressed adenomas and carcinomas show similar frequencies of promoter methylation for the majority of the genes. Normal tissues showed significantly lower frequencies of promoter methylation of *APC*, *p16^{INK4A}*, *GATA4* and *GATA5* (*P* values 0.02, 0.02, 1.1×10^{-5} and 0.008 respectively). P53 immunopositivity and chromosomal abnormalities occur predominantly in carcinomas (*P* values 1.1×10^{-5} and 4.1×10^{-10}). Since promoter methylation was already present in nonprogressed adenomas without chromosomal alterations, we conclude that promoter methylation can be regarded as an early event preceding *TP53* mutation and chromosomal abnormalities in colorectal cancer development.

Introduction

Colorectal cancer development is characterized by the growth of a benign precursor lesion of which eventually a small percentage will progress into a carcinoma¹. The genetic alterations underlying the adenoma to carcinoma transition have been extensively studied over the past two decades. Pioneering research of Vogelstein and co-workers has proposed a progression model in which genetic alterations as *APC* and *TP53* mutations and allelic loss of 5q and 18q play an important role²⁻⁶. Previously, we introduced the concept that chromosomal instability does not merely constitute genetic noise but occurs in non-random patterns. Accumulation of losses in 8p21-pter, 15q11-q21, 17p12-13 and 18q12-21 and gains in 8q23-qter, 13q14-31 and 20q13 are strongly associated with advanced lesions and can be used as indicator of progression towards malignancy^{7,8}. Recently, it has become clear that initiation and progression of cancer also involves epigenetic alterations such as DNA methylation and that genetic and epigenetic alterations interact in driving the development of cancer⁹. Colorectal cancer development is associated with epigenetic silencing of the DNA repair genes *hMLH1*¹⁰ and *O⁶MGMT*¹¹, the WNT signal transduction regulator *APC*¹², the Ras signaling molecule *RASSF1A*¹³, the transcription factors *GATA4* and *GATA5*¹⁴ and the cell cycle regulators *CHFR*^{15,16}, *p16^{INK4A}* and *p14^{ARF}*^{17,18}. Although extensive knowledge exists on epigenetic and genetic changes in colorectal cancer, little is known about the exact relationship between these two^{19,20}. In this cross-sectional study we address epigenetic and genetic (at the level of the single gene as well as at the level of whole chromosomes) alterations in colorectal cancers and its precursor lesions. Using a multi-gene approach we investigate the timing of promoter methylation and define how these epigenetic events are related to genetic events in colorectal cancer development.

Materials and Methods

Patient material

This study was performed on a subset (n=139) of colorectal adenoma and carcinoma tissues which has been analyzed for structural chromosomal abnormalities by comparative genomic hybridization (CGH) previously⁷. Part of this subset has also been analyzed for mutation status of *APC* (n=96) and *KRAS* (n=78)^{7,21}. We extended this series by adding 20 colorectal adenoma and carcinoma cases, bringing the overall total to 159 tissues. This series consists of 47 colorectal adenomas without signs of malignancy at time of resection (nonprogressed adenomas (nA)), 41 malignant polyps (colorectal adenomas containing a focus of carcinoma) and 38 additional solitary colorectal carcinomas (Cs). Of the 41 malignant polyps, the adenoma part, referred to as progressed adenomas (pA) (n=41), and the carcinoma part (Cmp) (n=33) were microdissected and analyzed separately. If present we added morphologically normal mucosa within the resection specimen (n=18) of patients with solitary carcinomas (Cs) to these series. For each tissue sample, DNA was extracted from fifteen 10-µm paraffin sections, dissecting the most tumor-rich areas, allowing a maximum of 20% non-tumor cell contamination.

Overall, the tissues were obtained from 95 patients, 46 males and 49 females (mean age of 67 years: range 40-89). Twenty-four patients exhibited multiple tumors; 4 patients presented with multiple adenomas, 1 patient presented with multiple carcinomas and 19 patients exhibited 1 or more adenomas adjacent to a carcinoma. The histological characteristics are listed in Table 1.

Table 1: Histological characteristics of 159 adenoma and carcinoma tissues

	adenomas	n	carcinomas	n
Tissue	nonprogressed adenoma (nA)	47	carcinoma part of malignant poly (Cmp)	33
	progressed adenoma (pA)	41	solitary carcinomas	38
Histologic type	tubular	38		
	tubulovillous	42		
	villous	5		
	serrated	3		
Degree of dysplasia	mild	13		
	moderate	50		
	severe	25		
Differentiation grade			Well	13
			Moderate	52
			Poor	6
TNM			I	23
			II	30
			III	17
			IV	1

TNM; tumor node metastasis

Promoter methylation analysis

DNA methylation in the CpG islands of the *hMLH1*, *O⁶MGMT*, *APC*, *p14^{ARF}*, *p16^{INK4A}*, *RASSF1A*, *GATA4*, *GATA5* and *CHFR* gene promoters was determined by chemical modification of genomic DNA with sodium bisulfite and subsequent methylation-specific PCR (MSP) as described in detail elsewhere²²⁻²⁴. Briefly, 1 µg of DNA was denatured by NaOH and modified by sodium bisulfite. DNA samples were then purified using Wizard DNA purification resin (Promega, Madison, USA) again treated with NaOH, precipitated with ethanol, and resuspended in H₂O. To facilitate MSP analysis on DNA retrieved from formalin-fixed, paraffin embedded tissue, DNA was first amplified with flanking PCR primers that amplify bisulfite-modified DNA but do not preferentially amplify methylated or unmethylated DNA. The resulting fragment was used as a template for the MSP reaction. Primer sequences have been described before^{14,22,25}. All PCRs were performed with controls for unmethylated alleles (DNA from normal lymphocytes), methylated alleles [normal lymphocyte DNA treated *in vitro* with *SssI* methyltransferase (New England Biolabs)], and a control without DNA. Ten µl of each MSP reaction were directly loaded onto nondenaturing 6% polyacrylamide gels, stained with

ethidium bromide, and visualized under UV illumination. The methylation status was assessable in 96% of the total number of analyses. To assess reproducibility, 234 MSP reactions have been performed in duplicate starting from DNA amplification with flanking PCR primers, the reproducibility was 90%. To exclude false priming, sequencing of the methylated amplicon of *APC* was performed, revealing extensive methylation of all amplicons, including the primer binding region.

P53 immunohistochemistry

P53 immunohistochemistry (n=146) was performed using the mouse monoclonal antibody DO7 (DAKO, Glostrup, Denmark). Immunoperoxidase staining for p53 in formalin-fixed, paraffin-embedded tissue sections was performed by a horseradish peroxidase labeled streptavidin-biotin method. Four μ m sections were mounted on 0.1% poly-L-lysine coated glass slides, deparaffinized, and rehydrated through graded alcohols to water. Endogenous peroxidase activity was blocked by incubation with 0.3% H₂O₂ in methanol. Sections were immersed in 10 mM sodium citrate buffer, pH 6.0, and subjected to heat-induced antigen retrieval with microwave. To block non-specific protein binding, sections were pre-incubated with normal rabbit serum (1:50, DAKO) for 10 min at room temperature. Mouse monoclonal antibody against p53 (1:500 DO7, DAKO) was applied, and tissue sections were incubated overnight at 4 °C. Then sections were rinsed with PBS, and treated with biotinylated rabbit anti-mouse IgG (1:500, DAKO) for 30 min at room temperature, rinsed with PBS, and then incubated with streptavidine-biotin-HRP complex (1:200, DAKO) for 1 hour at room temperature. After washing with PBS the complex was visualized with diaminobenzidine and H₂O₂ for 3 min. Sections were then counter stained with hematoxylin, dehydrated in graded alcohols, cleared in xylene and cover slipped. The area percentage of positive nuclei was scored with a point counting approach using a video overlay measuring system (Qprodit Leica, Cambridge, UK). An area percentage of 20 was used as threshold for positivity²⁶.

KRAS mutation analysis

KRAS mutation status of 78 colorectal adenoma and carcinoma tissues have been analyzed previously⁷. Fifty-two additional tissues were analyzed by PCR using an oligonucleotide 20-mer panel of codons 12 and 13 (TIB Molbiol, Advanced Biotechnology Center, Genova, Italy) as previously described²¹. Extracted DNA from peripheral blood lymphocytes from healthy donors was used as wild-type *KRAS* codon 12 GGT-gly and codon 13 GGC-gly controls, and extracted DNA from 6 different colon cancer cell lines was used as control for known *KRAS* mutations.

Array-CGH analysis

One hundred-thirty-nine colorectal adenoma and carcinoma tissues have been analyzed by conventional CGH previously⁷. The 20 additionally collected neoplasms were analyzed by array CGH analysis using 5 K BAC arrays^{27,28}. In short, we used a full-genome array printed in the house containing approximately 5000 clones with an average resolution of 1 Mb (<http://www.vumc.nl/microarrays/index.html>). After amplification of BAC clone DNA by ligation-mediated polymerase chain reaction (PCR) according to

Snijders *et al.*²⁸ all clones were printed in triplicate. Printing of clones was performed on codelinkTM slides (Amersham BioSciences, Roosendaal, NL) at a concentration of 1µg/µl, in 150mM sodium phosphate, pH 8.5, using a SpotArray72 printer (Perkin Elmer Life Sciences, Zaventum, BE). After printing slides were processed according to the manufacturer's protocol. Labeling and hybridization of tumor and reference DNAs were performed as described in detail previously²⁸ with some modifications, namely, hybridizations took place in a hybridization station (HybArray12TM – Perkin Elmer Life Sciences, Zaventum, BE) and slides were scanned with Agilent DNA Microarray scanner (Agilent technologies, Palo Alto, USA), omitting the DAPI staining step. Segmentation and quantification of the spots was done using Imagene 5.6 software (Biodiscovery Ltd, Marina del Rey, California). Local background median intensity was subtracted from the signal median intensity for both test and reference channels and a ratio tumor/reference was calculated. The ratios were normalized against the mode of all ratios of the autosomes. As the clones were spotted in triplicate, the median value of the corresponding three intensities was taken into account for each clone in the array. Clones from which the intensities of the three spots had a standard deviation > 0.2 were excluded. Furthermore, clones with more than 20% missing values in all carcinomas were excluded for further analysis. All the analyses were done excluding chromosome X, as in every hybridisation a sex-mismatched reference DNA was used for quality control of the experiment. Clone positions were considered according to freeze May2004. After using a smoothing algorithm²⁹, DNA copy number ratios obtained by array CGH were recoded as gains and losses at the resolution of whole chromosome arms compatible with the data obtained by chromosome CGH.

Data analysis

Differences in frequencies of gene methylation between the different stages of disease progression and associations between promoter methylation and mutations were evaluated by the Pearson's χ^2 or Fisher's exact test where appropriate. The total number of methylated genes, referred to as methylation index (MI), is defined as the number of genes methylated divided by the number of genes analyzed. The same accounts for the total number of gains and losses, referred to as chromosomal events, and the number of losses (8p21-pter, 15q11-q21, 17p12-13 and 18q12-21) and gains (8q23-qter, 13q14-31, and 20q13) associated with advanced lesions, referred to as cancer associated events⁷. The Mann-Whitney U and Kruskal Wallis nonparametric test were used for comparing means of continuous variables. The McNemar test for paired cases was used to test the methylation differences between a subset of solitary carcinomas (Cs) and paired normal tissue. Simple linear regression analysis was performed to investigate the correlation between the number of methylated genes and the number of chromosomal abnormalities. For all analyses SPSS software version 11.0 was used. All reported *P* values are two-sided, and a *P* value < 0.05 was considered statistically significant.

Results

Promoter methylation in relation to adenoma-carcinoma progression

In order to investigate the timing of promoter methylation in colorectal cancer development, we studied the frequency of promoter methylation of genes which function in regulating diverse cell functions in the colorectal adenoma and carcinoma tissues. Our data demonstrate that nonprogressed adenomas (nA), progressed adenomas (pA), carcinoma parts of malignant polyps (Cmp) and solitary carcinomas (Cs) showed similar frequencies of promoter methylation for the majority of genes (Table 2). No difference in mean methylation index (MI) (total number of methylated genes divided by the number of genes analyzed) between the different categories of neoplasms was observed. However, *p14^{ARF}* methylation was found in 71.1% and 73.5% of the nonprogressed adenomas (nA) and progressed adenomas (pA) respectively and decreases to 53.3% and 37.1% of the carcinoma parts of malignant polyps (Cmp) and solitary carcinomas (Cs) respectively (*P* value 0.006).

Table 2: Timing of promoter methylation, genetic and chromosomal alterations

	nA	pA	Cmp	Cs	<i>P</i> value
Number of cases	47	41	33	38	
Promoter methylation (%)					
<i>hMLH1</i>	44.7	31.7	36.4	55.3	
<i>O⁶MGMT</i>	66.7	72.5	54.5	60.5	
<i>RASSF1A</i>	19.1	24.4	24.2	29.7	
<i>APC</i>	61.7	51.2	33.3	44.7	
<i>p14^{ARF}</i>	71.1	73.5	53.3	37.1	0.006
<i>p16^{INK4A}</i>	28.9	36.6	36.4	31.6	
<i>GATA4</i>	77.3	77.5	75.8	86.5	
<i>GATA5</i>	95.7	87.8	84.4	82.9	
<i>CHFR</i>	48.6	56.7	50.0	55.9	
methylation index ^a	0.57	0.56	0.49	0.53	
Genetic alterations (%)					
p53 immunopositivity (n=146)	14.3	34.2	55.2	59.5	1.1 x 10 ⁻⁵
<i>APC</i> mutation (n= 96)	50 (17/34)	63.2 (24/38)	58.3 (7/12)	66.7 (8/12)	
<i>KRAS</i> mutation (n= 130)	34.3	40.0	28.6	28.1	
Chromosomal alterations ^a					
chromosomal events	6.2	12.4	12.2	10.6	4.1 x 10 ⁻⁶
CAE	0.9	3.0	3.3	3.0	3.6 x 10 ⁻¹⁰

^a values indicate mean

Since we observed that promoter methylation of the studied genes is already present in nonprogressed adenomas (nA), we were interested in the presence of promoter methylation in matching normal mucosa. For 18 solitary carcinomas (Cs) morphological normal tissue from the resection specimen was available (Table 3A).

Table 3A: Promoter methylation frequencies in 18 paired carcinoma and normal tissues

patient	tissue	<i>hMLH1</i>	<i>O⁶MGMT</i>	<i>RASSF1A</i>	<i>p14^{ARF}</i>	<i>p16^{INK4A}</i>	<i>APC</i>	<i>GATA4</i>	<i>GATA5</i>	<i>CHFR</i>
1	N									
	C									
2	N									
	C									
3	N									
	C									
4	N									
	C									
5	N									
	C									
6	N									
	C									
7	N									
	C									
8	N									
	C									
9	N									
	C									
10	N									
	C									
11	N									
	C									
12	N									
	C									
13	N									
	C									
14	N									
	C									
15	N									
	C									
16	N									
	C									
17	N									
	C									
18	N									
	C									

Results of promoter methylation in 18 paired carcinoma (C) and normal tissue (N). Black box indicates a methylated gene, white box indicates a unmethylated gene, grey box indicates a failed PCR

We performed a nonparametric test for matched pairs and compared the methylation profile of 18 solitary carcinomas to their corresponding normal mucosa. In 158 of the 162 (9 genes x 18 pairs) possible combinations the gene methylation status was assessable in the carcinoma as well as in the normal tissue. In 48.7% of the pairs (77/158) no difference in gene methylation was observed within pairs (N=C) (Table 3B).

Table 3B: Promoter methylation frequencies in 18 paired carcinoma and normal tissues

	<i>hMLH1</i>	<i>O⁶MGMT</i>	<i>RASSF1A</i>	<i>p14^{ARF}</i>	<i>p16^{INK4A}</i>	<i>APC</i>	<i>GATA4</i>	<i>GATA5</i>	<i>CHFR</i>
N (%)	50	38.9	16.7	33.3	0	16.7	16.7	16.7	27.8
C (%)	72.2	61.1	44.4	38.9	38.9	61.1	94.4	72.2	55.6
N=C (%)	66.7 (12/18)	44.4 (8/18)	38.9 (7/18)	50.0 (9/18)	61.1 (11/18)	44.4 (8/18)	22.2 (4/18)	46.7 (7/15)	64.7 (12/17)
C>N (%)	27.8 (5/18)	38.9 (7/18)	44.4 (8/18)	27.8 (5/18)	38.9 (7/18)	50 (9/18)	77.8 (14/18)	53.3 (8/15)	29.4 (5/17)
N>C (%)	5.6 (1/18)	16.7 (3/18)	16.7 (3/18)	22.2 (4/18)	0 (0/18)	5.6 (1/18)	0 (0/18)	0 (0/15)	5.6 (1/18)
<i>P</i> value					0.02	0.02	4.1 x 10 ⁻⁶	0.008	

Results of promoter methylation in 18 paired carcinoma (C) and normal tissue (N). N=C: no difference in gene methylation between carcinoma and normal tissue. C>N: gene methylated in carcinoma and unmethylated in normal tissue. N<C: gene methylated in normal tissue and unmethylated in carcinoma

In 66.2% (51/77) of these pairs both components were unmethylated while in 33.8% (26/77) the carcinoma as well as the corresponding normal tissue showed promoter methylation. In 43% (68/158) of all pairs gene methylation was present in the carcinoma while absent in the corresponding normal tissue (C>N), and only in 8.2% (13/158) of the pairs promoter methylation was only observed in the normal tissue (N>C).

The McNemar test for paired cases showed that promoter methylation of *APC*, *p16^{INK4A}*, *GATA4*, and *GATA5* occurred significantly more frequent in the carcinomas when compared to corresponding normal mucosa (*P* values 0.02, 0.02, 1.1×10^{-5} and 0.008 respectively) (Table 3B). Although promoter methylation of *hMLH1*, *O⁶MGMT* and *CHFR* was present in the normal tissues, this was predominantly when the carcinoma was also methylated. For example, promoter methylation of *hMLH1* was observed in 50% of the adenomas and 72.2% of the paired carcinomas (this high frequency could be explained by the fact that in 6 of the 18 pairs (33.3%) the carcinoma part showed microsatellite instability, data not shown. In 5 of these 6 pairs, the carcinoma as well as the paired normal tissue showed *hMLH1* methylation). In 8 of the 9 pairs in which normal tissue showed *hMLH1* promoter methylation, carcinoma tissue was methylated as well. In 6 cases a difference in methylation was present of which in 5 cases *hMLH1* was methylated in the carcinomas while unmethylated in the paired normal mucosa. Comparable patterns were observed for promoter methylation of *O⁶MGMT*, *RASSF1A* and *CHFR*. More difference between paired carcinoma and normal tissues was observed for *p14^{ARF}* methylation. In 4 of the 9 cases in which a difference within pairs was observed, normal tissue displayed gene methylation while *p14^{ARF}* was not methylated in the corresponding carcinoma.

Promoter methylation in relation to genetic alterations

In order to study the relationship between promoter methylation and genetic alterations we analyzed the mutation status of three key genes involved in development of colorectal cancer, i.e. *TP53*, *APC* and *KRAS*.

Disruption of the p53 pathway, amongst others, can occur by loss of function of *TP53* itself and by *p14^{ARF}* methylation³⁰. The frequency of *p14^{ARF}* methylation significantly decreased in tumor progression (Table 2). In contrast, aberrant p53 status, indicated by p53 immunopositivity, increased from 14.3% in the nonprogressed adenomas (nA) through 34.2% in the progressed adenomas (pA) to 55.2% and 59.5% in the carcinoma parts of malignant polyps

(Cmp) and solitary carcinomas (Cs) respectively (P value 1.1×10^{-5}). Case by case, $p14^{ARF}$ methylation shows an inverse relation with p53 immunopositivity, approaching statistical significance (P value 0.07).

For *APC* and *KRAS*, a similar pattern was found. *APC* was mutated in 50% and 63.2% of the nonprogressed and progressed adenomas (nA and pA) respectively, compared to 58.3% and 66.7% of the carcinoma parts of malignant polyps (Cmp) and solitary carcinomas (Cs)⁷. *KRAS* mutation was observed in 34.3 % and 40.0 % of the nonprogressed and progressed adenomas (nA and pA) respectively, and in 28.6% and 28.1% of the carcinoma parts of malignant polyps (Cmp) and solitary carcinomas (Cs) (Table 2). While neither the frequencies of *APC* mutation nor *APC* promoter methylation differ between the different stages of tumor development, case by case analysis indicated an inverse relation (P value 0.06). A similar pattern and inverse relation was observed for *KRAS* mutation and promoter methylation of *RASSF1A* and *hMLH1* (P values 0.01 and 0.001 respectively).

Promoter methylation in relation to chromosomal alterations

The timing and interrelationship of promoter methylation and chromosomal alterations in tumor progression were analyzed by studying promoter methylation in cases without chromosomal abnormalities and by relating gene methylation status to the mean number of chromosomal and cancer associated events (CAE). Simple linear regression analyses revealed no correlation between the MI and the number of chromosomal abnormalities or number of CAE. As described previously, the number of chromosomal abnormalities and especially the number of CAE are associated with progressed lesions (P values 4.1×10^{-6} and 3.6×10^{-10} respectively) (Table 2)⁷. Figure 1 shows that while the mean number of chromosomal events and CAE increases during tumor progression the mean MI remains stable. In 13 cases (11 nonprogressed adenomas (nA) and 2 solitary carcinomas (Cs)) no chromosomal alterations were observed. The 12 adenomas without chromosomal abnormalities did not differ in MI from adenomas with chromosomal abnormalities. Interestingly, the 2 solitary carcinomas (Cs) without chromosomal abnormalities (both cases exhibit microsatellite instability; data not shown) were characterized by a MI of 0.96, while the MI of carcinomas harboring chromosomal alterations was 0.56 (P value 0.006). No association between promoter methylation of a specific tested gene and the number of chromosomal abnormalities was observed.

One of the cancer associated chromosomal changes, deletion of 8p21, includes the *GATA4* gene, which was also a frequent target of epigenetic changes in these tumors. Promoter methylation as well as loss of heterozygosity could combine leading to loss of function of *GATA4*. *GATA4* methylation was found in respectively 77.3% and 77.5% of the nonprogressed adenomas (nA) and progressed adenomas (pA) and in 75.8% and 86.5% of the carcinoma parts of malignant polyps (Cmp) and solitary carcinoma (Cs) respectively. In contrast, the frequency of loss of 8p21-pter increases in tumor development from 12.8% and 39.0% of the nonprogressed adenomas (nA) and progressed adenomas (pA) respectively to 45.5% and 42.1% of the carcinoma parts of malignant polyps (Cmp) and solitary carcinoma (Cs) respectively (P value 0.005).

Figure 1: Promoter methylation and chromosomal alterations in colorectal cancer development. nA= nonprogressed adenoma; pA= progressed adenoma; Cmp= carcinoma part of a malignant polyp; Cs= solitary carcinoma. (A) Promoter methylation has been analyzed for 9 DNA repair- and tumor suppressor genes. The methylation index (total number of methylated genes divided by the number of genes analyzed) stays stable during tumor development, while (B) the total number of chromosomal alterations (chromosomal events), analyzed on genomic level, and (C) the number of cancer associated events (losses at 8p21-pter, 15q11-q21, 17p12-13, 18q12-21, and gain at 8q23-qter, 13q14-31 and 20q13) increase. * P value 4.1×10^{-6} , † P value 3.6×10^{-10}

The frequency in which loss of 8p21-pter is combined with *GATA4* methylation (*GATA4* M/8p-) increases during tumor development from 9% in nonprogressed adenomas (nA) to 25%, 30% and 32% in progressed adenomas (pA), carcinoma parts of malignant polyps (Cmp) and solitary carcinoma (Cs) respectively. The frequency in which only *GATA4* is methylated (*GATA4*/8p) is stable and occurs in 68.2% of the nonprogressed adenomas (nA) and 52.2%, 45% and 54% of the progressed adenomas (pA), carcinoma parts of malignant polyps (Cmp) and solitary carcinoma (Cs) respectively. Loss of 8p21-pter without concomitant methylation of *GATA4* (*GATA4* U/8p-) is infrequent occurring in 4.5% and 15% of the nonprogressed adenomas (nA) and progressed adenomas (pA) and in 15% and 8.1% of the carcinoma parts of malignant polyps (Cmp) and solitary carcinoma (Cs).

Discussion

In this study we attempt to elucidate the timing and interrelation of promoter methylation and genetic alterations in colorectal cancer (CRC) development. Therefore we studied genetic and epigenetic events known to be associated with colorectal cancer development.

Considering the timing of epigenetic events in tumor progression, our results indicate that promoter methylation of the studied genes can be regarded as an early event in colorectal carcinogenesis. A high frequency of promoter methylation of multiple DNA repair- and tumor suppressor genes is already present in adenomas without any histological signs of progression, and malignant lesions showed similar frequencies of methylation. Even in morphologically normal mucosa from patients with solitary carcinomas (Cs) promoter methylation of *hMLH1*, *O⁶MGMT*, *RASSF1A*, *p14^{ARF}* and *CHFR* was observed, but, with exception of *p14^{ARF}* methylation, in lower frequencies compared to the carcinomas. *P16^{INK4A}*, *APC*, *GATA4*, and *GATA5* methylation occurred predominantly in the carcinomas. Promoter methylation in normal tissues was in most cases consistent with the methylation profile of the paired carcinoma.

However, additional studies involving normal colonic mucosa from individuals without cancers are required to determine the exact timing of promoter methylation of the studied genes.

Interestingly, hypermethylation of *p14^{ARF}* was more frequently present in nonprogressed adenomas (nA) and progressed adenomas (pA) when compared to carcinoma parts of malignant polyps (Cmp) and solitary carcinomas (Cs). This observation can possibly be explained by the concept that the transition from an adenoma to a carcinoma can be considered as a transition from a heterogeneous cellular population to one that is more homogeneous⁵. Even though promoter methylation is a dynamic process, this indicates that *p14^{ARF}* methylation is not necessarily associated with a definitive growth advantage.

Furthermore, since the tumor suppressor function of *p14^{ARF}* is dependent upon the presence of functional p53³¹, *p14^{ARF}* methylation is possibly of greater importance in early stages of disease progression where *TP53* mutations are not highly prevalent. This is supported by the observation that the frequency of p53 immunopositivity, as a marker of *TP53* mutations, increases during colorectal cancer development.

The concept that epigenetic alterations occur most frequently during the early stages of tumor development as well as the presence of promoter methylation of *hMLH1* and *O⁶MGMT* in normal colonic tissues of patients with colon cancers has also been shown by others³²⁻³⁴. Baylin and Ohm recently hypothesized that the early epigenetic alterations predispose cells to acquire the genetic abnormalities that proceed the neoplastic process³⁴. In addition, Tlsty *et al.* showed that hypermethylation of the *p16^{INK4A}* promoter in mammary epithelial cells is associated with entrance into a state of unrestricted proliferation accompanied by chromosomal instability^{35,36}. The actual mechanism involved is unknown but as epigenetical silencing of mismatch repair (MMR) genes causes a mutator phenotype³⁷ one would hypothesize that promoter methylation of "stability genes", such as *p16^{INK4A}* which retains proper cell cycle control, can initiate chromosomal instability. In this study however, promoter methylation of *p16^{INK4A}* shows no association with chromosomal instability. Also no association between promoter methylation of the mitotic checkpoint control gene *CHFR* and an increased number of chromosomal abnormalities was observed. These results are consistent with a study of Bertholon *et al.*³⁸ which showed that methylation of *CHFR* is not associated with chromosomal instability in cell lines. Apparently, in CRC, promoter methylation of other control genes needs to be evaluated to determine if epigenetic changes are indeed associated with the initiation of chromosomal instability.

Furthermore, we studied the relationship between epigenetic and genetic alterations in tumor development and inverse relations between promoter methylation and gene mutation within important regulatory pathways were observed. We confirmed previous reports that *p14^{ARF}* methylation shows an inverse correlation to *TP53* mutation in CRC^{9,31,39}, which has also been observed in bladder cancer⁴⁰ and non-small cell lung cancer⁴¹. In head and neck squamous cell carcinomas (HNSCC) an inverse correlation between *TP53* mutation status and cyclin A1 methylation, another downstream target of *TP53* has been described⁴². Similar relations have been shown for *APC* promoter methylation and mutation¹² and *KRAS* mutation and *RASSF1A* methylation⁴³ indicating that gene mutation and promoter methylation do not

frequently occur simultaneously in the same pathway, but rather may act in a mutual exclusive or complimentary fashion.

A different approach to study the relationship between epigenetic and genetic silencing of a gene was to examine the relationship between promoter methylation of a gene, *GATA4*, and deletion of the chromosomal location of the gene, loss of 8p21-pter. Both events occur frequently in tumor development, but no association was observed. Hypermethylation of *GATA4* was not restricted to tissues with or without chromosomal loss of 8p21-pter. *GATA4* methylation occurs prior to loss of 8p21-pter and the number of cases in which both events were present increased during tumor progression.

In addition, no association between promoter methylation of a gene and the number of chromosomal alterations was observed. An observation which is in agreement with a recent study on hepatocellular carcinogenesis in which no correlation between the degree of chromosomal structural alterations and that of aberrant promoter methylation was present⁴⁴. Furthermore, in non-progressed adenomas without chromosomal abnormalities, high frequencies of promoter methylation were already present. Together, these observations suggest that promoter methylation of the selected DNA repair- and tumor suppressor genes precedes chromosomal abnormalities in colorectal cancer development.

In summary, the data indicate that promoter methylation of the selected genes can be considered as an early event which occurs prior to *TP53* mutations and chromosomal instability. The association between gene methylation and pre-malignant lesions is highly relevant for methylation-marker based colorectal cancer screening. The observation that the presence of promoter methylation in normal tissues corresponds to the methylation profile of paired carcinomas suggests that methylation levels in normal colonic mucosa could serve as marker of risk of development of CRC. Given that aberrant DNA methylation can also be detected in stool DNA^{45,46}, studying methylation as common event in pre-malignant lesions is promising to provide novel specific biomarkers for risk assessment and secondary prevention³⁶.

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CHAPTER 5|

Integrated analysis of chromosomal, microsatellite and epigenetic instability in colorectal cancer identifies specific associations between promoter methylation of pivotal tumor suppressor and DNA repair genes and specific chromosomal alterations

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Carcinogenesis 29(2), 343-9 (2008)

Abstract

Colorectal cancer (CRC) is a complex and heterogeneous disease in which genomic instability and DNA promoter methylation play important roles. The aim of this study was to investigate the relationship between chromosomal instability (CIN), microsatellite instability (MSI) and promoter methylation of colorectal cancer associated genes. Therefore 71 CRCs were analyzed for CIN and MSI by comparative genomic hybridization and the mononucleotide marker BAT-26 respectively. Promoter methylation of the tumor suppressor- and DNA repair genes *hMLH1*, *O⁶MGMT*, *APC*, *p14^{ARF}*, *p16^{INK4A}*, *RASSF1A*, *GATA4*, *GATA5* and *CHFR* was analyzed using methylation-specific PCR (MSP). These integrative analyses showed that in CIN+ CRCs, promoter methylation of *GATA4* and *p16^{INK4A}* was inversely related to chromosomal loss at 15q11-21 and gain at 20q13 respectively (*P* values 3.8×10^{-2} and 4.5×10^{-2} respectively). Interestingly, promoter methylation of *RASSF1A*, *GATA4*, *GATA5* and *CHFR* as well as a high methylation index was positively related to chromosomal gain at 8q23-qter (*P* values 1.5×10^{-2} , 3.8×10^{-2} , 3.9×10^{-2} , 4.9×10^{-2} and 8.2×10^{-3} respectively). MSI was associated with *BRAF* mutation, promoter methylation of *hMLH1*, *APC* and *p16^{INK4A}* and a high methylation index (total number of methylated genes) (*P* values 2.4×10^{-2} , 2.5×10^{-3} , 1.8×10^{-2} , 4.6×10^{-2} and 1.0×10^{-2} respectively). Therefore we conclude that promoter methylation of pivotal tumor suppressor and DNA repair genes is associated with specific patterns of chromosomal changes in colorectal cancer, which is different from methylation patterns in MSI tumors.

Introduction

Colorectal cancer (CRC) is a complex and heterogeneous disease in which numerous genetic and epigenetic alterations have been identified. Multiple molecular pathways are likely to underlie colorectal carcinogenesis in which genetic instability is thought to be a driving force¹⁻³.

Approximately 85% of all CRCs are characterized by CIN which describes a condition of aneuploidy and an increased rate of loss of heterozygosity (LOH)⁴. Despite extensive research, the mechanism driving the process of numerical and structural chromosomal instability in CRC is not known.

Previously we have shown that CIN does not constitute random genetic noise, but occurs in patterns of associated chromosomal changes^{5,6}. Seven specific chromosomal changes, losses in 8p21-pter, 15q11-q21, 17p12-13 and 18q12-21 and gains in 8q23-qter, 13q14-31, and 20q13 are strongly associated with the progression of colorectal adenomas towards carcinomas and can be considered as cancer associated events (CAE)⁵.

In addition, approximately 10-15% of sporadic CRCs, and all tumors from patients with hereditary nonpolyposis colorectal cancer (HNPCC), are characterized by MSI⁷. This form of genetic instability is associated with defective mismatch repair (MMR) which is commonly achieved by promoter methylation of the DNA mismatch repair gene *hMLH1* in sporadic CRCs⁸⁻¹¹, while in patients with HNPCC germline mutations of MMR genes take place⁷. Defective mismatch repair leads to accumulating frameshift mutations at simple repeated nucleotide sequences. Some of these mutations affect genes that are implicated in colorectal carcinogenesis such as *TGFβRII*, *IGFIIR* and *BAX*, thereby leading to a mutator phenotype¹²⁻¹⁵.

Emerging evidence indicates that also "epigenetic instability" is involved in colorectal cancer development¹⁶⁻¹⁸. Besides altered chromatin structure and histone modifications, epigenetic instability is characterized by hypermethylation of multiple CpG islands, a phenotype also referred to as the CpG island methylator phenotype (CIMP). Although the concept of CIMP is under debate¹⁹ and different definitions of CIMP have been used²⁰⁻²³, emerging evidence supports the existence of a group of colorectal cancers with a high prevalence of methylated CpG islands^{17,20,24}. Although the relative importance of this pathway in tumor progression is not well understood, CIMP positive tumors are thought to represent a distinct group of carcinomas which show a strong association with MSI and *BRAF* mutations^{21,25} and an inverse relationship to CIN^{26,27}.

We previously showed that DNA promoter methylation of tumor suppressor and DNA repair genes *hMLH1*, *O⁶MGMT*, *APC*, *p14^{ARF}*, *p16^{INK4A}*, *RASSF1A*, *GATA4*, *GATA5* and *CHFR* is a frequent and early event in CRC²⁸. However, it is not directly clear how these methylation events are related to the different genetic phenotypes in CRC. Therefore, in the present study we investigated the relationship between promoter methylation of established tumor suppressor and DNA repair genes and chromosomal and microsatellite instability in a group of 71 CRCs. Data were complemented with analysis of *BRAF* mutation status.

Materials and Methods

Patient material

This study included 71 CRCs of which a subset has been analyzed for chromosomal abnormalities by comparative genomic hybridization (CGH) previously⁵. The CRC tissues were obtained from 67 patients, 34 males and 33 females (mean age of 67 years: range 44-89). Archival material was used in compliance with the institutions ethical regulations. Four patients had synchronous carcinomas. Since this can influence results, all analyses were performed omitting these CRCs as well. Since no difference in significant results was observed, only results of the total series of 71 CRC are reported. The clinical pathologic characteristics are listed in Table 1.

Table 1: Clinicopathologic characteristics of CIN and MSI carcinomas

	total	CIN- (%)	CIN+ (%)	MSI- (%)	MSI+ (%)
Number of cases	71	22.5	77.5	83.6	16.4
Age ^a		71.9 (± 18.9)	67.3 (± 9.8)	67.4 (± 9.8)	72.8 (± 15.7)
Size ^a		41.8 (± 16.9)	35.1 (± 13.0)	36.2 (± 18.8)	41.6 (± 17.4)
Sex					
Male	37	50	51.9	50	45.5
Female	34	50	48.1	50	54.5
Differentiation grade (%)					
Well	18.3	25.0	16.4	19.6	18.2
Moderate	73.2	68.8	74.5	75	54.5
Poor	8.5	6.3	9.1	5.4	27.3
TNM stage (%) ^b					
1	32.4	6.3	40	37.5	0
2	42.3	68.8	34.5	33.9	90.9
3	23.9	25.0	23.6	26.8	9.1
4	1.4	0.0	1.8	1.8	0
Tumor location ^c					
Proximal colon	24	68.3	24.1	23.2	100
Distal colon	46	31.3	75.9	76.8	0

CIN-, chromosomal stable; CIN+, chromosomal unstable; MSI-, microsatellite stable; MSI+, microsatellite unstable.

^a values indicate mean; ^b Tumor Node Metastasis; ^c tumor location of 1 carcinoma is not designated

** P value < 0.01; *** P value < 0.001

CGH analysis

Fifty-one CRC tissue samples have been analyzed by conventional CGH previously⁵. Chromosomal gains or losses were interpreted when the fluorescence ratio was significantly higher or lower than 1.0, as evaluated by the 95% confidence interval.

Twenty additionally collected CRCs were analyzed by array CGH analysis using 5 K BAC arrays²⁸⁻³⁰. Briefly, we used a full-genome in house printed array containing approximately 5000 clones with an average resolution of 1 Mb. Protocols used for labelling, hybridization and feature extraction are available on <http://internet-extra.vumc.nl/microarrays/>. After applying a smoothing algorithm to the array CGH data³¹, DNA copy number ratios obtained by ar-

ray CGH were recoded as gains and losses at the resolution of whole chromosome arms compatible with the data obtained by chromosome CGH. Since the presence of two or more specific chromosomal alterations (losses at 8p, 15q, 17p and 18q and gains in 8q, 13q, and 20q) is strongly associated with CRC progression⁵, we defined chromosomal instability (CIN) by the presence of two or more of these cancer associated events (CAE).

Microsatellite instability analysis

The MSI status of each tumor was evaluated by amplification of BAT-26, a single Poly(A) tract previously shown to be highly sensitive and specific for MSI^{32,33}. A subset of CRCs (n=46) was also analyzed for MSI by amplifying four additional mononucleotide repeats in a pentaplex reaction as described previously³⁴. This pentaplex includes the mononucleotide repeats BAT-25, BAT-26, NR-21, NR-24 and NR-27. Primer sequences were as described previously³⁴. Each sense primer was end-labelled with one of the fluorescent markers FAM, HEX or NED. Pentaplex PCR was performed with an initial 5 min denaturation step at 94°C, followed by 35 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, with a final extension at 72°C for 7 min. Amplified PCR products were run on an Applied Biosystems PRISM 3100 automated capillary electrophoresis DNA sequencer. Allelic sizes were estimated using Genescan 2.1 software (Applied Biosystems, Foster City, LA, USA). The accordance between the BAT-26 analysis and pentaplex data was 100%. For the complete study population, MSI was therefore defined as instability of the BAT-26 locus.

Promoter methylation analysis

DNA methylation in the CpG islands of *hMLH1*, *O⁶MGMT*, *APC*, *p16^{INK4A}*, *p14^{ARF}*, *RASSF1A*, *GATA4*, *GATA5* and *CHFR* gene promoters was determined by chemical modification of genomic DNA with sodium bisulfite and subsequent methylation-specific PCR (MSP) as described in detail elsewhere^{28,35,36}. Primer sequences are as described previously and available upon request³⁷⁻³⁹. Unsupervised hierarchic cluster analysis (see also data analysis section) was performed to cluster CRCs based on the methylation status of the above mentioned genes and to study patterns of promoter methylation.

Detection of BRAF codon 599 mutations

The common *BRAF* V600E mutation in exon 15 was analyzed by a semi-nested PCR and subsequent RFLP analysis as described previously⁴⁰⁻⁴². The semi nested amplification step (primer sequences and PCR conditions are available upon request) was added in order to optimize the amplification of formalin-fixed, paraffin-embedded DNA.

Data analyses

Differences in frequencies, e.g of gene methylation between different groups of carcinomas, were evaluated by the Pearson's χ^2 or Fisher's exact test where appropriate. The Mann-Whitney U nonparametric and Kruskal-Wallis test were used for comparing means of the continuous variables between groups of CRCs. We calculated means of the total number of methylated

genes per case, referred to as methylation index (MI), which is defined as the number of genes methylated divided by the number of genes analyzed.

All reported P values are two-sided, and a P value < 0.05 was considered statistically significant, using SPSS software version 12.0. All statistical tests were corrected for multiple comparisons using the Bonferroni method.

In order to group CRCs based on the patterns of promoter methylation of *hMLH1*, *O⁶MGMT*, *APC*, *p16^{INK4A}*, *p14^{ARF}*, *RASSF1A*, *GATA4*, *GATA5* and *CHFR*, we performed unsupervised hierarchic cluster analysis using Spotfire software version 8.2 with the setting "Ward's method" for clustering, "Half square Euclidean distance" as similarity measure and row interpolation to handle missing values.

Results

CIN and MSI

We first categorized all CRCs based on their type of genomic instability, i.e. CIN and MSI.

We previously showed that the acquisition of 2 or more cancer associated events (CAE), defined as losses in 8p21-pter, 15q11-q21, 17p12-13 and 18q12-21 and gains in 8q23-qter, 13q14-31, and 20q13, is strongly associated with CRC progression⁵. Therefore we defined chromosomal instability (CIN+) as the presence of 2 or more CAE, which was present in 55 of 71 CRCs (77.5%). In this CIN+ group losses in 8p, 15q, 17p and 18q and gains in 8q, 13q, and 20q, were present in 54.5%, 49.1%, 67.5%, 61.8%, 54.5%, 49.1% and 43.6% of CRCs respectively (Table 2).

MSI status, defined by BAT-26 instability, was assessable in 67 of 71 CRCs (94%). Eleven of 67 CRCs (16.4%) were unstable at the BAT-26 locus and classified as microsatellite instable (MSI+), while 56 of 67 CRCs (83.6%) displayed microsatellite stability (MSI-).

Although MSI+ CRCs displayed some chromosomal alterations (mean 5.7) a higher number of chromosomal alterations was observed in MSI- CRCs (mean 12.2, P value 2.7×10^{-3}). When we examined associations between specific chromosomal alterations and microsatellite status, MSI- was associated with chromosomal losses in 1p, 15q, 17p and 18q and gain in 13q (P values respectively 1.8×10^{-2} , 4.3×10^{-2} , 1.3×10^{-2} , 1.7×10^{-3} , 3.6×10^{-3}). Four of these five chromosomal alterations belong to the group of CAE which were significantly more frequently present in MSI- CRCs (3.5 CAE) when compared to MSI+ CRCs (0.9 CAE, P value 5.0×10^{-5}). Although not statistically significant, also CAEs loss of 8p and gain of 8q and 20q also occur more frequently in MSI- CRCs when compared to MSI+ CRCs.

In order to investigate whether an overlap exists between CIN and MSI CRCs we correlated CIN status to MSI status. We classified the 67 CRCs, for which both CIN and MSI status could be determined, into 4 groups: CIN+/MSI- group ($n=50$), CIN+/MSI+ group ($n=2$), CIN-/MSI- group ($n=6$) and CIN-/MSI+ group ($n=9$). In this way, 88% of CRCs could be characterized by either CIN or MSI and only in 3% ($n=2$) of the CRCs an overlap between the CIN and MSI pathway was present.

Table 2: Epigenetic and genetic events in CIN and MSI carcinomas

	total	CIN- (%)	CIN+ (%)		MSI- (%)	MSI+ (%)	
Number of cases	71	22.5	77.5		83.6	16.4	
Promotor methylation							
<i>hMLH1</i>	46.5	68.8	40.0	*	41.1	90.9	**
<i>O⁶MGMT</i>	57.7	68.8	54.4		54.5	57.1	
<i>RASSF1A</i>	26.8	25.0	27.8		25.5	45.5	
<i>APC</i>	39.4	56.3	34.5		32.1	72.7	*
<i>p14^{ARF}</i>	40.8	53.3	42.0		40.4	60.0	
<i>p16^{INK4A}</i>	33.8	56.3	27.3	*	30.4	63.6	*
<i>GATA4</i>	80.3	93.8	77.8		76.4	100.0	
<i>GATA5</i>	79.9	93.8	80.4		81.1	90.0	
<i>CHFR</i>	40.8	78.6	45.0	*	46.5	77.8	
Methylation index ^a	0.52	0.66	0.47	**	0.47	0.72	**
Genetic alterations							
8p-	43.7	6.3	54.5	***	48.2	18.2	
15q-	38	0	49.1	***	42.9	9.1	*
17p-	54.9	12.5	67.3	***	58.9	18.2	*
18q-	52.1	18.3	61.8	**	60.7	9.1	**
8q+	43.7	6.3	54.5	***	46.4	18.2	
13q+	50.7	6.3	63.6	***	57.1	9.1	**
20q+	35.2	6.3	43.6	**	39.3	9.1	
Number of chromosomal events ^a	11.4	4.3	13.4	***	12.2	5.7	**
Number of CAE ^a	2.9	0.6	3.9	***	3.5	0.9	***

CIN-, chromosomal stable; CIN+, chromosomal instable; MSI-, microsatellite stable; MSI+, microsatellite instable; methylation index, total number of methylated genes divided by the number of genes analyzed; CAE, cancer associated events (losses at 8p, 15q, 17p, 18q and gain at 8q,13q and 20q). ^a values indicate mean

* P value < 0.05; ** P value < 0.01; *** P value < 0.001

Promoter methylation analysis

We next included gene promoter methylation into the analysis. As shown previously²⁸, 71 CRCs were analyzed for promoter methylation of *hMLH1*, *O⁶MGMT*, *RASSF1A*, *APC*, *p16^{INK4A}*, *p14^{ARF}*, *GATA4*, *GATA5* and *CHFR*. We selected these genes since they are reported to be functionally and frequently methylated in CRC carcinogenesis. Promoter methylation was present in 46.5%, 57.7%, 26.8%, 39.4%, 40.8%, 33.6%, 80.3%, 79.9% and 40.8% of CRCs respectively (Table 2).

Furthermore, relations between promoter methylation events were frequently observed for *p16^{INK4A}*, *GATA4*, *GATA5* and *CHFR*. Promoter methylation of *p14^{ARF}* was associated with methylation of all genes analyzed, except from *hMLH1* and *APC*. Promoter methylation *hMLH1* was solely associated with *CHFR* promoter methylation (P value 2.7×10^{-2}). The same accounts for *APC* promoter methylation, which only showed an inverse relation to *O⁶MGMT* promoter methylation (P value 2.4×10^{-3}) (data not shown).

Except from the association between *hMLH1* and *CHFR* methylation, all relationships were independent of MSI or CIN status and were also observed when we analyzed interrelations in CIN+ CRCs separately. Similar patterns of promoter methylation were observed when we performed an unsupervised hierarchical cluster analysis, based on promoter methylation of above mentioned genes. Cluster analysis identified 3 groups of CRCs (Figure 1).

Figure 1: Unsupervised hierarchical clustering of CRCs based on promoter methylation of 9 DNA repair- and tumor suppressor genes, selected 3 groups with different methylation profiles. A black box represents a methylated gene promoter, a white box represents an unmethylated promoter and a grey box illustrates a failed PCR amplification.

We calculated the mean methylation index (MI), the number of methylated genes divided by the number of genes analyzed per case, of each cluster. Cluster I ($n=15$, 21.1%) showed the highest frequency of promoter methylation (MI: 0.82), cluster II and III could be characterized by a significantly lower MI index of 0.53 ($n=31$, 43.7%) and 0.31 ($n=25$, 35.2%) respectively (P value 5.7×10^{-10}). Although the majority of the genes show the highest frequency of promoter methylation in cluster I, *O⁶MGMT* is most frequently methylated in cluster II (P value 7.1×10^{-6}) and *APC* is frequently methylated in the cluster I as well as in cluster III, whereas only 3.2% of CRCs in cluster II show *APC* promoter methylation (P value 3.7×10^{-7}) (Table 3).

CIN and MSI in relation to promoter methylation

We next studied how promoter methylation was related to the CIN and MSI. We determined the mean methylation index (MI) of the 4 subgroups of CRCs defined above, and observed that the CIN-/MSI+ and CIN+/MSI+ groups of CRCs showed the highest MI (both 0.72), while CIN-/MSI- and CIN+/MSI- CRCs showed a MI of 0.58 and 0.46 respectively (P value 4.0×10^{-2}) (Figure 2).

In addition to the relationship between MSI and a high mean MI, MSI+ was associated with promoter methylation of *hMLH1* (P value 2.5×10^{-3}), *APC* (P value 1.8×10^{-2}) and *p16^{INK4A}* (P value 4.6×10^{-2}) (Table 2). Although not statistically significant, promoter methylation of *RASSF1A*, *p14^{ARF}*, *GATA5* and *CHFR* occurred more frequently in MSI+ CRCs. *GATA4* was methylated in 100% of the MSI+ cases versus 76.4% of the MSI- cases. *O⁶MGMT* methylation on the other hand did not differ between MSI+ and MSI- CRCs.

Table 3: Epigenetic and genetic events in clusters of methylated genes

	total	cluster I (%)	cluster II (%)	cluster III (%)	
Number of cases	71	21.1	43.7	35.2	
Promotor Methylation (%)					
<i>hMLH1</i>	46.5	100	35.5	28.0	***
<i>O⁶MGMT</i>	57.7	46.7	90.3	24.0	***
<i>RASSF1A</i>	26.8	73.3	13.3	16	***
<i>APC</i>	39.4	80	3.2	60	***
<i>p14^{ARF}</i>	40.8	92.9	50	8.7	***
<i>p16^{INK4A}</i>	33.8	66.7	41.9	4	***
<i>GATA4</i>	80.3	100	83.9	66.7	
<i>GATA5</i>	79.9	100	96.7	56.6	**
<i>CHFR</i>	40.8	85.7	61.9	21.1	***
Methylation index ^a	0.52	0.82	0.53	0.31	***
Genetic alterations (%)					
8p-	43.7	33.3	48.4	44.0	
15q-	38	20.0	38.7	48.0	
17p-	54.9	33.3	61.3	60.0	
18q-	52.1	33.3	51.6	64.0	
8q+	43.7	46.7	51.6	32.0	
13q+	50.7	33.3	51.6	60.0	
20q+	35.2	26.7	32.3	44.0	
CIN		53.3	80.6	88	
MSI		53.3	6.9	4.3	***
Number of chromosomal events ^a	11.4	9.8	11.3	12.4	
Number of CAE ^a	2.9	2.3	3.4	3.5	

CIN-, chromosomal stable; CIN+, chromosomal instable; MSI-, microsatellite stable; MSI+, microsatellite instable; methylation index, total number of methylated genes divided by the number of genes analysed; CAE, cancer associated events (losses at 8p, 15q, 17p, 18q and gain at 8q,13q and 20q). ^a values indicate mean

** P value < 0.01; *** P value < 0.001

CIN+ CRCs displayed a significantly lower MI when compared to CIN- CRCs (MI: 0.47 and 0.66 respectively, P value 6.3×10^{-3}) and an inverse relation to promoter methylation of *hMLH1*, *p16^{INK4A}* and *CHFR* (P values 4.2×10^{-2} , 3.1×10^{-2} , 3.0×10^{-2} respectively). Furthermore, *p16^{INK4A}* methylation showed an inverse relationship to CAEs chromosomal loss of 18q and gain of 20q and the total number of CAE (P value 5.7×10^{-3} , 1.9×10^{-2} and 3.9×10^{-2} respectively). In addition, *GATA4* methylation showed an inverse relation to CAE chromosomal loss of 15q and, although not statistically significant, to the total number of chromosomal abnormalities (P value 1.2×10^{-2} and 5.9×10^{-2} respectively) (data not shown).

Figure 2: Promoter methylation in distinct CRC groups. Promoter methylation has been analyzed for 9 DNA repair- and tumor suppressor genes. Mean methylation index (total number of methylated genes divided by the number of genes analyzed) per group; CIN-, chromosomal stable; CIN+, chromosomal instable; MSI-, microsatellite stable; MSI+, microsatellite instable.
* P value 0.04

Although MSI+ CRCs show a higher MI when compared to CIN+ CRCs, promoter methylation events are present in CIN+ CRCs. Therefore we studied the relationship between gene promoter methylation events and chromosomal alterations in CIN+/MSI- CRCs ($n=50$). Also in this group the inverse relationship between $p16^{INK4A}$ and gain of 20q (P value 4.5×10^{-2}) and $GATA4$ methylation and loss of 15q (P value 3.8×10^{-2}) was observed. Furthermore, a positive relationship between promoter methylation of $RASSF1A$, $GATA4$, $GATA5$ and $CHFR$ and CAE gain of 8q was present (P values 1.5×10^{-2} , 3.8×10^{-2} , 3.9×10^{-2} and 4.9×10^{-2} respectively). Although not statistically significant, the same accounts for promoter methylation of $hMLH1$, O^6 MGMT, APC , $p16^{INK4A}$ and $p14^{ARF}$. Additionally, CRCs with gain of 8q show a higher MI (0.55) when compared to CRC without 8q gain (MI: 0.38, P value 8.2×10^{-3}). No other associations between CAEs and MI were observed.

BRAF mutation shows an inverse relation to CIN

BRAF mutation status was determined in 57 out of 71 CRCs, of which sufficient DNA was available. Seven CRCs (12.3%) exhibited the V599E *BRAF* mutation.

We investigated the association between *BRAF* mutation and CIN and observed an inverse relation approaching statistical significance (P value 5.4×10^{-2}) (Table 4). In addition, *BRAF* mutated CRCs display significantly less CAE when compared to wild-type *BRAF* CRCs (1.6 CAE and 3.3 CAE respectively; P value 2.9×10^{-2}). Furthermore, *BRAF* mutation was associated with MSI+, since 36.4% of the MSI cases showed a *BRAF* mutation and only 6.8% of the MSI- cases (P value 2.4×10^{-2}). All *BRAF* mutated CRCs showed *hMLH1* promoter methylation as well as an association with promoter methylation of $p14^{ARF}$ and $CHFR$ (P values 1.1×10^{-2} , 3.6×10^{-2} and 2.7×10^{-2} respectively).

Table 4: CIN, MSI and promoter methylation in relation to *BRAF* mutation

	<i>BRAF</i> wt (%)	<i>BRAF</i> mt (%)	
Number of cases	50	7	
Promotor Methylation			
<i>hMLH1</i>	46.0	100.0	*
<i>O⁶MGMT</i>	56.0	57.1	
<i>RASSF1A</i>	30.6	42.9	
<i>APC</i>	34.0	57.1	
<i>p14^{ARF}</i>	38.3	85.7	*
<i>p16^{INK4A}</i>	32.0	57.1	
<i>GATA4</i>	88.0	85.7	
<i>GATA5</i>	85.4	100.0	
<i>CHFR</i>	48.7	100.0	*
Methylation index ^a	0.51	0.75	**
CIN	80.0	42.9	*
MSI	14.6	57.1	*
Number of chromosomal events*	11.4	7.6	
Number of CAE ^a	3.3	1.6	*

CIN-, chromosomal stable; CIN+, chromosomal unstable; MSI-, microsatellite stable; MSI+, microsatellite unstable; methylation index, total number of methylated genes divided by the number of genes analysed; CAE, cancer associated events (losses at 8p, 15q, 17p, 18q and gain at 8q, 13q and 20q).

Wt, wild-type; Mt, mutant

^a values indicate mean, *P value < 0.05; ** *P* value < 0.01

Discussion

The aim of the present study was to investigate the association between chromosomal instability (CIN), microsatellite instability (MSI) and DNA promoter methylation of multiple DNA repair- and tumor suppressor genes reported to be involved and frequently methylated in CRC carcinogenesis.

In order to perform such a study, clear definitions for the genetic phenotypes are needed. MSI was defined by instability of the BAT-26 locus, as was previously shown to be highly sensitive and specific for MSI-H^{32,33}. Defining CIN was less obvious since no generally accepted definition of CIN exists, despite the common use of the term CIN in the literature. Biologically, CIN is a dynamic process and is usually defined as an accelerated rate of chromosome missegregation during cell division³. Since no common definition of CIN is present, we defined CIN, in this study, as the presence of 2 or more cancer associated events (CAE). CAE are defined as chromosomal alterations, losses in 8p21-pter, 15q11-q21, 17p12-13 and 18q12-21 and gains in 8q23-qter, 13q14-31, and 20q13, which show a strong association with progression of adenomas towards carcinomas⁵. Using this definition, CIN was observed in 77.5% of CRCs, a percentage which is in line with several other reports using other definitions^{1,3,43}.

When we studied the association between CIN and MSI, MSI showed an inverse relation to chromosomal losses in 1p, 15q, 17 and 18q and gain in 13q, of which 4 CAEs. Chromosomal loss in 1p has previously been shown to be strongly associated with aneuploidy and also the inverse relationships between MSI and chromosomal losses in 15q, 17p and 18q and gain in 13q are in agreement with earlier reports⁴⁴⁻⁴⁷. Although chromosomal aberrations are

not completely absent in MSI+ CRCs^{45,46}, with the definition of CIN used in the present study, the overlap with MSI was only 3%.

In this study we investigate promoter methylation of genes relevant to pathogenesis of CRC, i.e. promoter methylation of *hMLH1*, *O⁶MGMT*, *APC*, *p14^{ARF}*, *p16^{INK4A}*, *RASSF1A*, *GATA4*, *GATA5* and *CHFR* and observed some interesting findings. Although most of the genes showed concordant methylation, *APC* promoter methylation showed an inverse relation to *O⁶MGMT* methylation and *hMLH1* was solely associated with *CHFR* promoter methylation. Thereby, we confirmed the association between *hMLH1* and *CHFR* methylation which has been previously shown in both CRC and gastric cancer^{39,48,49}.

We performed a hierarchical cluster analysis to study patterns of promoter methylated genes and observed that not all genes show the highest frequency of promoter methylation in the group with the highest number of methylated genes. *O⁶MGMT* was most frequently methylated in a group of CRCs displaying moderate frequencies of methylated genes while *APC* promoter methylation was most frequently observed in the groups of CRCs with the highest and lowest frequencies of methylated genes. These results are in agreement with recent reports which show that *O⁶MGMT* promoter methylation is associated with CIMP-low CRCs⁵⁰ and that *APC* promoter methylation is inversely related to features of CIMP⁵¹. Together these results indicate that promoter methylation of specific genes might occur in different pathways.

When we studied the association between promoter methylation and genetic alterations, MSI was associated with *hMLH1*, *p16^{INK4A}* and *APC* promoter methylation. The association between promoter methylation of *hMLH1* and MSI, as well as the associations with *BRAF* mutation, is well described⁸⁻¹¹ and also the association of *p16^{INK4A}* methylation and MSI has been reported previously²⁰. However, the relationship between *APC* methylation and MSI is not directly clear. *APC* is a key regulator of the WNT signaling pathway and downregulation of *APC* is one of the earliest events observed in colorectal carcinogenesis which leads to activation of WNT signaling. Inactivation of *APC* is accomplished by promoter methylation, LOH or mutation. Although genetic and epigenetic events can collaborate for inactivation of this gene⁵², we and others have previously observed that *APC* mutation and promoter methylation occur in distinct CRCs^{28,53}. This is in line with the observation of Samowitz *et al.* which showed an association between *APC* mutations and microsatellite stable (MSS) tumors⁵⁴ whereas this study shows a relationship between *APC* promoter methylation and MSI CRCs.

Furthermore, a recent study has shown an inverse relationship between CIMP, defined by promoter methylation of MINTs, *p14^{ARF}*, *p16^{INK4A}* and *hMLH1*, and CIN analyzed by loss of heterozygosity (LOH) analysis⁵⁵. We also observed lower frequencies of methylated genes in CIN CRCs when compared to MSI CRCs and an inverse relationship between promoter methylation of *p16^{INK4A}* and CAEs chromosomal loss at 18q12-21 and gain at 20q13 as well as to the total number of CAE was observed. Moreover, *GATA4* promoter methylation showed an inverse relation to CAE chromosomal loss at 15q11-q21 which indicates that promoter methylation and CIN occur in distinct tumors.

However, we also observed a statistical significant positive relationship between CAE chromosomal gain of 8q23-qter and promoter methylation of *RASSF1A*, *GATA4*, *GATA5* and *CHFR*. Although not statistically significant, the same accounts for promoter methylation of *hMLH1*, *O⁶MGMT*, *APC*, *p16^{INK4A}*

and *p14^{ARF}*. Amplification of this chromosomal region involves the proto-oncogene *c-MYC* which encodes a transcription factor playing a critical role in regulating cell growth, proliferations, apoptosis and differentiation through its ability to activate or repress transcription⁵⁶. The finding that c-Myc represses transcription through recruitment of a DNA methyltransferase corepressor, which leads to methylation and silencing of target genes⁵⁷, might provide a mechanistical link between amplification of 8q23-qter and an increased number of methylated genes in CRC.

In summary, our results indicate that CIN and MSI in CRCs can be considered as two alternative mechanisms. Although promoter methylation demonstrates a strong relationship to MSI, promoter methylation of pivotal tumor suppressor- and DNA repair genes are observed in CIN CRCs and are associated with a specific pattern of chromosomal changes.

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CHAPTER 6|

CHFR promoter CpG island methylation is an indicator of poor prognosis in stage II microsatellite stable colorectal cancer

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Submitted for publication

Abstract

Unraveling the prognostic significance of promoter CpG island methylation in colorectal cancer (CRC) is complicated by factors affecting the course of the disease, such as genetic alterations and adjuvant therapy. Here we evaluate the prognostic value of promoter CpG island methylation in CRC patients treated with surgery alone.

CRCs (n=173) were analyzed for promoter CpG island methylation of 19 tumor suppressor- and DNA repair genes, i.e. *hMLH1*, *p16^{INK4A}*, *p14^{ARF}*, *O⁶MGMT*, *RASSF1A*, *APC*, *HLTF*, *GATA4*, *GATA5*, *CHFR*, *ADAM23*, *RAB32*, *JPH3*, *FOXL2*, *BNIP3*, *NEURL*, *CACNA2*, *THBS1* and *TFPI2*, and CIMP¹ genes *CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3* and *SOCS1* using methylation-specific PCR (MSP). Microsatellite instability (MSI) was determined by analyzing allelic size variants of five mononucleotide MSI markers. The exon 15 V600E *BRAF* mutation was analyzed by RFLP analysis, and *KRAS* mutation in exon 12 and 13 was analyzed by direct sequencing.

Using unsupervised hierarchical clustering based on methylation patterns of 19 tumor suppressor- and DNA repair genes, three CRC subgroups, cluster 1 (CL1, 57% (98/173) of CRCs), cluster 2 (CL2, 25% (43/173) of CRCs) and cluster 3 (CL3, 18% (32/173) of CRCs) were identified, of which CL3 had the highest mean number of methylated genes (methylation index 0.25, 0.49 and 0.69 respectively, $p < 0.001$). After stratification for tumor stage, MSI and *BRAF* status, no statistically significant differences in survival between CL1, CL2 and CL3 were detected. The same accounted for CIMP using the definition according to Weisenberger *et al*¹. However, promoter CpG island methylation of *CHFR* was associated with a poor prognosis in stage II, MSS, *BRAF* wild-type CRCs (HR=2.80 (CI =1.24-6.33), $p = 0.01$). This finding could be confirmed in a second population-based series of CRCs (HR=2.024 (CI =1.06-3.87), $p = 0.03$).

Thus, *CHFR* promoter CpG island methylation is associated with a poorer prognosis in stage II, MSS, *BRAF* wild-type CRCs.

Introduction

Accurate staging of colorectal cancer (CRC) is essential for optimal management of the disease. Although patients within the same TNM stage can demonstrate considerable variation in outcome, the TNM staging system is still the gold standard predicting prognosis and guiding clinical management of CRC. Adjuvant chemotherapy is recommended for all stage III CRC patients. In Europe, the majority of stage II CRC patients undergo surgery alone, despite the recognition that a subgroup with a poor prognosis would probably benefit from adjuvant chemotherapy. Molecular classification of CRC, by detecting (epi)genetic alterations associated with a poor prognosis, might aid in selecting CRC patients who could benefit from adjuvant therapy.

CRC is characterized by activation of oncogenes and inactivation of tumor suppressor genes controlling cell growth, apoptosis, replicative potential, angiogenesis, invasion and metastasis². Frequently observed alterations affecting these pathways include chromosomal instability (CIN), microsatellite instability (MSI), coding sequence mutations in *APC*, *TP53*, *KRAS* and *PI3KCA*³⁻⁵ and promoter CpG island hypermethylation. Frequent promoter CpG island methylation, annotated as CpG island methylator phenotype (CIMP)^{6,7}, has been reported to occur more commonly in female and older CRC patients as patients with proximally located CRCs. Furthermore CIMP is associated with mucinous histology, poor differentiation, MSI and *BRAF* mutations^{1,6,8-11}. CIMP has been analyzed with respect to prognosis of CRC, however, data are inconsistent, likely because of confounding factors affecting the natural course of the disease, such as genetic aberrations (MSI and *BRAF* and *KRAS* mutations) and chemotherapy protocols¹²⁻¹⁴. CRC patients with MSI tumors have a better prognosis than patients with microsatellite stable (MSS) carcinomas¹⁵. Furthermore, there are reports suggesting that MSI CRCs do not benefit from 5-FU-based adjuvant therapy¹⁶. *KRAS* and *BRAF* mutations are associated with clinical resistance in CRC patients treated with the epidermal growth factor receptor (EGFR)-targeted monoclonal antibodies¹⁷⁻¹⁹.

The aim of the present study was to evaluate the prognostic significance of promoter CpG island methylation in CRC, independent of MSI, *BRAF*- and *KRAS* mutation status and adjuvant chemotherapy. Therefore, we analyzed promoter CpG island methylation of CIMP genes, as recently defined by Weisenberger *et al.*¹, as well as 19 additional tumor suppressor- and DNA repair genes, functioning in a diversity of signaling pathways and methylated in CRC, and investigated their prognostic value.

Material and methods

Patient population

Samples were collected from colorectal cancer patients that had been entered in two multi-center prospective clinical trials between 1979 and 1981 in the Netherlands. One trial was designed to compare patient survival after treatment of colon cancer by conventional surgery or the no-touch isolation technique²⁰. The second trial was conducted to compare survival in rectal cancer patients with or without preoperative radiotherapy. In the current study, we

included only the patients who did not receive preoperative radiotherapy. At the time the trial was conducted, only surgical removal of the tumors was performed, and adjuvant chemotherapy was not yet standard practice, thereby excluding adjuvant treatment bias. After surgery, tumor tissues and lymph nodes had been fixed in buffered formalin, sectioned, and embedded in paraffin. Experienced pathologists documented the histopathological characteristics of the carcinomas, including tumor stage, differentiation grade, size, (lymph-)angioinvasion, perineural invasion and lymphnode involvement. Tumor stage was defined according to the TNM staging system. For both trials, follow up took place every 3 months during the first three years and every 6 months between three and five years after initial diagnosis and surgery. Standard protocols were followed, with routine blood counts and chemistry studies (including CEA levels) at each visit and liver ultrasound, chest x-ray and colonoscopy annually, to evaluate both recurrence of disease and disease-related death. After the initial five year follow up period, only the time and cause of death were registered. Follow-up was complete for all patients. In the present study, failure was defined as death due to recurrent disease, excluding postoperative mortality within 30 days and non-disease related death.

For molecular analyses, tumor tissue samples from 173 patients with primary CRC were available. The distribution of age, gender, tumor location, stage, event frequency and mean follow-up time are provided in Table 1. To validate the survival analysis data, an independent population-based series of 151 paraffin-embedded stage II MSS CRCs was derived from the prospective Netherlands Cohort Study on Diet and Cancer (NLCS). The clinical characteristics of this cohort are as described previously^{21,22}.

Promoter CpG island methylation-, MSI- and BRAF- and KRAS analysis

Genomic DNA was extracted from CRC tissues using PureGene™ Genomic DNA Isolation Kit (Gentra Systems) according to the manufacturer's protocol. Promoter CpG island methylation of *hMLH1* (mutL homolog1, colon cancer, nonpolyposis type 2 (E.Coli)), *p16^{INK4A}* (cyclin dependent kinase inhibitor 2A), *p14^{ARF}* (cyclin dependent kinase inhibitor 2A), *O⁶MGMT* (O-6-methylguanine-DNA methyltransferase), *RASSF1A* (Ras association domain family member 1), *APC* (adenomatous polyposis coli), *HLTF* (helicase-like transcription factor), *GATA4* (GATA binding protein 4), *GATA5* (GATA binding protein 5), *CHFR* (checkpoint with forkhead and ring finger domain), *ADAM23* (ADAM metallopeptidase domain 23), *Rab32*, a member *RAS oncogene family* (*RAB32*), *JPH3* (junctophilin 3), *FOXL2* (forkhead box L2), *BNIP3* (BCL2-adenovirus E1B 19kDa interacting protein 3), *NEURL* (neuralized homolog (Drosophila)), *CACNA2* (calcium channel, voltage dependent, alpha2-delta subunit 1), *THBS1* (thrombospondin 1), *TFPI2* (tissue factor inhibitor 2) and the CIMP gene panel *CACNA1G* (calcium channel, voltage-dependent, T type, alpha-1G subunit), *IGF2* (insulin-like growth factor 2 (somatomedin A)), *NEUROG1* (neurogenin 1), *RUNX3* (runt-related transcription factor 3) and *SOC31* (suppressor of cytokine signaling 1) was determined using sodium bisulfite modification of genomic DNA using the EZ DNA methylation kit (ZYMO research Co., Orange, CA).

Table 1: Clinicopathologic characteristics of CRC series

	Total
Age*	
<69 years	86/173 (50%)
>69 years	87/173 (50%)
Gender	
Male	82/173 (47%)
Female	91/173 (53%)
Tumor location	
Proximal colon	62/173 (36%)
Distal colon	52/173 (30%)
Rectum	59/173 (34%)
TNM Stage	
I	4/173 (2%)
II	100/173 (58%)
III	50/173 (29%)
IV	19/173 (11%)
Event frequency**	64 (38%)
Mean follow up time	5,6 years

*Age: ≤69 years = age below or similar to mean age (=69 years) within study population; >69 years = age above mean age (=69 years) within study population)

TNM; tumor node metastasis

** colorectal cancer specific death

Methylation-specific PCR (MSP) was performed as described in detail elsewhere^{23,24}. In brief, to facilitate MSP analysis on DNA retrieved from formalin-fixed, paraffin-embedded tissue, DNA was first amplified with flanking PCR primers that amplify bisulfite modified DNA but do not preferentially amplify methylated or unmethylated DNA. The resulting fragment was used as a template for the MSP-reaction. All PCRs were performed with a control for unmethylated alleles (normal lymphocyte DNA) and a positive control for methylated alleles (SssI methyltransferase (New England Biolabs) treated normal lymphocyte DNA) and a negative control without DNA. Each PCR reaction was loaded onto a 2% agarose gel, stained with Gelstar® (Cambrex Bioscience Rockland Inc, USA) and visualized under UV illumination. Primers and PCR conditions are provided in Supplementary Table S1.

Microsatellite instability (MSI) was determined by a pentaplex PCR, using the mononucleotide MSI markers BAT-26, BAT-25, NR-21, NR-22 and NR-24, as previously described²⁵. MSI was defined positive when three or more of five markers (BAT-26, BAT-25, NR-21, NR-22 and NR-24) showed allelic size variants.

The common V600E *BRAF* mutation in exon 15 was analyzed by a semi-nested PCR and subsequent RFLP analysis, *KRAS* mutations were analyzed as described previously^{26,27}.

Data analysis

CIMP was determined using the CpG island panel as suggested by Weisenberger *et al.*¹. CRCs are defined as CIMP positive when >3/5 analyzed markers (*CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3*, *SOCS1*) are methylated.

Unsupervised clustering (using Spotfire DecisionSite® for Functional Genomics), based on the similarity of methylation of 19 CpG islands (CIMP panel genes excluded), was performed by using half square euclidian distance (Wards method linkage rule). Methylation Index (MI = number of methylated promoter CpG islands divided by number of promoter CpG islands successfully analyzed) was calculated using the promoter CpG islands of 19 tumor suppressor- and DNA repair genes as well as the CIMP panel.

Correlations between methylation-, clinicopathological- and molecular characteristics were determined by the Pearson's χ^2 and Fisher's exact test, where appropriate. To evaluate the relationship between promoter CpG island methylation frequency and patient survival, Kaplan-Meier survival curves were calculated and stratified for tumor stage, MSI and *BRAF* mutation status. Statistical differences between groups were determined by using the Log-rank test. The endpoint for analyses was overall survival starting from the day of surgery. Independent variables predicting survival were evaluated by the multiple stepwise regression analyses using Cox Regression. The Cox-regression model included the variables: CIMP, cluster membership, *CHFR* promoter CpG island methylation, gender and age. All *P* values are two sided and *P* value <0.05 were considered statistically significant. SPSS 15.0 software was used for data analyses.

Results

(Epi)genetic characterization of CRCs

We first characterized the study population of 173 CRCs (clinical characteristics are described in Table 1), based on promoter CpG island methylation of CIMP genes and 19 additional genes, reported to be functionally and frequently methylated in colorectal carcinogenesis, as well as MSI, *BRAF* and *KRAS* mutation status (Table 2).

The frequency of promoter CpG island methylation ranged from 5% (*THBS1*) to 87% (*TFPI2*) and was as follows: *hMLH1* (30%, 52/17), *p16^{INK4A}* (43%, 72/166), *p14^{ARF}* (44%, 76/172), *O⁶MGMT* (32%, 55/173), *RASSF1A* (20%, 34/172), *APC* (35%, 60/173), *HLTF* (41%, 68/168), *GATA4* (56%, 90/162), *GATA5* (58%, 93/161), *CHFR* (43%, 71/166), *ADAM23* (42%, 64/152), *RAB32* (12%, 21/170), *JPH3* (67%, 115/171), *FOXL2* (34%, 56/165), *BNIP3* (55%, 94/171), *NEURL* (19%, 33/170), *CACNA2* (29%, 45/156), *THBS1* (5%, 8/147), *TFPI2* (87%, 125/144), *CACNA1G* (18%, 30/170), *IGF2* (13%, 20/154), *NEUROG1* (42%, 70/165), *RUNX3* (32%, 52/163) and *SOCS1* (61%, 103/170) (Table 2).

Table 2: (Epi)genetic alterations in relation to clinicopathologic characteristics

Total (%)	Age		Gender		Tumor location			Differentiation		CRC Stage			
	<69 years	>69 years	Male	Female	Proximal colon	Distal colon	rectum	Well/ Moderate	Poor	I	II	III	IV
hMLH1 52/173 (30%)	31/86 (36%)	21/87 (24%) .09	23/82 (28%)	29/91 (32%) .6	26/62 (42%)	8/52 (15%)	18/59 (31%) .009	26/96 (27%)	26/77 (34%) .3	1/4 (25%)	30/100 (30%)	13/50 (26%)	8/19 (42%) .6
	p16^{INK4A} 72/166 (43%)	32/84 (38%)	40/82 (49%) .2	31/77 (40%)	41/89 (46%) .5	30/60 (50%)	13/50 (26%)	29/56 (52%) .01	38/92 (41%)	34/74 (46%) .5	0/4 (0%)	39/95 (41%)	26/49 (53%)
p14^{ARF} 76/172 (44%)		40/86 (47%)	36/86 (42%) .5	33/81 (41%)	43/91 (47%) .4	29/62 (47%)	19/51 (37%)	28/59 (48%) .5	42/96 (44%)	34/76 (45%) .9	1/4 (25%)	43/99 (43%)	23/50 (46%)
	O⁶MGMT 55/173 (32%)	25/86 (29%)	30/87 (35%) .4	24/82 (29%)	31/91 (34%) .5	21/62 (34%)	17/52 (33%)	17/59 (29%) .8	29/96 (30%)	26/77 (34%) .6	1/4 (25%)	32/100 (32%)	16/50 (32%)
RASSF1A 34/172 (20%)		18/86 (21%)	16/86 (19%) .7	18/82 (22%)	16/90 (18%) .5	7/62 (11%)	17/52 (33%)	10/58 (17%) .01	15/95 (16%)	19/77 (25%) .1	0/4 (0%)	22/99 (22%)	10/50 (20%)
	APC 60/173 (35%)	29/86 (34%)	31/87 (36%) .8	26/82 (32%)	34/91 (37%) .4	19/62 (31%)	21/52 (40%)	20/59 (34%) .5	34/96 (35%)	26/77 (34%) .8	1/4 (25%)	36/100 (36%)	17/50 (34%)
HLTF 68/168 (41%)		37/85 (44%)	31/83 (37%) .5	31/81 (38%)	37/87 (43%) .6	24/61 (39%)	18/50 (36%)	26/57 (46%) .6	29/93 (31%)	39/75 (52%) .006	2/4 (50%)	40/96 (42%)	20/49 (41%)

Total (%)	Age		Gender		Tumor location			Differentiation		CRC Stage			
	<69 years	>69 years	Male	Female	Proximal colon	Distal colon	rectum	Well/ Moderate	Poor	I	II	III	IV
GATA4 90/162 (56%)	43/82 (52%)	47/80 (59%) .4	41/75 (55%)	49/87 (56%) .8	34/59 (58%)	22/48 (46%)	34/55 (62%) .2	50/91 (54%)	40/71 (56%) .9	3/4 (75%)	51/91 (56%)	26/49 (53%)	10/18 (56%) .9
	GATA5 93/161 (58%)	45/80 (56%)	48/81 (59%) .7	38/76 (50%)	55/85 (65%) .06	35/59 (59%)	24/47 (51%)	34/55 (62%) .5	48/88 (55%)	45/73 (62%) .4	2/4 (50%)	51/92 (55%)	32/48 (67%)
CHFR 71/166 (43%)		38/83 (46%)	33/83 (40%) .4	31/78 (40%)	40/88 (46%) .5	31/62 (50%)	13/49 (27%)	27/55 (49%) .02	36/91 (40%)	35/75 (47%) .4	0/3 (0%)	39/97 (40%)	26/48 (54%)
	ADAM23 64/152 (42%)	30/78 (39%)	34/74 (46%) .4	26/72 (36%)	38/80 (48%) .2	26/53 (49%)	11/44 (25%)	27/55 (49%) .02	37/83 (45%)	27/69 (39%) .5	0/4 (0%)	33/83 (40%)	27/48 (56%)
RAB32 21/170 (12%)		8/84 (49%)	13/86 (15%) .3	9/80 (11%)	12/90 (13%) .7	12/61 (20%)	4/51 (8%)	5/58 (9%) .1	11/94 (12%)	10/76 (13%) .8	1/4 (25%)	8/98 (8%)	9/49 (18%)
	JPH3 115/171 (67%)	52/84 (62%)	63/87 (72%) .1	53/81 (65%)	62/90 (69%) .6	43/61 (71%)	35/51 (69%)	37/59 (63%) .6	60/94 (64%)	55/77 (71%) .3	2/4 (50%)	69/99 (70%)	34/49 (69%)
FOXJ2 56/165 (34%)		28/82 (34%)	28/83 (34%) 1.0	25/78 (32%)	31/87 (36%) .6	25/60 (42%)	13/48 (27%)	18/57 (32%) .3	27/91 (30%)	29/74 (39%) .2	0/3 (0%)	30/96 (31%)	20/47 (43%)

Total (%)	Age		Gender		Tumor location			Differentiation		CRC Stage			
	<69 years	>69 years	Male	Female	Proximal colon	Distal colon	rectum	Well/ Moderate	Poor	I	II	III	IV
BNIP3													
94/171 (55%)	46/86 (54%)	48/85 (57%)	46/81 (57%)	48/90 (53%)	40/61 (66%)	21/51 (41%)	33/59 (56%)	51/95 (54%)	43/76 (57%)	1/4 (25%)	49/99 (50%)	37/50 (74%)	7/18 (39%)
	.7		.7		.04			.7		.008			
NEURL													
33/170 (19%)	22/84 (26%)	11/86 (13%)	17/80 (21%)	16/90 (18%)	19/61 (31%)	5/51 (10%)	9/58 (16%)	16/93 (17%)	17/77 (22%)	0/4 (0%)	18/99 (18%)	15/48 (31%)	0/19 (0%)
	.03		.6		.01			.4		.02			
CACNA2													
45/156 (29%)	21/79 (27%)	24/77 (31%)	21/75 (28%)	24 (30%)	22/52 (42%)	5/45 (11%)	18/59 (31%)	22/86 (26%)	23/70 (33%)	0/3 (0%)	26/91 (29%)	14/45 (31%)	5/17 (29%)
	.5		.8		.003			.3		.7			
THBS1													
8/147 (5%)	1/73 (1%)	7/74 (10%)	2/69 (3%)	6/78 (8%)	5/53 (9%)	2/43 (5%)	1/51 (2%)	5/80 (6%)	3/67 (5%)	0/4 (0%)	3/84 (4%)	3/43 (7%)	2/16 (13%)
	.03		.2		.2			.6		.5			
TFPI2													
125/144 (87%)	64/73 (88%)	61/71 (86%)	61/73 (84%)	64/71 (90%)	45/53 (85%)	35/39 (90%)	45/52 (87%)	70/79 (89%)	55/65 (85%)	3/4 (75%)	68/80 (85%)	38/43 (88%)	16/17 (94%)
	.8		.2		.8			.5		.7			
CACNA1G													
30/170 (18%)	14/86 (51%)	16/84 (49%)	10/80 (13%)	20/90 (22%)	17/59 (29%)	4/52 (8%)	9/59 (15%)	15/93 (55%)	15/77 (45%)	0/4 (0%)	14/97 (14%)	13/50 (26%)	3/19 (16%)
	.6		.1		.01			.6		.3			
MSI													
19/169 (11%)	8/84 (10%)	11/85 (13%)	9/80 (11%)	10/89 (11%)	16/61 (26%)	2/51 (4%)	1/57 (2%)	10/93 (11%)	9/76 (12%)	0/3 (0%)	11/99 (11%)	6/48 (13%)	2/19 (11%)
	.5		1.0		<0.001			.8		.9			

Total (%)	Age		Gender		Tumor location			Differentiation		CRC Stage				
	<69 years	>69 years	Male	Female	Proximal colon	Distal colon	rectum	Well/ Moderate	Poor	I	II	III	IV	
BRAF mt														
14/161 (9%)	8/79 (10%)	6/82 (7%) .5	7/74 (10%)	7/87 (8%) .8	6/59 (10%)	3/48 (6%)	5/54 (9%) .8	7/88 (8%)	7/73 (10%) .7	0/3 (0%)	10/93 (11%)	3/47 (6%)	1/18 (6%) .7	
KRAS mt														
41/157 (26%)	14/77 (18%)	27/80 (34%) .03	18/73 (25%)	23/84 (27%) .7	17/58 (29%)	11/45 (24%)	13/54 (24%) .7	22/85 (26%)	19/72 (26%) .9	0/2 (0%)	25/93 (27%)	11/45 (24%)	5/17 (29%) .8	
MSI; microsatellite instability, mt; mutation														

Table 3: CIMP and CL1, CL2 and CL3 in relation to clinicopathologic characteristics

Total (%)	Age		Gender		Tumor location			Differentiation		CRC Stage			
	≤69 years	>69 years	Male	Female	Proximal colon	Distal colon	Rectum	Well/Moderate	Poor	I	II	III	IV
CIMP													
39/168 (23%)	17/85 (20%)	22/83 (26%)	15/78 (20%)	24/90 (27%)	20/58 (66%)	5/52 (10%)	14/58 (24%)	19/93 (20%)	20/75 (27%)	0/4 (0%)	18/97 (19%)	17/48 (35%)	4/19 (21%)
		.3		.3			.008		.3				.4
CL1													
98/173 (57%)	52/86 (61%)	46/87 (53%)	50/82 (61%)	48/91 (53%)	29/62 (47%)	39/52 (75%)	30/59 (51%)	61/96 (64%)	37/77 (48%)	3/4 (75%)	58/100 (58%)	25/50 (50%)	12/19 (63%)
CL2													
98/173 (57%)	52/86 (61%)	46/87 (53%)	50/82 (61%)	48/91 (53%)	29/62 (47%)	39/52 (75%)	30/59 (51%)	61/96 (64%)	37/77 (48%)	3/4 (75%)	58/100 (58%)	25/50 (50%)	12/19 (63%)
CL2													
43/173 (25%)	19/86 (22%)	24/87 (28%)	19/82 (23%)	24/91 (26%)	16/62 (26%)	11/52 (21%)	16/59 (27%)	19/96 (20%)	24/77 (31%)	1/4 (25%)	26/100 (26%)	14/50 (28%)	2/19 (11%)
CL3													
32/173 (18%)	15/86 (17%)	17/87 (20%)	13/82 (16%)	19/91 (21%)	17/62 (27%)	2/52 (4%)	13/59 (22%)	16/96 (17%)	16/77 (21%)	0/4 (0%)	16/100 (16%)	11/50 (22%)	5/19 (26%)
	.6			.5			.007		.1				.6

CIMP: CpG island methylation phenotype as defined by Weisenberger *et al.* 1. CL 1, 2 and 3: groups of CRCs indentified by unsupervised hierarchical clustering based on methylation patterns of 19 tumor suppressor and DNA repair genes

Using the Weisenberger criteria to define CIMP, 23% (39/168) of CRCs could be classified as CIMP (Table 3), which is in agreement with previous reports^{1,28,29}. Unsupervised hierarchical cluster analysis identified three clusters, CL1 (57% (98/173) of CRCs), CL2 (25% (43/173) of CRCs) and CL3 (18% (32/173) of CRCs) (Figure 1) of which CL3 showed the highest number of methylated genes (MI) (mean MI, CL1=0.25, CL2=0.49, CL3=0.69, $p<0.001$).

MSI, *BRAF* and *KRAS* mutations were detected in 11% (19/169), 9% (14/161) and 26% (41/157) of CRCs respectively (Table 2).

Figure 1: CRCs clusters CL1 (57%), CL2 (25%), CL3 (18%) obtained by unsupervised hierarchical clustering of promoter CpG island methylation of *hMLH1*, *p16^{INK4A}*, *p14^{ARF}*, *O⁶MGMT*, *RASSF1A*, *APC*, *HLTF*, *GATA4*, *GATA5*, *CHFR*, *ADAM23*, *RAB32*, *JPH3*, *FOXL2*, *BNIP3*, *NEURL*, *CACNA2*, *THBS1*, *TFPI2*.

Black box indicates methylated gene, white box indicates unmethylated gene, grey box indicates failed PCR. After clustering identification of patients was done for CIMP, MSI, *BRAF*- and *KRAS* mutations as visualized. Black box indicates positive, white box indicates negative, grey box indicates missing value.

Molecular interrelations

By studying the relationship between different molecular alterations in CRC, strong associations between epigenetic alterations were observed. CIMP showed a strong association with promoter CpG island methylation of 16/19 additional genes, (*hMLH1* ($p=0.002$), *p16^{INK4A}* ($p<0.001$), *p14^{ARF}* ($p=0.001$), *O⁶MGMT* ($p=0.01$), *HLTF* ($p=0.002$), *GATA4* ($p=0.002$), *GATA5* ($p<0.001$), *CHFR* ($p<0.001$), *ADAM23* ($p=0.001$), *RAB32* ($p<0.001$), *JPH3* ($p=0.04$), *FOXL2* ($p=0.003$), *BNIP3* ($p=0.002$), *NEURL* ($p<0.001$), *CACNA2* ($p<0.001$) and *THBS1* ($p<0.001$)) (Supplementary Table S2). As shown in Figure 1, CRCs grouped in CL3 were most often classified as CIMP as well ($p<0.001$) (Table 4).

MSI was significantly associated with CIMP ($p<0.001$), CL3 ($p<0.001$) and promoter CpG island methylation of 11/19 genes (*hMLH1* ($p<0.001$), *p16^{INK4A}* ($p=0.04$), *p14^{ARF}* ($p=0.005$), *GATA5* ($p=0.04$), *CHFR* ($p=0.02$), *RAB32* ($p<0.001$), *FOXL2* ($p=0.001$), *BNIP3* ($p=0.023$), *NEURL* ($p=0.003$), *CACNA2* ($p<0.001$), *THBS1* ($p<0.001$)) (Table 4 and Supplementary Table S2).

Furthermore, *BRAF* mutations were positively correlated with promoter CpG island methylation of *hMLH1* ($p=0.03$), *RAB32* ($p=0.02$), *FOXL2* ($p=0.01$), *NEURL* ($p=0.002$) and *CACNA2* ($p=0.004$). Mutations in *KRAS* were positively correlated with promoter CpG island methylation of *ADAM23* and *BNIP3* ($p=0.03$ and $p=0.03$, respectively, Supplementary Table S2).

Table 4: Correlations between CIMP, CL1, CL2 and CL3 and MSI and *BRAF* mutation

	MSI	MSS	<i>BRAF</i> Mutant	<i>BRAF</i> Wild-type	CIMP
CIMP	12/19 (63%)	26/145 (18%) <0.001	7/14 (50%)	7/14 (50%) .05	
CL1	3/19 (16%)	94/150 (63%)	5/14 (36%)	83/147 (57%)	6/39 (15%)
CL2	4/19 (21%)	38/150 (25%)	3/14 (21%)	39/147 (27%)	8/39 (21%)
CL3	12/19 (63%)	18/150 (12%) <0.001	6/14 (43%)	25/147 (17%) .06	25/39 (64%) <0.001

CIMP; CpG island methylation phenotype as defined by Weisenberger *et al.*¹. CL 1,2 and 3; groups of CRCs identified by unsupervised hierarchical clustering based on methylation patterns of 19 tumor suppressor and DNA repair genes. MSI; microsatellite instability, MSS; microsatellite stability.

Correlations between molecular- and clinicopathological characteristics

Since it has been shown that CIMP correlates with specific clinicopathologic features, we studied the correlation between (epi)genetic alterations and age, gender, tumor stage, -location and -differentiation as well (Table 2 and 3). These analyses showed that promoter CpG island methylation of *NEURL* and *THBS1* was correlated with older age years ($p=0.02$ and 0.03 respectively). Comparing promoter CpG island methylation status to tumor location, we observed that promoter CpG island methylation of *hMLH1* ($p=0.009$), *p16^{INK4A}* ($p=0.01$), *CHFR* ($p=0.02$), *ADAM23* ($p=0.02$), *BNIP3* ($p=0.04$), *NEURL* ($p=0.01$), *CACNA2* ($p=0.003$), *CACNA1G* ($p=0.01$), *IGF2* ($p=0.004$), *RUNX3* ($p=0.01$) was associated with a proximal tumor location. In contrast, *RASSF1A* promoter methylation was associated with a distal tumor location ($p=0.01$). Promoter CpG island methylation of *ADAM23* ($p=0.02$), *BNIP3* ($p=0.008$) was associated with tumor stage and most frequently occurred in stage II and III CRCs compared to stage I and stage IV. Promoter CpG island methylation of *NEURL* ($p=0.02$) was most prominent in stage III compared to other stages of CRC.

CIMP as well as MSI was associated with a proximal colon tumor location ($p=0.008$ and $p<0.001$ respectively). The same accounted for CL3 CRCs which were more frequently located in the proximal colon (17/62, 27%) and rectum (13/59, 22%), when compared to distal colon tumors (2/52, 4%) ($p=0.007$) (Table 3).

For *BRAF*- and *KRAS* mutation no statically significant associations were observed.

Prognostic value of promoter CpG island methylation in CRC

The aim of this study was to evaluate the prognostic value of promoter CpG island methylation of CIMP- and 19 additional tumor suppressor- and DNA repair genes. Since tumor stage, MSI, *BRAF*- and *KRAS* mutations might influence results, we analyzed whether these variables were of prognostic significance. Disease outcome was significantly influenced by tumor stage ($p<0.001$, Figure 2A), MSI (91% 10-year survival) and *BRAF* mutations (100% 10-years survival).

Figure 2: Survival curves for A) overall population (n=167) for stage I (n=3), stage II (n=96), stage III (n=49), stage IV (n=19), $p<0.001$. Survival curves in stage II, MSS and *BRAF* wild-type tumors of (B) CIMP+ (n=12) and CIMP- (n=61), $p=0.2$; (C) CL1 (n=45), CL2 (n=22), CL3 (n=9), $p=0.1$; (D) *CHFR* unmethylated (n=43) and *CHFR* methylated (n=30) CRCs, $p=0.02$.

However, due to the limited number of MSI (n=11/95) and *BRAF* mutations (n=8/89) in stage II CRCs this observation was not statistically significant (data not shown). *KRAS* mutations were of no prognostic value. Therefore,

survival analyses were stratified for stage, MSI status, *BRAF* mutation status and not for *KRAS* mutations.

No significant survival differences between CIMP and non-CIMP CRCs were observed within Stage II (Figure 2B) or Stage III (data not shown), MSS, *BRAF* wild-type tumors. The same was observed for CL3 CRCs (Figure 2C).

When analyzing individual genes for their prognostic value, *CHFR* methylation was identified as an indicator of poor prognosis in stage II MSS, *BRAF* wild-type CRCs ($p=0.02$) (Figure 2D). The Cox-regression multivariate model within a population of MSS, *BRAF* wild-type, stage II CRCs, showed that *CHFR* methylation was associated with a poor prognosis (HR=2.80 (CI =1.24-6.33), $p=0.01$) (Table 5A).

We were able to validate the *CHFR* findings in an independent validation cohort of stage II, MSS, *BRAF* wild-type CRCs (HR=2.024 (CI =1.06-3.87), $p=0.03$) (Table 5B).

Table 5A Cox proportional hazard model in MSS, *BRAF* wild-type, stage II tumors (n=66)

	Event: CRC mortality HR (95%CI)	P value
<i>CHFR</i> M*	2.802 (1.23-6.40)	0.01
Age years	1.030 (0.98-1.08)	0.2
Gender Female**	0.723 (0.327-1.597)	0.4

HR, Hazard Ratio=Relative Risk; M = Methylated. *Reference group = *CHFR* unmethylated cases;

**Reference group = Man

Table 5B Cox proportional hazard model in MSS, *BRAF* wild-type, stage II tumors (n=151)

	Event: CRC mortality HR (95%CI)	P value
<i>CHFR</i> M*	2.024 (1.06-3.87)	0.03
Age years	0.992 (0.919-1.071)	0.8
Gender Female**	1.503 (0.784-2.880)	0.2

HR, Hazard Ratio=Relative Risk; M = Methylated. *Reference group = *CHFR* unmethylated cases;

**Reference group = Man

Discussion

The aim of this study was to evaluate the prognostic value of promoter CpG island methylation in CIMP genes as defined by Weisenberger *et al*¹ as well as in 19 additional tumor suppressor- and DNA repair genes in CRC.

This study was performed in a series of 173 CRCs. At the time the study was conducted, therapy consisted of surgical removal of the CRCs only, and adjuvant chemotherapy was not yet standard practice. Since CIMP CRCs have been reported to respond differently to adjuvant chemotherapy, this would have introduced a bias which we were able to avoid.

In this series, CIMP was observed in 23% of CRCs and was strongly associated with proximal tumor location, MSI and *BRAF* mutation. Since also other genes, which function in a diversity of signaling pathways, are methylated in

CRC, we studied the prognostic effect of promoter CpG island methylation of 19 additional genes as well. Besides studying the prognostic value of individual genes, we identified a subgroup of CRCs with extensive promoter methylation of these genes. Using unsupervised hierarchical cluster analysis, 3 subgroups of CRCs were identified of which cluster 3 (CL3) showed the highest number of methylated genes. This subgroup consisted of 18% of CRCs which showed a strong correlation to CIMP, MSI and proximal tumor location as well. Although the Weisenberger gene panel showed a stronger correlation to *BRAF* mutation, proximal and MSI CRCs were identified by CL3 as well.

Furthermore, while it has been reported that the Weisenberger panel of CIMP markers outperforms other markers for CIMP regarding its correlation to clinicopathologic features, studies on the prognostic value of CIMP show conflicting results. In the present study CIMP nor cluster membership had prognostic power after stratifying for tumor stage, MSI and *BRAF* mutation status. Different studies have suggested that CIMP CRCs show a significant survival benefit from anticancer agent 5-Fluorouracil (5-FU)³⁰, while MSI CRCs do not, which seems contradictory³¹. However, other studies have reported conflicting results and a poor outcome for CIMP CRCs after treatment with 5-FU^{12,14}. This difference might be explained by the use of different markers sets but also by differences in stratification for genetic alterations and treatment.

Moreover, while most studies on promoter CpG island methylation focus on the prognostic power of CIMP, we were interested in the prognostic value of promoter CpG island methylation of individual genes as well. Most interestingly, these analysis showed that promoter CpG island methylation of *checkpoint with forkhead and ring finger domain*, *CHFR*, was associated with a poor prognosis in stage II, MSS and *BRAF* wild-type CRCs (HR=2.80 (CI =1.24-6.33), p=0.01). Also in non small cell lung cancers, *CHFR* promoter CpG island methylation is associated with a poor prognosis³². Furthermore, we were able to reproduce our results in a population-based independent series of MSS stage II, *BRAF* wild-type CRCs. This is important to overcome multiple comparison issues with data analysis. Epigenetic alterations seem promising prognostic markers for CRC, but these issues should be taken into account when assessing the clinical use.

CHFR encodes an ubiquitin ligase that regulates both entry into metaphase and chromosome segregation later in mitosis to maintain genomic stability^{33,34}. Therefore *CHFR* is considered to be a tumor suppressor gene, which is methylated in a variety of solid tumors³⁵⁻⁴⁰ among which 43% of CRCs. Although *CHFR* promoter CpG island methylation was thought to be associated with chromosomal instability, a strong correlation with MSI and *hMLH1* promoter CpG island methylation was shown in CRC³⁵. The prognostic power of *CHFR* promoter methylation, however, is different in MSS- compared to MSI CRCs. Although the underlying mechanism remains to be elucidated, assessing *CHFR* promoter CpG island methylation in MSS CRCs might be relevant for clinical practice.

Besides a potential role for *CHFR* as prognostic marker, *CHFR* promoter methylation has been proposed as biomarker for response to microtubule inhibitor taxanes in endometrial-⁴¹ cervical-⁴², oral squamous cell-⁴³ and gastric cancer⁴⁴. Although taxanes are not implemented in CRC treatment because they failed to demonstrate a significant clinical benefit in Phase II trials^{45,46}, CRCs with *CHFR* promoter methylation might benefit from taxanes.

In summary, here we show that promoter CpG island methylation of *CHFR* is a strong indicator of poor patient survival in stage II, MSS and *BRAF* wild-type CRC. Although molecular markers are not yet applied to daily clinical practice, future studies on the role of *CHFR* promoter CpG island methylation as prognostic- and predictive marker might contribute to the development of personalized CRC treatment.

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Supplementary Table S1. Primer sequences and PCR conditions

Gene	Primer	Sense primer	Antisense primer	Amplicon size (bp)	Annealing temp (°C)	PCR cycles
<i>CANA1G</i>	flank	5'-GGT YGG TTT TTT TTT ATT TTG TT-3'	5'-CRC CRC TAC CCC CCT TTT C-3'	122	56	35
<i>CANA1G</i>	U	5'-TTT TTT TGT TTT GTG TTT AGG TTT T-3'	5'-CCC TCT CAA AAC AAC TTC ACC A-3'	71	64	35
<i>CANA1G</i>	M	5'-TCG TTT CGC GTT TAG GTT TC-3'	5'-CTC GAA ACG ACT TCG CCG-3'	62	64	35
<i>IGF2</i>	flank	5'-GTA GTT TYG GGT YGT TTT TTT TT-3'	5'-AAA AAA CRA AAT AAA AAC TAC ACC C-3'	164	56	35
<i>IGF2</i>	U	5'-GGA GTG GTT TTG GTG TTG TTA TT-3'	5'-CCC AAC TCA ATT TAA ACC AAC A-3'	90	66	35
<i>IGF2</i>	M	5'-GCG GTT TCG GTG TCG TTA TC-3'	5'-CCA ACT CGA TTT AAA CCG ACG-3'	86	68	30
<i>NEUROG1</i>	flank	5'-GTT YGG GTA TTT GTA TAA TTT ATG TT-3'	5'-AAC RCC CTA ACC AAC TTA ACC C-3'	143	56	35
<i>NEUROG1</i>	U	5'-TGT ATA ATT TAT GTT TGT GGG AGG TT-3'	5'-CCA ACA ATA ATT ACA AAC ACA CTC CA-3'	73	66	35
<i>NEUROG1</i>	M	5'-ATT TAT GTT CGC GGG AGG TC-3'	5'-GAC GAT AAT TAC GAA CAC ACT CCG-3'	65	66	35
<i>RUNX3</i>	flank	5'-GTT YGA TGG TGG AYG TGT TGG-3'	5'-AAT CCC RCA CTC ACC TTA AAA AC-3'	139	56	35
<i>RUNX3</i>	U	5'-GTT GGT GGA TTA TGT AGG TGA GTT T-3'	5'-ACT CAC CTT AAA AAC AAC AAA CA-3'	115	66	35
<i>RUNX3</i>	M	5'-GCG GAT TAC GTA GGC GAG TTC-3'	5'-ACC TTA AAA ACG ACG AAC AAC G-3'	107	66	35
<i>SOC1</i>	flank	5'-GTT YGT GGT TAT TTT TTT GGT G-3'	5'-TAA ATC CCR AAA CCA TCT TCA C-3'	83	56	35
<i>SOC1</i>	U	5'-GGG TAT TTT TTT GGT GTG TGA TAG TT-3'	5'-CCA AAA CCA TCT TCA CAC TAA AAA CA-3'	71	66	35
<i>SOC1</i>	M	5'-TTT TTT TGG TGC GCG ATA GTC-3'	5'-GAA ACC ATC TTC ACG CTA AAA ACG-3'	64	66	35
<i>MLH1</i>	flank	5'-TTT TGA YGT AGA YGT TTT ATT AGG GT-3'	5'-AAA ACR ATA AAA CCC TAT ACC TAA TCT ATC-3'	154	56	35
<i>MLH1</i>	U	5'-TGT GTG TTT GTT TGT TAT ATA TTG TTT-3'	5'-ACC ACC TCA TCA TAA CTA CCC ACA-3'	98	60	35
<i>MLH1</i>	M	5'-GTT CGT CGT TCG TTA TAT ATC GTT C-3'	5'-CCT CAT CGT AAC TAC CCG CG-3'	89	60	35
<i>p16^{INK4A}</i>	flank	5'-GGG TTG GTT GGT TAT TAG AGG GT-3'	5'-RAC CRT AAC CAA CCA ATC AAC C-3'	148	56	35
<i>p16^{INK4A}</i>	U	5'-GTT GGT TAT TAG AGG GTG GGG TGG ATT GT-3'	5'-AAC CAA AAA CTC CAT ACT CCC CAC CA-3'	124	62	25
<i>p16^{INK4A}</i>	M	5'-TTA TTA GAG GGT GGG GCG GAT CGC-3'	5'-GAA AAC TCC ATA CTA CTC CCC GCC G-3'	115	62	25
<i>p14^{ARF}</i>	flank	5'-GYG TTG TTT ATT TTT GGT GTT AAA GG-3'	5'-AAA TAT AAA CCA CRA AAA CCC TCA CT-3'	152	56	35
<i>p14^{ARF}</i>	U	5'-TTT TTG GTG TTA AAG GGT GGT GTA GT-3'	5'-CAC AAA AAC CCT CAC TCA CAA CAA-3'	132	60	25
<i>p14^{ARF}</i>	M	5'-GTG TTA AAG GCG GGC GTA GC-3'	5'-AAA ACC CTC ACT CGC GAC GA-3'	122	60	25
<i>MGMT</i>	flank	5'-GYG TTT YGG ATA TGT TGG GAT AGT T-3'	5'-AAA CTC CRC ACT CTT CCR AAA AC-3'	135	56	35
<i>MGMT</i>	U	5'-TTT GTG TTT TGA TGT TTG TAG GTT TTT GT-3'	5'-AAC TCC ACA CTC TTC CAA AAA CAA AAC A-3'	93	60	30
<i>MGMT</i>	M	5'-TTT CGA CGT TCG TAG GTT TTC GC-3'	5'-GCA CTC TTC CGA AAA CGA AAC G-3'	81	60	30

Gene	Primer	Sense primer	Antisense primer	Amplicon size (bp)	Annealing temp (°C)	PCR cycles
RASSF1A	flank	5'-GTT TAG TTT GGA TTT TGG GGG AG-3'	5'-CCC RCA ACT CAA TAA ACT CAA ACT C-3'	144	56	35
RASSF1A	U	5'-GGG GTT TGT TTT GTG GTT TTG TTT-3'	5'-AAC ATA ACC CAA TTA AAC CCA TAC TTC A-3'	81	64	30
RASSF1A	M	5'-GGG TTC GTT TTG TGG TTT CGT TC-3'	5'-TAA CCC GAT TAA ACC CGT ACT TCG-3'	76	64	30
APC	flank	5'-TGG GYG GGG TTT TGT GTT TTA TT-3'	5'-TAC ROC CAC ACC CAA CCA ATC-3'	136	56	35
APC	U	5'-GTG TTT TAT TGT GGA GTG TGG GTT-3'	5'-CCA ATC AAC AAA CTC CCA ACA A-3'	108	60	25
APC	M	5'-TAT TGC GGA GTG CGG GTC-3'	5'-TCG ACG AAC TCC CGA CGA-3'	98	60	25
HLTF	flank	5'-GTA GGT ATY GTA GTY GTA TTT TTG GG-3'	5'-CAA AAC ACA AAA AAA AAA ACA ACT CC-3'	155	56	35
HLTF	U	5'-GGT TTT GTG GTT TTT TTG TGT GTT T-3'	5'-CCC CAC TAC CAT TCA AAA ACA ACA-3'	103	65	30
HLTF	M	5'-GTG GTT TTT TCG CGC GTT C-3'	5'-CGC TAC CAT TCA AAA ACG ACG-3'	96	65	30
GATA4	flank	5'-GGG AGT TTT TYG TAT AGT TTY GTA G-3'	5'-COR ACC RCC TCC AAA TCC CCA AC-3'	167	56	35
GATA4	U	5'-TTT GTA TAG TTT TGT AGT TTG TGT TTA GT-3'	5'-CCC AAC TCA CAA CTC AAA TCC CCA-3'	140	64	30
GATA4	M	5'-GTA TAG TTT CGT AGT TTG CGT TTA GC-3'	5'-AAC TCG CGA CTC GAA TCC CCG-3'	136	68	25
GATA5	flank	5'-TAG ATA YGG AGT TYG TTT TTA GGT TAG-3'	5'-ORA AAC CCR AAC CAA TAC AAC TAA AC-3'	160	56	35
GATA5	U	5'-TGG AGT TTG TTT TTA GGT TAG TTT TTG GT-3'	5'-CAA ACC AAT ACA ACT AAA CAA ACA AAC CA-3'	147	64	25
GATA5	M	5'-AGT TCG TTT TTA GGT TAG TTT TCG GC-3'	5'-CCA ATA CAA CTA AAC GAA CGA ACC G-3'	140	64	25
CHFR	flank	5'-TTT TYG TTT TTT TTG TTT TAA TAT AAT ATG G-3'	5'-CRC TCA CCA AAA ACR ACA ACT AAA AC-3'	167	56	35
CHFR	U	5'-GAT TGT AGT TAT TTT TGT GAT TTG TAG GTG AT-3'	5'-AAC TAA AAC AAA ACC AAA AAT AAC CCA CA-3'	115	66	30
CHFR	M	5'-GTT ATT TTC GTG ATT CGT AGG CGA C-3'	5'-CGA AAC CGA AAA TAA CCC GCG-3'	100	66	30
ADAM23	flank	5'-GGG GGG TTG TTG YGT TTA G-3'	5'-CAA ACA AAA AAA AAA AAA AAA AC-3'	147	56	35
ADAM23	U	5'-GTT TTG TGT TTT TTG TGT TGG TTT TTT T-3'	5'-AAA ACC AAA CCA AAA ACA AAC CCA-3'	99	66	30
ADAM23	M	5'-CGT GTT TTT TGC GTT GGT TTT TTC-3'	5'-CGG AAC CGA AAA CAA ACC CG-3'	91	66	30
RAB32	flank	5'-GGA GAG GAA AGT TTA GTT GGG TT-3'	5'-CCC AAA ACC CRC CAA TAC TC-3'	156	56	35
RAB32	U	5'-GGG TTT GGT TGG GTT TTA GAG GT-3'	5'-ACC CAC CAA TAC TCA CAA CCC A-3'	133	64	35
RAB32	M	5'-TTC GGT CGG GTT TTA GAG GC-3'	5'-GCC AAT ACT CGC GAC CCG-3'	126	64	35
JPH3	flank	5'-GGT TTT TAA TAT GGT GTA GTY GTT AG-3'	5'-CCC CCC ACC TAC RAC TAC C-3'	110	56	35

Gene	Primer	Sense primer	Antisense primer	Amplicon size (bp)	Annealing temp (°C)	PCR cycles
<i>JPH3</i>	U	5'-GGT TTT TAA TAT GGT GTA GTT GTT AGT GTT-3'	5'-CCC CCC ACC TAC AAC TAC CAC A-3'	103	64	35
<i>JPH3</i>	M	5'-TTT AAT ATG GTG TAG TCG TTA GCG TC-3'	5'-CCC ACC TAC GAC TAC CGC G-3'	103	64	35
<i>FOX12</i>	flank	5'-ATA AAA AGT GAT TTG GAG ATG AAT T-3'	5'-CTC TAT TCT AAT TCR TAT AAA CTC CAC-3'	114	56	35
<i>FOX12</i>	U	5'-TGA TTT GGA GAT GAA TTT GTT TGT GT-3'	5'-AAT TCA TAT AAA CTC CAC CAA ATT CCA-3'	97	65	20
<i>FOX12</i>	M	5'-TTG GAG ATG AAT TCG TTC GTG C-3'	5'-CGT ATA AAC TCC ACC GAA TTC CG-3'	89	65	20
<i>BNIP3</i>	flank	5'-TTY GTT TTG TTT TGT GAG TTT TTT-3'	5'-CCR AAC TAC AAA ATA TAC TTC AAC TAC-3'	129	56	35
<i>BNIP3</i>	U	5'-GGT TTT GTT TAG TTT GGG AGT G-3'	5'-CCT CAA CTA CAA ACA ATA AAA CA-3'	74	61	35
<i>BNIP3</i>	M	5'-TTT CGT TTA GTT CGG GAG CG-3'	5'-CAA CTA CGA ACG ATA AAA CG-3'	69	61	35
<i>NEURL</i>	flank	5'-GGT TTA GGG TTT TGT TTG TGG-3'	5'-CCT CAA CTT CCT TTC TCT AAA CTT-3'	132	56	35
<i>NEURL</i>	U	5'-TTG TTT GTG GTT TTT GTT TTT AT-3'	5'-CTT TCT CTA AAC TTC ACA ATC CTT AAC A-3'	111	65	35
<i>NEURL</i>	M	5'-GTT TGT GGT TTT CGT TTT CGT TAC-3'	5'-TCT AAA CTT CGC GAT CCT TAA CG-3'	104	65	35
<i>CACNA2</i>	flank	5'-GYG GAG YGG AGT AGG TAG TTT-3'	5'-AAA ACR CCR AAA CCC CC-3'	159	56	35
<i>CACNA2</i>	U	5'-TTG TTT GTT TTG TGT AGT TTT TTG TGG TT-3'	5'-CCA AAA CCC CCC AAA ACA CA-3'	118	62	35
<i>CACNA2</i>	M	5'-CGC GTA GTT TTT CGC GGT C-3'	5'-CCC CCC GAA ACG CG-3'	102	62	35
<i>THBS1</i>	flank	5'-GGA GTT TAG ATT GGT TTT TAT TTT T-3'	5'-CCC TAA ACT CRC AAA CCA ACT C-3'	149	56	30
<i>THBS1</i>	U	5'-TAT TTT TTG TTT TTT GTT TGG TTG TT-3'	5'-ACT AAC AAA ACA AAA AAA CCA CAC A-3'	99	64	30
<i>THBS1</i>	M	5'-TTC GTT TTT TGT TCG GTC GTC-3'	5'-ACC GAA AAA ACC GCG CG-3'	86	64	30
<i>TFPI2</i>	flank	5'-GTG TAT GAA TTA GTT ATT TTT TAG GTT T-3'	5'-CTA AAC AAA ACR TCC RAA AAA AC-3'	144	56	35
<i>TFPI2</i>	U	5'-TTA GTT ATT TTT TAG GTT TTG TTT TGG T-3'	5'-AAA AAC ACC TAA CAA AAA AAA ATA CAC A-3'	118	64	35
<i>TFPI2</i>	M	5'-ATT TTT TAG GTT TCG TTT CGG C-3'	5'-GCC TAA CGA AAA AAA ATA CGC G-3'	106	66	35

Supplementary Table S2. Correlations between promoter CpG island methylation and MSI, BRAF, KRAS and CIMP

	MSI	MSS	BRAF		KRAS		CIMP+	CIMP-
			mutant	wild-type	mutant	wild-type		
hMLH1	13/19 (68%)	37/150 (25%) <0.001	8/14 (57%)	41/147 (28%) .03	11/41 (27%)	38/116 (33%) .5	20/39 (51%)	32/129 (25%) .002
p16^{INK4A}	12/18 (67%)	59/144 (41%) .04	7/14 (50%)	63/141 (45%) .7	19/39 (49%)	49/111 (44%) .6	30/37 (81%)	42/125 (34%) <0.001
p14^{ARF}	14/19 (74%)	59/149 (40%) .005	7/14 (50%)	64/146 (44%) .7	22/40 (55%)	48/116 (41%) .1	27/39 (69%)	49/128 (38%) .001
O⁶MGMT	5/19 (26%)	49/150 (33%) .6	3/14 (21%)	50/147 (34%) .4	14/41 (34%)	40/116 (35%) 1.0	19/39 (49%)	35/129 (27%) .01
RASSF1A	2/19 (11%)	31/149 (21%) .3	1/14 (7%)	32/146 (22%) .2	9/40 (23%)	22/116 (19%) .6	10/38 (26%)	24/129 (19%) .3
APC	8/19 (42%)	51/150 (34%) .5	3/14 (21%)	54/147 (37%) .3	17/41 (42%)	38/116 (33%) .3	13/39 (33%)	46/129 (36%) .8
HLTF	11/19 (58%)	55/145 (38%) .1	6/14 (43%)	60/142 (42%) 1.0	15/37 (41%)	48/115 (42%) .9	24/38 (63%)	44/125 (35%) .002
GATA4	13/18 (72%)	75/140 (54%) .1	11/14 (79%)	76/139 (55%) .09	24/38 (63%)	58/110 (53%) .3	30/38 (79%)	60/120 (50%) .002
GATA5	15/19 (79%)	75/138 (54%) .04	9/14 (64%)	81/138 (59%) .7	23/39 (59%)	64/107 (60%) 1.0	33/38 (87%)	60/120 (50%) <0.001

	MSI	MSS	BRAF		KRAS		CIMP+	CIMP-
			mutant	wild-type	mutant	wild-type		
CHFR	13/19 (68%)	57/143 (40%) .018	9/13 (69%)	60/142 (42%) .061	17/39 (44%)	50/112 (45%) .909	29/39 (74%)	42/122 (34%) <0.001
ADAM23	10/16 (63%)	51/132 (39%) .067	8/12 (67%)	54/131 (41%) .089	21/36 (58%)	39/103 (38%) .033	25/38 (66%)	38/111 (34%) .001
RAB32	9/19 (47%)	11/147 (8%) <0.001	6/13 (46%)	15/145 (10%) <0.001	4/41 (10%)	17/115 (15%) .418	17/39 (44%)	4/126 (3%) <0.001
JPH3	16/19 (84%)	95/148 (64%) .082	10/13 (77%)	100/146 (69%) .528	33/41 (81%)	75/115 (65%) .069	32/39 (82%)	82/127 (65%) .039
FOXL2	13/19 (68%)	41/142 (29%) .001	9/13 (69%)	46/141 (33%) .014	17/40 (43%)	35/111 (32%) .211	21/39 (54%)	34/122 (28%) .003
BNIP3	15/19 (79%)	76/148 (51%) .023	9/14 (64%)	79/145 (55%) .481	28/40 (70%)	58/115 (50%) .032	30/39 (77%)	62/127 (49%) .002
NEURL	9/19 (47%)	23/147 (16%) .003	7/13 (54%)	25/145 (17%) .002	7/39 (18%)	23/116 (20%) .797	20/39 (51%)	12/126 (10%) <0.001
CACNA2	14/17 (82%)	29/135 (22%) <0.001	8/12 (67%)	36/133 (27%) .004	14/38 (37%)	27/104 (26%) .205	26/38 (68%)	19/115 (17%) <0.001
THBS1	4/17 (24%)	3/126 (2%) <0.001	2/12 (17%)	6/129 (5%) .080	3/35 (9%)	5/99 (5%) .450	7/38 (18%)	1/108 (1%) <0.001
TFPI2	15/17 (88%)	106/123 (86%) .816	12/14 (86%)	106/122 (87%) 1.0	28/35 (80%)	87/97 (90%) .151	35/39 (90%)	89/104 (86%) .513

CIMP⁺, CpG island methylation phenotype as defined by Weisenberger *et al.*¹; MSI⁺, microsatellite instability, MSS; microsatellite stability.

CHAPTER 7|

Promoter CpG island hypermethylation- and H3K9me3 and H3K27me3-mediated epigenetic silencing targets the *Deleted in Colon Cancer (DCC)* gene in colorectal carcinogenesis without affecting neighboring genes on chromosomal region 18q21

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Carcinogenesis 30(6), 1041-8 (2009)

Abstract

Chromosomal loss of 18q21 is a frequent event in colorectal cancer (CRC) development, suggesting that this region harbors tumor suppressor genes (TSGs). Several candidate TSGs, among which Methyl-CpG-Binding Domain Protein 1 (*MBD1*), CpG-Binding Protein (*CXXC1*), Sma- and Mad related protein 4 (*SMAD4*), Deleted in Colon Cancer (*DCC*), and Methyl-CpG-Binding Domain Protein 2 (*MBD2*) are closely linked on a 4-MB DNA region on chromosome 18q21. As TSGs can be epigenetically silenced, this study investigates whether *MBD1*, *CXXC1*, *SMAD4*, *DCC* and *MBD2* are subject to epigenetic silencing in CRC.

Methylation-specific PCR and sodium bisulfite sequencing of these genes show that *DCC*, but not *MBD1*, *CXXC1*, *SMAD4* and *MBD2*, has promoter CpG island methylation in CRC cell lines and tissues (normal mucosa (29.5% (18/61), adenomas (81.0% (47/58)) and carcinomas (82.7% (62/75)) (P value 8.6×10^{-9})) which is associated with reduced *DCC* expression, independent of 18q21 loss analyzed by multiplex ligation-dependent probe amplification. Reduced gene expression of *CXXC1*, *SMAD4* and *MBD2* correlates with 18q21 loss in CRC cell lines (P values 0.04, 0.02 and 0.02 respectively). Treatment with the demethylating agent 5-Aza-2'-deoxycytidine, but not with the histone deacetylase inhibitor Trichostatin A exclusively restored *DCC* expression in CRC cell lines. Chromatin immunoprecipitation studies reveal that the *DCC* promoter is marked with repressive histone-tail marks H3K9me3 and H3K27me3, whereas activity related H3K4me3 was absent. Only active epigenetic marks were detected for *MBD1*, *CXXC1*, *SMAD4* and *MBD2*.

This study demonstrates specific epigenetic silencing of *DCC* in CRC as a focal process not affecting neighboring genes on chromosomal region 18q21.

Introduction

Colorectal cancer (CRC) development is characterized by the growth of a benign precursor lesion from which a small percentage will progress into a carcinoma. Genetic alterations underlying the adenoma to carcinoma transition have been extensively studied over the past two decades. Chromosomal loss of 18q has been reported to occur in 60-70% of CRCs, suggesting this region harbors tumor suppressor genes (TSGs) involved in colorectal carcinogenesis¹⁻³. Although chromosomal loss of 18q often affects a large section of the chromosomal arm, a minimal loss region on 18q21, including *Deleted in Colon Cancer (DCC)* has been identified^{2,4}. Near to *DCC*, within the same 4-Mb chromosomal region, lie several other candidate TSGs, including *Sma- and Mad related protein 4 (SMAD4)*⁵, *Methyl-CpG-Binding Domain Protein 1 (MBD1)*, *CpG-Binding Protein CXXC1* and *Methyl-CpG-Binding Domain Protein 2 (MBD2)*^{6,7}.

Since loss of function for TSGs requires biallelic inactivation, these genes have been examined for the presence of mutations in the remaining allele. For *DCC*⁸, *MBD1*, *CXXC1* and *MBD2*⁶ mutations are relatively rare events (<5%) in CRC. *SMAD4* has some reports of mutations (15%)⁹, which may be higher in tumors with distant metastasis (35%)¹⁰, but even this mutational rate does not match the frequency of loss (60-70%) at 18q21. While haplo-insufficiency might provide another explanation for the observed low mutation frequencies¹¹, this raises the question of whether alternative mechanisms might account for inactivation of these TSGs.

In addition to genetic changes in cancer, epigenetic modifications including promoter CpG island methylation are associated with gene silencing and serve as mechanism to inactivate tumor suppressor- and DNA repair genes¹². Promoter CpG island methylation is often associated with histone modifications¹³. Histone 3 trimethyl lysine 9 (H3K9me3) or lysine 27 (H3K27me3) marks are associated with methylated DNA and transcriptional silencing¹⁴. In contrast, acetylation of histone H3¹⁵ and trimethylation of histone H3 at lysine 4 (H3K4me3) is associated with unmethylated DNA and gene expression and can be considered as marks of active chromatin¹³.

While chromosomal region 18q21 deletion occurs frequently in colorectal carcinogenesis and has been extensively studied for genetic mechanisms potentially leading to gene inactivation¹⁶, little is known about epigenetic silencing of genes in this region. Therefore, in this study we investigate whether epigenetic mechanisms serve as second hit in inactivating genes on chromosome 18q21 in colorectal cancer.

Materials and Methods

Cell lines, study population and tissues

CRC cell lines (CACO2, COLO205, HT29, SW480, HCT116, RKO and LS174T and SW48) were cultured in DMEM (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (Hyclone).

MBD1, *CXXC1*, *SMAD4*, *DCC* and *MBD2* promoter CpG island methylation was investigated in a well-characterized series of colorectal carcinomas. The series consists of formalin-fixed, paraffin-embedded CRC tissues (n=75) of

patients over 50 years of age, 37 males and 38 females (mean age 71.0, range 50-88, at time of diagnosis) which were retrospectively collected from the tissue archive of the Dept. of Pathology of the Maastricht University Medical Center. When present, also normal (n=61) and adenoma (n=58) tissue was collected from these patients. Histological normal biopsy material from patients undergoing endoscopy for non-specific abdominal complaints (n=48) were selected (23 males, 25 females, age 63.9, range 50-87 years at time of diagnosis). Clinicopathologic characteristics are listed in Supplemental Table 1. This study was approved by the Medical Ethical Committee (MEC) of the Maastricht University Medical Center.

DNA isolation and bisulfite treatment

A 5µm section of each tissue block was stained with haematoxylin and eosin and revised by a pathologist (AdB). Five sections of 20µm were deparaffinated prior to DNA-isolation. DNA was extracted using the Puregene® DNA isolation kit (Gentra systems) according to the manufacturer's instructions. Sodium bisulfite modification of 500 ng genomic DNA was performed using the EZ DNA methylation kit (ZYMO research Co, Orange, CA) according to the manufacturer's instructions.

Promoter CpG island methylation analyses: sodium bisulfite sequencing and methylation-specific PCR (MSP)

For sequencing of sodium bisulfite-converted DNA, PCR products were amplified and cloned using the TOPO-TA cloning kit (Invitrogen, Breda, the Netherlands). Single colonies were sequenced using an automated sequencer (Applied Biosystems, Foster City, CA).

MSP analysis on bisulfite treated DNA were performed as described in detail elsewhere^{17,18}. To facilitate MSP analysis on DNA retrieved from formalin-fixed, paraffin embedded tissue, DNA was first amplified with flanking PCR primers which was used as a template for the MSP reaction. All primer sequences and conditions are shown in Supplemental Table 2.

Multiplex Ligation-dependent Probe Amplification (MLPA)

MLPA was performed as described before^{19,20}. In brief, an oligonucleotide MLPA probe set was designed for 45 genes on chromosomes 3, 5, 8p, 9, 11q, 15q, 18q, and 19q. This mix contained probes for eight genes located on chromosome 18q, including probes for *DCC* and *SMAD4*. Additionally, eleven reference probes (two probes on chromosome 2p; three probes on chromosome 4q; two probes on chromosome 12; and four probes on chromosome 16) were included for quality control and normalization purposes. Data analysis was performed using excel-based software developed at MRC-Holland, which provides a reliable and robust normalization for MLPA fragment data files²¹. The data was normalized using the median of the reference probes. As reference DNA, a pool of DNA extracted from normal paraffin embedded tissue samples of different organs (10 colons, 10 stomach, 4 kidneys, 4 liver and 4 spleen) was used. DNA copy number ratios were obtained by dividing the median area under the peak for each probe in the sample by the median area under the peak of at least three reference DNA samples. Each sample was run at least three times, and the median of the different runs were used for analysis. Finally, all DNA copy number ratios were normalized by setting

the median copy number ratio of the reference probes to 1.0. A ratio less than 0.8 was considered as a deletion, and a ratio higher than 1.2 as a gain.

Quantitative reverse transcription PCR

Total RNA was isolated using the RNeasy Mini kit (Qiagen) following the manufacturer's instructions. Possible genomic DNA contaminations were removed by DNase treatment with the RNase-free DNase set (Qiagen). cDNA synthesis was performed using the Iscript cDNA synthesis kit (Bio-Rad). Quantitative reverse transcription-PCR was performed as described previously²² using SYBR Green PCR master mix (Applied Biosystems, Nieuwekerk a/d IJssel, The Netherlands). *MBD1*, *CXXC1*, *SMAD4*, *DCC* and *MBD2* expression levels were normalized to *CYCLOPHILIN* and average expression levels in normal colon tissues (n=10) for each tissue sample using the following equation: relative expression = $2^{-(\Delta Ct)}$, where ΔCt = average Ct (tissue sample) - average Ct (*CYCLOPHILIN*) - average Ct (normal colon tissues). Since alternative splicing of *DCC* exons 6-7 and 18-23 has been reported^{23,24}, *DCC* RT-primers are located in exon 2-3.

To investigate re-expression of *MBD1*, *CXXC1*, *SMAD4*, *DCC* and *MBD2* following inhibition of DNA methyltransferases or inhibition of histone deacetylation, HCT116, RKO cells and HT29 cells were treated with 5 μ M 5-Aza-2'deoxyctidine (Aza) (Sigma) and with 300 nM Trichostatin A (TSA) (Sigma) for 96 and 18 hours respectively.

As a positive control for gene expression following inhibition of DNA methyltransferases re-expression of *GATA4* and *GATA5* were used. As a positive control for gene expression following treatment with TSA, previously described control gene *FABP4*^{15,25} was analyzed.

Chromatin immunoprecipitation (ChIP) assays

ChIPs were performed for cell lines HCT116 and HT29 cells and analyzed essentially as described previously²⁶. Briefly, HCT116 and HT29 cells were fixed in 1% formaldehyde. Samples were immunoprecipitated with antibodies for either HA as a negative control (sc-805; Santa Cruz), H3K9me3 (07-442;Upstate), H3K27me3 (07-449; Upstate) and H3K4me3 (ab8580; Abcam). Immunoprecipitated DNA was quantified by real-time PCR. Enrichment was calculated as percentage of input DNA. HA was used as negative control and subtracted from enrichment values. For *MBD1*, *CXXC1*, *SMAD4* and *MBD2*, three primer pairs were designed covering -600 bp to transcription start site (TSS). For *DCC*, six additional primer pairs were designed to investigate the region -597bp to +467 bp relative to TSS. Control PCRs for each antibody immunoprecipitation were performed using primers for *GAPDH* and *P16^{INK4A}* (monoallelic methylation in HCT116 and biallelic methylation in HT29 (data not shown)) as negative and positive control dependent on the antibody used.

Data analysis

We used the Pearson's χ^2 or Fisher's Exact test, students t-test, Kruskal-Wallis, Mann-Whitney or Wilcoxon rank test where appropriate to compare categorical and continuous data, respectively. Since a significant difference in age was observed between CRC cases and controls (P value 1.1×10^{-4}), logistic regression analyses were used to adjust for age. All quoted P values

are two-sided, and a P value < 0.05 was considered statistically significant. Where appropriate, the Bonferroni method was used to correct for multiple comparisons. Data analysis was done using SPSS software (version 12.0.1).

Results

DCC promoter is methylated in colorectal cancer cell lines, while MBD1, CXXC1, SMAD4 and MBD2 are unmethylated

We first analyzed promoter CpG island methylation of 5 TSGs genes within a 4-Mb region in proximity to *DCC* (46.0 Mb to 49.9 Mb) including *MBD1*, *CXXC1*, *SMAD4*, *DCC* and *MBD2*. Using public ENSEMBL and EMBOSS CpG plot/CpG software we observed that *MBD1*, *CXXC1*, *SMAD4*, *DCC* and *MBD2* each have a CpG island-associated promoter region (GC content $> 60\%$, ratio of observed CpG / expected CpG > 0.6 and minimum length 200 bp²⁷). For an initial assessment of the methylation status of these promoter CpG islands, methylation-specific PCR (MSP) was performed on colorectal cancer (CRC) cell lines CACO2, COLO205, HT29, SW480, HCT116, RKO, LS174T and SW48. Since *DCC* has 2 CpG rich regions, region 1 located -95 bp to +170 bp and region 2 located +608 bp and +962 bp relative to the transcription start site (TSS), both regions were analyzed. *MBD1*, *CXXC1*, *SMAD4* and *MBD2* promoter CpG island methylation status was analyzed using primers for the CpG islands spanning the TSS (primers, locations and PCR conditions are provided in Supplemental Table 2). All CRC cell lines have complete (CACO2, COLO205, HT29, SW480, HCT116, RKO, SW48) or partial (LS174T) *DCC* DNA methylation in region 1, while region 2 showed complete *DCC* methylation for all cell lines. In contrast, CpG-rich regions within *MBD1*, *CXXC1*, *SMAD4* and *MBD2* promoters were unmethylated in all cell lines analyzed (data not shown).

To study in more detail the DNA methylation of these promoter regions, sodium bisulfite sequencing of individual clones of CRC cell lines HCT116 and RKO and 3 paired normal and adjacent CRC tissues showed that both *DCC* CpG islands are densely hypermethylated in CRC tissues while normal tissues showed less methylated CpG dinucleotides (Figure 1). This difference is most apparent for the promoter region of *DCC*, in which normal tissues 1, 4 and 8 show, respectively, 7.3%, 1% and 3.1% of CpGs methylated, while in the more 3' region, 40.3%, 22.2% and 25% of CpG's are methylated. Since the promoter region demonstrated the most tumor specific methylation and epigenetic changes are most tightly associated with transcription near the TSS, further *DCC* promoter methylation analysis examined the CpG rich region crossing the TSS. In contrast to *DCC*, the *MBD1*, *CXXC1*, *SMAD4* and *MBD2* gene promoter regions are almost completely unmethylated at all CpG dinucleotides.

Figure 1: Sodium bisulfite sequencing results of *MBD1*, *CXXC1*, *SMAD4*, *DCC* and *MBD2* gene promoters on cell lines HCT116 and RKO and normal and adjacent tumor tissues of 3 patients (1,4 and 8). Each row represents an individual cloned allele and each circle indicates a CpG dinucleotide. Black circle; methylated CpG site, white circle; unmethylated CpG site, grey circle; not determined.

Reduced CXXC1, SMAD4 and MBD2 gene expression associates with 18q21 loss

To determine the effect of promoter CpG island methylation and loss of 18q21 on gene expression, we analyzed DNA copy number changes of 8 loci on region 18q harboring *SMAD4* (46.8 Mb) and *DCC* (48.1 Mb) in detail by multiplex ligation dependent probe amplification (MLPA)²⁸ in CRC cell lines (Figure 2A). These analyses revealed that cell lines CACO2, COLO205, HT29 and SW480 had loss of the complete region tested, including the *DCC* locus. This was not observed for cell lines HCT116, RKO, LS174T and SW48 which had no loss of 18q21.

Next we studied *MBD1*, *CXXC1*, *SMAD4*, *DCC* and *MBD2* gene expression and the relation to 18q21 deletion in CRC cell lines CACO2, COLO205, HT29, SW480, HCT116, RKO, LS174T and SW48. Expression of *CXXC1*, *SMAD4* and *MBD2* was significantly higher in cell lines HCT116, RKO, LS147T and SW48 in which 18q21 was retained, compared to CACO2, COLO205, HT29 and SW480 cells in which 18q21 is deleted (Mann-Whitney, *P* values 0.04, 0.02 and 0.02 respectively) (Figure 2B). *MBD1* expression was detectable in all CRC cell lines but did not correlate to 18q21 loss. In contrast, *DCC* mRNA levels were nearly undetectable in all CRC cell lines, while clearly present in positive control brain tissue²³ and normal colon tissue, suggesting that complete DNA methylation observed in these cell lines correlated with absent expression.

Figure 2: A) Heatmap representation of Multiplex Ligation-dependent Probe Amplification (MLPA) which shows loss of 8 loci on chromosomal region 18q in cell lines CACO2, COLO205, HT29 and SW480. A ratio less than 0.8 was considered as a deletion, a ratio higher than 1.2 as a gain. B) Quantitative reverse transcription PCR of *MBD1*, *CXXC1*, *SMAD4*, *DCC*, and *MBD2* on CRC cell lines. Gene expression levels are relative to the mean expression level of the normal colon tissues (n=10), which is set to equal 1. Data represent mean and standard error of mean of 2 or 3 independent experiments. * Mann-Whitney, *P* value 0.04. ** Mann-Whitney *P* value 0.02

DCC promoter methylation is a frequent and early event in colorectal carcinogenesis

Next, promoter CpG island methylation of these 18q21 genes was studied in a well characterized series of formalin-fixed, paraffin-embedded primary CRCs (n=75). When available, adenoma- (n=58) and normal tissue (n=61) of the same patients was analyzed as well. This analysis showed that *DCC* promoter methylation is a common event in CRC, which occurred in 82.7% (62/75) of CRCs, 81% of adenomas (47/58) and 29.5 % of normal colon mucosa of CRC patients (16/61) (*P* value 8.6×10^{-9}) (Table 1A). For 50 patients normal, adenoma and carcinoma tissue was available allowing a detailed analysis of *DCC* promoter methylation in colorectal carcinogenesis (Table 1B).

Table 1A: Promoter methylation analyses (MSP) on normal- and CRC tissues

tissue	<i>MBD1</i>	<i>CXXC1</i>	<i>SMAD4</i>	<i>DCC</i>	<i>MBD2</i>
Normal colon				10/48 (20.8%)	
CRC tissues					
normal	0/31	0/32	0/32	16/61 (29.5%)	0/31
adenoma	1/33	1/34	2/32	47/58 (81%)	1/32
carcinoma	1/33	5/32	1/29	62/75 (82.7) *	1/32

* *P* value 8.6×10^{-9}

Table 1B: DCC promoter CpG island methylation in CRC patients

	CRC patient		
	normal	adenoma	carcinoma
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
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45			
46			
47			
48			
49			
50			

Black- and white boxes represent methylated- and unmethylated genes respectively.

Figure 3: Integrative analysis of *DCC* promoter CpG island methylation and chromosomal loss of region 18q21 and their relation to transcriptional genes silencing in CRC tissues.

A) Heatmap representation of Multiplex Ligation-dependent Probe Amplification (MLPA) which shows loss of 8 loci on chromosomal region 18q (A) and their relation to *DCC* promoter methylation. Black- and white boxes represent methylated (M)- and unmethylated (U) genes respectively.

B) Quantitative reverse transcription PCR of *MBD1*, *CXXC1*, *SMAD4*, *DCC*, and *MBD2* on paired normal and carcinoma tissues of 10 patients. Figure shows gene expression levels of normal and carcinoma tissues relative to the mean expression level of the normal colon tissues, which is set to equal 1.

These analyses showed that for 13 patients, carcinoma-, adenoma- and normal tissues were synchronously methylated indicating that *DCC* promoter methylation occurs early in CRC development. *DCC* DNA promoter methylation was also studied in age-matched normal colorectal mucosa tissues of 48 non-cancer patients of which 20.8% (10/48) was methylated. *MBD1*, *CXXC1*, *SMAD4* and *MBD2* gene promoters were unmethylated in the vast majority of CRCs (32/33, 27/32, 28/29,31/32 respectively), adenoma (32/33, 33/34, 30/32,31/32 respectively) and normal tissues (31/31, 32/32, 32/32, 31/31 respectively). (Table 1A). Remarkably, in 1 carcinoma *CXXC1*, *SMAD4*, *DCC* and *MBD2* were synchronously methylated.

18q21 copy number change complements DCC promoter methylation

Next we studied how 18q21 deletion and DNA promoter methylation collaborate in transcriptional gene silencing in a second series of normal and adjacent CRC tissues of patients (n=20) of which frozen tissue was available. In this series *DCC* promoter methylation was detected in 75% (15/20) of CRCs (Figure 3A). In the paired normal tissues, only 5% (1/20) was methylated. *MBD1*, *CXXC1*, *SMAD4* and *MBD2* promoters were all unmethylated in these tumors.

Deletion of 18q21 was detected in 40% (8/20) of cancer tissues and in none of the normal tissues. In all detectable 18q21 deletions the *DCC* locus was included and in 7 of these 8 CRCs all probes on 18q showed DNA copy number loss. Thereby, we show that *DCC* is affected by promoter CpG island methylation and/or loss of the *DCC* locus in 90% of CRCs. No specific correlation between DNA promoter methylation and 18q21 copy number loss was observed.

The effect of *DCC* promoter methylation and 18q21 loss on gene expression was studied in 10 of the 20 patient samples for which RNA was available (Figure 3B). In this subset *DCC* promoter methylation was observed for all CRCs, and 1 normal tissue (patients 16).

Although inter-individual differences in gene expression levels were observed, quantitative RT-PCR analyses showed that *DCC* expression was significantly reduced in all CRCs when compared to matching normal tissues. Very low *DCC* transcript levels were detected in both normal and carcinoma tissue of patient 16 which corresponds to the methylated status of both tissues. Apart from tumor T11, in which *DCC* expression was more selectively targeted, most of the carcinoma tissues showed reduced expression of *MBD1*, *CXXC1*, *SMAD4* and *MBD2* as well. In T20, characterized by loss of 18q21, a strong reduction in expression of all genes was observed. However, in contrast to the correlations observed in cell lines, reduced gene expression of *CXXC1*, *SMAD4* and *MBD2* was not restricted to tumors with loss of 18q21, indicating the presence of other transcriptional silencing mechanisms for *MBD1*, *CXXC1*, *SMAD4* and *MBD2* in CRC.

Demethylating agents, but not HDAC inhibitors, restore DCC expression

In order to directly investigate the roles of DNA promoter methylation or histone deacetylation in transcriptional silencing of 18q21 genes, HCT116, RKO and HT29 cells were treated with the DNA methyltransferase inhibitor 5-Aza-2'deoxyctidine (Aza) and the histone deacetylase (HDAC) inhibitor Trichostatin A (TSA). Aza restored *DCC* expression in HCT116, RKO and HT29 cells (Figure 4A). Of the unmethylated genes, *MBD1* showed a modest increase in expression after treatment with Aza, while no change in expression was observed for *CXXC1*, *SMAD4* and *MBD2* (Figure 4B). TSA was unable to reactivate *DCC* or increase expression of *MBD1*, *CXXC1*, *SMAD4* and *MBD2*. These results indicate that promoter CpG island methylation, but not histone deacetylation alone, accounts for transcriptional silencing of *DCC*. *MBD1*, *CXXC1*, *SMAD4* and *MBD2* expression is not dependent upon these processes.

Figure 4: A) Treatment with 5 μ M 5-aza-2'-deoxycytidine (Aza) for 96 hours, but not 300 nM Trichostatin A (TSA) for 18h restores *DCC* expression in HCT116, RKO and HT29 cells. C; control, A; treatment with Aza, T; treatment with TSA. B) Quantitative reverse transcription PCR of *MBD1*, *CXXC1*, *SMAD4* and *MBD2* on HCT116, RKO and HT29 cells treated with Aza and TSA respectively. Gene expression levels in untreated control were set to equal 1. Data represent mean and standard error of mean of 2 or 3 independent experiments.

DCC promoter CpG island methylation is associated with inactive chromatin marks

We then analyzed the presence of inactive (H3K9me3 and H3K27me3) and active (H3K4me3) chromatin marks on *MBD1*, *CXXC1*, *SMAD4*, *DCC* and *MBD2* promoter regions with chromatin immunoprecipitation (ChIP) for cell line HCT116 (18q21 retention) and cell line HT29 (18q21 copy number loss). ChIP in HCT116 demonstrated that the entire *DCC* promoter and adjacent 3' region have enrichment of the repressive histone-tail marks H3K9me3 and H3K27me3, and depletion of the active chromatin mark H3K4me3 (Figure 5A). As a control, HCT116 had enrichment of H3K9me3 and H3K4me3 but not H3K27me3 on the *P16^{INK4A}* promoter which is consistent with a recent report by Kondo *et al.*²⁹ and with the known active and silent alleles in this cell line³⁰. Conversely, the *MBD1*, *CXXC1*, *SMAD4* and *MBD2* promoters have enrichment of the active H3K4me3 mark, but not of the repressive histone-tail marks H3K9me3 and H3K27me3. In cell line HT29, a similar pattern was observed (Figure 4B), with active marks for all genes, except *DCC*, and repressive marks at *DCC*, although the pattern was slightly different with enrichment for only H3K27me3 and not H3K9me3 at *DCC*. Thus, ChIP analyses showed a clear distinction for the *DCC* gene, with the *DCC* promoter surrounded by inactive chromatin, while *MBD1*, *CXXC1*, *SMAD4* and *MBD2*, have enrichment for active histone tail-marks and no signs of epigenetic gene silencing.

Figure 5: Chromatin immunoprecipitation (ChIP) studies in HCT116 (A) and HT29 cells (B) reveal that the *DCC* promoter is enriched with repressive histone-tail marks (H3K9me3 and H3K27me3), while *MBD1*, *MBD2*, *CXXC1* and *SMAD4* gene promoters are associated with the active chromatin mark H3K4me3. Immunoprecipitated DNA was quantified by real-time PCR. Enrichment was calculated as percentage of input DNA. HA was used as a negative control and subtracted from enrichment values. Control PCRs for each antibody immunoprecipitation were performed using primers for *GAPDH* and *P16^{INK4A}* as negative and positive control dependent on the antibody used. Figure shows a representative experiment for two or three ChIPs per antibody.

Discussion

In this study we investigated epigenetic silencing of 5 genes, *MBD1*, *CXXC1*, *SMAD4*, *DCC* and *MBD2*, located on a 4 Mb region on chromosome 18q21; a chromosomal region which becomes frequently lost during colorectal carcinogenesis¹. Although a variety of studies have investigated genetic silencing of genes on this chromosomal region, the contribution of epigenetic modifications is still unclear.

With promoter CpG island methylation analysis we showed for the first time, that the *DCC* promoter is densely methylated in CRC cell lines and primary CRC tissues. *DCC* promoter CpG island methylation has been described in oral squamous cell carcinomas³¹, head and neck squamous cell carcinomas³², breast cancer³³, gastric cancer³⁴ and esophageal squamous cell carcinomas^{35,36}. In CRC, research on inactivation of *DCC* has mainly focused on loss of heterozygosity (LOH) and gene mutation. *DCC* is affected by DNA promoter methylation and/or loss of the *DCC* locus in 90% of CRCs. While 18q21 LOH mainly occurs in advanced lesions³, *DCC* promoter methylation occurs early in CRC development, being present in 80% of adenoma and carcinoma tissues and 29% of normal tissues.

To the contrary *MBD1*, *CXXC1*, *SMAD4*, and *MBD2* promoter CpG island methylation is rare in CRC. However, all five 18q21 genes show reduced gene expression in CRC cell lines and primary tissues compared to normal colon. *CXXC1*, *SMAD4* and *MBD2* reduced expression in cell lines was associated with 18q21 copy number loss, but in primary cancer tissues low mRNA levels were not restricted to CRCs with 18q21 deletion, indicating the presence of other targeting mechanisms. However, none of the genes responded to treatment with histone deacetylase inhibitor Trichostatin A (TSA) and matching DNA promoter methylation results, demethylating agent 5-aza-2'-deoxycytidine (Aza) exclusively restored *DCC* expression in CRC cell lines. ChIP studies revealed that only *DCC* promoter CpG island methylation is associated with repressive histone-tail marks H3K9me3 and H3K27me3 and not with active chromatin mark H3K4me3. The opposite was observed for unmethylated *MBD1*, *CXXC1*, *SMAD4* and *MBD2* promoters which are enriched with active histone-tail mark H3K4me3 and not with H3K9me3 and H3K27me3. Therefore we conclude that promoter CpG island methylation and histone modifications specifically target *DCC* on chromosomal region 18q21, while neighboring genes *MBD1*, *CXXC1*, *SMAD4* and *MBD2* remain unaffected. In general, epigenetic gene inactivation is considered a gene- and tissue specific process³⁷. However, Frigola *et al.* recently showed that epigenetic modifications can cover large chromosomal regions in CRC as well, a mechanism termed long range epigenetic silencing (LRES)³⁸. In this study, genes located on a 4-Mb domain on chromosomal region 2q14 are coordinately epigenetically suppressed by H3K9 methylation, which occurs independently of promoter CpG island methylation. Recently, the same mechanism was observed for chromosomal region 3p22 which flanks the *hMLH1* gene³⁹ and chromosomal region 5q32.2⁴⁰. Here we show for chromosomal region 18q21 that this type of long range epigenetic silencing was not observed in cell lines HCT116 (18q21 retention), and HT29 (18q21 loss). Interestingly, in 1 primary CRC *CXXC1*, *SMAD4*, *DCC* and *MBD2* were synchronously methylated, however this was not observed for the other tumors (n=31) nor in any colorectal cancer cell line.

The observation that only *DCC* is inactivated by promoter CpG island hypermethylation- and H3K9me3 and H3K27me3-mediated epigenetic silencing, while *MBD1*, *CXXC1*, *SMAD4* and *MBD2* are not, raises questions about which underlying mechanism leads to this gene specificity. Recent studies have shown that genes which become *de novo* methylated in cancer are often bivalently marked by both active (H3K4me3) and repressive (H3K27me3) histone marks in human embryonal stem cells^{14,41}. In adult cancer cells H3K4me3 is diminished and TSGs generate a fully repressive chromatin state¹⁴. H3K27me3-marked loci are often occupied by Polycomb group repressive complexes (PRC). Interestingly, in a genome-wide ChIP-ChIP assay which identified target genes of the human Polycomb group protein SUZ12 in human embryonic stem (ES) cells⁴², the *DCC* locus was found enriched for H3K27me3 and SUZ12 in its promoter region, whereas *MBD1*, *CXXC1*, *SMAD4* and *MBD2* were not. As is clear for other cancer associated hypermethylated genes¹⁴, these findings seem to suggest an inherent susceptibility of *DCC* for aberrant methylation during cancer progression.

In summary, this study shows how genetic and epigenetic alterations collaborate in transcriptional silencing of *DCC*. While chromosomal loss of 18q21 frequently involves the entire chromosomal region, promoter CpG island hypermethylation- and H3K9me3 and H3K27me3-mediated epigenetic silencing specifically targets the *DCC* gene. Long range epigenetic gene silencing has been observed on chromosomal region 2q14, 3p22 and 5q32.2, but on 18q21 a rather fine-tuning role for epigenetic gene silencing is observed.

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Supplemental Table 1: Clinicopathologic characteristics of normal, adenoma and carcinoma tissues

		CRC tissues			P value
		normal colon (n=48)	normal (n=61)	Adenoma (n=58)	
age (mean, range)		63.86 (50-87)	71.2 (51-88)	71.52 (51-88)	1.1 x 10 ⁻⁴
sex (%)					
	M	47.9	55.7	53.4	49.3
	F	52.1	44.3	46.6	50.7
localisation (%)					
	proximal	25	43.1	32.7	47.9
	distal	75	56.9	67.3	52.1
Histological type (%)					
	tubular			62.1	
	villous			1.7	
	tubulo-villous			36.2	
Dysplasia (%)					
	low grade			87.9	
	high grade			12.1	
Differentiation (%)					
	well				12
	moderate				76
	poor				12
TNM (%)					
	I				13.3
	II				32
	III				44
	IV				10.7

TNM; Tumor, Node, Metastasis

Supplemental Table 2: Primer locations, sequences and conditions

Experiment	Gene	Temp	Cycles	type	Location	Primer sequence
MSP	MBD1	56	35	MSP_Fup/down	-386 to -228	GTATYGGTTGTGGAGAT GAGGGAAGGGGAGGTGTAGGTGAAG- TTTTTTTGATT
		68	35	MSP_U/as		GAGGGCGTAGGCGTAAGTTTTTTTC- GATC
	CXXC1	68	35	MSP_Ms/as		ATGTACGGAATTAATTATTAAGATGG
		56	35	MSP_Fup/down	-303 to -171	GAATTAATTATTAAGATGGTGGTGT
		62	35	MSP_U/as		ATTATTAAGATGGCGGCGTTC
		62	35	MSP_Ms/as		GYGTTTTTGGATAATTTTTTTGTAA
	SMAD4	56	35	MSP_Fup/down	-296 to +117	TTTGTAAATGAGATGTTAATTTTTTTGGT
		66	35	MSP_U/as		TAACGAGATGTTAATTTTTTCGGC
		66	35	MSP_Ms/as		GTGTAGTATGGTTTTAATTTTAGTTTG- TATAATTGTTGT
		66	35	MSP_U/as		TAGTACGGTTTTAATTTTTTAGTTCGTAT- ATCGTTGGC
bisulfite sequencing	DCC1	56	35	MSP_Fup/down	+28 to +180	TATTTTYGGAGTTTTTTTGTTAG TTTTTGTTTAGTGATTTTTTAAG- TAATT
		68	30	MSP_U/as		TGTTTAGCGGTATTTTAAAGTAATC TGTTTAGCGGTATTTTAAAGTAATC
	DCC2	56	35	MSP_Fup/down	+728 to +841	YGTTGTTYGAGAAGG
		60	35	MSP_U/as		GAAGGTGGAGATAAGATGTTGTTTATAGTG
		66	35	MSP_Ms/as		CGGAGATAAGATGTCGTTTATAGC
		66	35	MSP_Fup/down	+5 to +187	GTAGGYGGAAGTAGTTTTTTTGT
	MBD2	62	40	MSP_Ms/as	-413 to -124	TTATTTATTTGTGAATTTTTTGT
		58	40	Bsseq up/down	-342 to -57	TTGAGGTTAGGTTTAGGTTTAGAT
		61	40	Bsseq up/down	-208 to +108	TTTATATTTTTTTTTTAGTTTTTTATT
		56	40	Bsseq up/down	-72 to +217	GTAAGTGGTTTTTTTTTTTTTTT
bisulfite sequencing	DCC1	60	40	Bsseq up/down	+677 to +952	TATGATATTTTTTAGTTGTTGGTTAGTT
		64	40	Bsseq up/down	-79 to +226	
	DCC2	56	35	MSP_Fup/down	+728 to +841	CAAAATAAAATAAAAAATAAAATATAC
		60	35	MSP_U/as		AAAAATAAAATAAAAAATAAAATACAA
		66	35	MSP_Ms/as		AAATAAAATACGAAAAATAATACGACG
		66	35	MSP_Fup/down	+5 to +187	RAACCTTAAATCCCAAACCC
	MBD2	62	40	MSP_Ms/as	-413 to -124	CACAAAAACACCTCTCTACCCAAACA
		58	40	Bsseq up/down	-342 to -57	GACCTCTACCCGAAACG
		61	40	Bsseq up/down	-208 to +108	TACCTTTAATAAAAAATCTCC
		56	40	Bsseq up/down	-72 to +217	CTCTAATATACTTTCCRAAAC
bisulfite sequencing	DCC1	56	40	Bsseq up/down	-72 to +217	AAAAACAAAAAACCTCTCC
		60	40	Bsseq up/down	+677 to +952	AAAAATAAAATAAAAAATAAAATATAC
	DCC2	56	35	MSP_Fup/down	+728 to +841	CAAAATAAAATAAAAAATAAAATATAC
		60	35	MSP_U/as		AAAAATAAAATAAAAAATAAAATACAA
		66	35	MSP_Ms/as		AAATAAAATACGAAAAATAATACGACG
		66	35	MSP_Fup/down	+5 to +187	RAACCTTAAATCCCAAACCC
	MBD2	62	40	MSP_Ms/as	-413 to -124	CACAAAAACACCTCTCTACCCAAACA
		58	40	Bsseq up/down	-342 to -57	GACCTCTACCCGAAACG
		61	40	Bsseq up/down	-208 to +108	TACCTTTAATAAAAAATCTCC
		56	40	Bsseq up/down	-72 to +217	CTCTAATATACTTTCCRAAAC

Experiment	Gene	Temp	Cycles	type	Location	Primer sequence	
qRT-PCR	MBD1	60	40	RT_PCR	exon 2-3	GGACGCTCAGACACCTATTACCA	GTTTGAAGTCGAAGAGGTGAGAT
	CXXC1	60	40	RT_PCR	exon 7-8	CTACAGCCACACCTGAGCCA	TTCTGTGTGCTCATCCAGG
	SMAD4	60	40	RT_PCR	exon 12-13	GGAAACATCCCTGGCCC	AACTCATCCTGAGTATGCA TAAGC
	DCC	60	40	RT_PCR	exon 2-3	GGGACTTTTACCAATGTGAGGCA	TTCTGTCTGTGAAAGAACCTCAGT
ChIP	MBD2	60	40	RT_PCR	exon 7-8	AACCCTGCTGTGGCTTAAC	CCATCAGTGCTTCTTCCAATTT
	GAPDH	60	40	ChIP	-43 to +52	CGCCCCGGTTTCTATAAAT	CCTGGCGACGCAAAAGAAG
	P16 ^{INK4A}	60	40	ChIP	-388 to -280	ACCCCGATTCAATTTGGCAG	AAAAAGAAATCCGCCCCCG
	MBD1_1	60	40	ChIP	-484 to -383	GGGACCCACGAACACAGATA	AACCGGCAAGGAAGCTG
	MBD1_2	60	40	ChIP	-413 to -289	GCAGGCGGAAGCAGC	ATGGCTTTCCAGCCTCAA
	MBD1_3	60	40	ChIP	-74 to +6	AGAGGCAGGCAGCGGACTT	CGGCGCCTCCTCTGAA
	CXXC1_1	60	40	ChIP	-518 to -390	GAGGAGTAAAGCCGGAAGAGT	AGTCTCTTTCCCTTCCACATGT
	CXXC1_2	60	40	ChIP	-420 to -313	CATTAAATACATGTGAAGGGGAAA	TCAGGCAGAGAGGTTCACAGGTA
	CXXC1_3	60	40	ChIP	-218 to -104	AATTCGACCCACGTACGGAAT	CGTACGGCGCTGCTTG
	SMAD4_1	60	40	ChIP	-520 to -425	GGCTGCCAGAAAGAGAAGGAA	AGTCGACAGCTCTGGGGAGAA
	SMAD4_2	60	40	ChIP	-468 to -312	GAACTCCTACAACCTAGCCACCTT	GAGTGGCGTCCAGTAAGTGTT
	SMAD4_3	60	40	ChIP	-206 to -48	CCAGGCCAGGTCCAGATT	TCCGGGTAATTTACGGGTTTG
	DCC_1	60	40	ChIP	-597 to -497	GAAGAGAGAACACACGACATGAGA	ACACACGCGGACAGGACAAG
	DCC_2	60	40	ChIP	-469 to -372	GAAGGGAGTTGGATCAGGTGATT	GGAGTGGCCAGGATGTTTAC
	DCC_3	60	40	ChIP	-308 to -186	CAACACGCTCGCTGCAAA	TTGCCTGGTCTCGGTATCAAT
	DCC_4	60	40	ChIP	-153 to -46	TTTTTCCCTAAGCCATAGTCTGTCT	CGTACTACACATTGATCCGGAGAA
	DCC_5	60	40	ChIP	-63 to +43	CCCTTCAGCCCTCCACTT	GGGGTGGAGCGCTCTCACT
	DCC_6	60	40	ChIP	-6 to +110	GCGGACACCCCACTAACCAAGT	TGGAGATGCTGTGGTTTCTT
	DCC_7	60	40	ChIP	-78 to +174	GGCTCTCGGAAGAAAAACCAA	GCATTAAAGGTGAGGCTGTT
	DCC_8	60	40	ChIP	-222 to +374	CTCCTGGATGTGGTTTATTGATGA	GCAGGCAGCAAAAGTTCCA
	DCC_9	60	40	ChIP	-228 to +467	GGGCTCGGGATCTCTTGGG	TCGTCTCTTCTCCACCTCTT
	MBD2_1	60	40	ChIP	-548 to -434	TAGGCTGGACTTTCAACATAATGG	TAGAATGACCAGAAATCCCTAAATCC
	MBD2_2	60	40	ChIP	-359 to -264	AAGGTTCCAGGAGAACTGTCTCAAT	ATAATGAAGGAAATGAAGCAAGTGTC
	MBD2_3	60	40	ChIP	-224 to -114	GGCTATCACCCCTTAAATGAAT	TCACCATTATGTGATTTACCTATGT

CHAPTER 8|

Gene silencing through promoter CpG island methylation and c-Myc overexpression in colorectal cancer: is there a connection?

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To be submitted

Abstract

Promoter CpG island methylation is a common epigenetic modification in colorectal cancer (CRC) and is known to be a nonrandom, tissue specific process. The exact mechanism behind the generation of gene specific promoter CpG island methylation patterns is not clear. A role for transcription factor c-Myc has been proposed since c-Myc has been shown to recruit DNA methyltransferases (DNMTs) to target promoter *CDKN1A* and thereby induces DNA promoter methylation¹. Although proto-oncogene *c-MYC* is over-expressed in >70% of CRCs and amplification of chromosomal region 8q23-qter, which harbors *c-MYC*, is associated with a high number of methylated genes², c-Myc induced target gene promoter CpG island methylation has not been described in CRC. Therefore, this study aims to identify genes transcriptionally silenced by c-Myc induced promoter methylation in CRC.

In a previous *c-MYC* transfection study, genes repressed by c-Myc overexpression and re-expressed after treatment with demethylation agent 5-aza-2'-deoxycytidine (Aza), were identified among which *CXCL14*, *DNAJA4*, *DKK3*, *TGFβi* and *TRIM59*. Promoter CpG island methylation analysis showed that all 5 of these genes were methylated in CRC cell lines. Quantitative reverse transcription PCR showed an inverse relation to the presence of DNA promoter methylation. However, no relationship between promoter CpG island methylation and high c-Myc transcript levels was observed. Although chromatin immunoprecipitation (ChIP) showed the presence of c-Myc on the promoter region of *DNAJA4*, *TGFβi* and *TRIM59*, this was associated with gene transcription and enrichment of active chromatin mark H3K4me3.

In conclusion, we were indeed able to find genes that were repressed by promoter CpG island methylation in conditions of c-Myc overexpression, however, the presence of c-Myc and promoter CpG islands methylation could not be observed concomitantly. This suggests that c-MYC might be important for the establishment, but not the maintenance of CpG island methylation.

Introduction

DNA promoter methylation is a common epigenetic modification in colorectal cancer (CRC) and is known to be associated with transcriptional silencing of DNA repair- and tumor suppressor genes³. The occurrence of DNA methylation in the genome is a nonrandom, tissue specific process⁴. The exact mechanism behind the generation of these gene specific DNA methylation patterns is not clear at this time. Several explanations for gene specific promoter methylation have been proposed.

One proposed mechanism involves local DNA sequence features contributing to the susceptibility of CpG islands to become methylated^{5,6}. Another mechanism is provided by Polycomb group proteins which premark genes in progenitor- or stem cell state by targeting H3K27 histone methylation predisposing to de novo promoter CpG island methylation during cancer development⁷. A recent study demonstrated that genes with a methylation-prone sequence motif and genes characterized by Polycomb group protein occupancy in embryonic stem cells are strongly related⁸.

An alternative hypothesis suggests that transcription factors or other DNA binding proteins recruit DNA methyltransferase enzymes (DNMTs) to specific loci. DNMTs themselves have little sequence specificity and therefore require sequence-specific binding proteins, such as transcription factors or gene repressors, to target specific promoters⁹. This mechanism has been shown for the oncogenic transcription factor PML-RAR¹⁰ and has been proposed for the proto-oncogene *c-MYC* (hereafter referred to as *MYC*) as well¹.

MYC encodes a transcription factor which is overexpressed in a wide range of human malignancies¹¹. In 70% of colorectal cancers, Myc is overexpressed at least in part by upregulated WNT signaling, but also by amplification of its chromosomal region 8q23-qter¹². The oncogenic potential of Myc results from the ability to both activate and repress transcription of genes regulating cell growth and proliferation^{13,14}.

Transcriptional activation by Myc occurs via recruitment of chromatin modifying complexes¹⁵. Myc is capable of binding specific DNA sequences called Enhancer Box sequences (E-boxes)¹⁶ and recruiting co-activator complexes containing TRRAP and histone acetyltransferases (HATs)¹⁷ leading to a transcriptional permissive state.

Transcriptional repression by Myc is less well understood, but is thought to involve interference with positively acting transcription factors. For example, when Myc binds transcriptional activator Miz-1, as has been shown for genes as *CDKN1A*¹⁸, *CDKN2B*¹⁹ and *MAD4*²⁰, the binding to its own co-activator is blocked and thereby transcriptional activation inhibited¹⁹. The Myc-Myc box III (MBIII) element, which regulates protein stability, is important for gene repression of target gene *CDKN1A*, by its ability to recruit histone deacetylases (HDACs) to chromatin²¹.

Besides the ability of Myc to interact with HATs and HDACs, an alternative mechanism proposed by Brenner *et al.* involves recruitment of DNMTs by Myc to target promoter *CDKN1A*, which results in DNA methylation within the locus¹. *CDKN1A* promoter CpG island methylation, however, has not been reported to occur frequently in CRC²² or in other human tumors. Although we recently reported an association between amplification of chromosomal region 8q23-qter and a significantly high number of methylated genes in CRCs², until now there are no additional reports on Myc mediated promoter

CpG island methylation in CRCs. Therefore the aim of this study is to identify genes targeted by Myc induced promoter CpG island methylation in CRC.

Method

Cell lines, Aza and TSA treatment and primary cancer tissues

CRC cell lines HCT116, LOVO, RKO, SW480, HT29, LS174T and CACO2 were cultured in DMEM (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (Hyclone). To investigate re-expression of putative Myc target genes following inhibition of DNA methyltransferases, HCT116, RKO and HT29 cells were treated with 5 μ M 5-aza-2'-deoxycytidine (Aza) (Sigma) or 300 nM Trichostatin A (Sigma) for 18 hours respectively. Both media and the drug were replaced every 24 hrs.

Promoter CpG island methylation and expression analyses were performed on a series of fresh frozen CRC tissues (n=25) and adjacent normal colon mucosa of patients over 50 years of age, 15 males and 10 females (mean age 71.9, range 51-87, at time of diagnosis) which were retrospectively collected from the tissue archive of the department of Pathology of the Maastricht University Medical Center. This study was approved by the Medical Ethical Committee (MEC) of the Maastricht University Medical Center.

DNA isolation and bisulfite treatment

A 5 μ m section of each tissue block was stained with haematoxylin and eosin and revised by a pathologist (AdB). DNA was extracted using the Puregene® DNA isolation kit (Gentra systems) according to the manufacturer's instructions. Sodium bisulfite modification of 500 ng genomic DNA was performed using the EZ DNA methylation kit (ZYMO research Co, Orange, CA) according to the manufacturer's instructions.

Quantitative reverse transcription PCR

Total RNA was isolated using the RNeasy Mini kit (Qiagen) following the manufacturer's instructions. Possible genomic DNA contaminations were removed by DNase treatment with the RNase-free DNase set (Qiagen). cDNA synthesis was performed using the Iscript cDNA synthesis kit (Bio-Rad). Quantitative reverse transcription PCR was performed as described previously²³ using SYBR Green PCR master mix (Applied Biosystems, Nieuwekerk a/d IJssel, The Netherlands). *MYC*, *CXCL14*, *DNAJA4*, *DKK3*, *TGF β i* and *TRIM59* expression levels were normalized to *CYCLOPHILIN* and the average expression in a pool of normal colon tissues, for each tissue sample using the following equation: relative expression = $2^{-(\Delta Ct)}$, where ΔCt = average Ct (tissue sample) - average Ct (*CYCLOPHILIN*) - average CT (average normal colon tissues). As a positive control for gene expression following inhibition of DNA methyltransferases re-expression of *GATA4* and *GATA5* was determined. As a positive control for gene expression following treatment with Trichostatin A (TSA), previously described control gene *FABP4*^{24,25} was analyzed.

Promoter CpG island methylation analyses: sodium bisulfite sequencing and methylation-specific PCR (MSP)

For sequencing of sodium bisulfite-converted DNA, PCR products were amplified and cloned using the TOPO-TA cloning kit (Invitrogen, Breda, the Netherlands). Single colonies were sequenced using an automated sequencer (Applied Biosystems, Foster City, CA).

MSP analysis on bisulfite treated DNA were performed as described in detail elsewhere^{26,27}. All primer sequences and conditions are available upon request.

Western Blot

Cytoplasmic and nuclear fractions were prepared for Western blot analysis by using the NE-PER Nuclear and Cytoplasmic Extraction kit (Pierce, Rockford, IL). For each cell line, 10 µg of protein was mixed with 4X loading dye, reducing reagent (Invitrogen Corporation, Carlsbad, CA) boiled for 10 minutes and loaded onto a 4-12% gradient bis-acrylamide gels (Invitrogen Corporation, Carlsbad, CA). The gel was transferred onto a nitrocellulose membrane which was then incubated overnight in blocking solution (5% non-fat dry milk in PBS/0.1% Tween 20). Primary antibody against Myc (N262) was purchased from SantaCruz biotechnology (CA, USA). β-actin antibody (1/10000; catalogue number: A5441 (Sigma-Aldrich, St. Louis, MO)) was used as a loading control. Rabbit secondary (1/30000) and anti mouse (1/10000) was from Amersham Biosciences, Pittsburgh, PA. ECL or femto ECL (Pierce Biotechnology, Inc. Rockford, IL) was used as a detection reagent.

Chromatin immunoprecipitation (ChIP) assays

ChIPs were performed on HCT116, RKO and LS174T cells and analyzed essentially as described previously²⁸. Briefly, HCT116, RKO and LS174T cells were fixed in 1% formaldehyde. Samples were immunoprecipitated with antibodies for either HA as a negative control (sc-805; Santa Cruz), Myc (N-262, sc-764; Santa Cruz), H3K9me3 (07-442;Upstate), H3K27me3 (07-449; Upstate), and H3K4me3 (ab8580; Abcam). Immunoprecipitated DNA was quantified by real-time PCR. Enrichment was calculated as percentage of input DNA. HA was used as negative control and subtracted from enrichment values. For all genes primers were designed for the region -400 to transcription start site (TSS). Control PCRs for each antibody immunoprecipitation were done using primers for *GAPDH*, *P16^{INK4A}* and *CDKN1A* as negative and positive control dependent on the antibody used.

Data analysis

We used the Pearson's χ^2 to compare categorical data. Data analysis was done using SPSS software (version 15).

Results

Identification of Myc induced promoter CpG island methylated target genes

In a previous study, we compared the transcriptome of the small cell lung cancer (SCLC) cell line H209, which expresses low to undetectable levels of Myc mRNA, and *MYC* transfected cells (209^{MYC}) in order to identify genes downregulated by Myc overexpression (Figure 1) (Licchesi *et al*, in preparation). Genes downregulated by a process involving DNA promoter methylation, were identified by treating 209^{MYC} cells with demethylating agent 5-aza-2'-deoxycytidine (209^{MYC+Aza}). Genes which showed increased gene expression were selected for further investigation. By this method, 326 genes were identified as being regulated by Myc and Aza, with these genes having a greater than 2 fold change downregulation in H209^{MYC}/H209 as well as greater than 2 fold change upregulation in H209^{MYC+Aza}/H209^{MYC}. A CpG island screen (GC content > 60%, ratio of observed CpG / expected CpG > 0.6 and minimum length 200 bp²⁹) showed that around 166/326 genes were found to have a CpG island within a 2kb region of the promoter. Three of these putative Myc target genes, *DnaJ* (*Hsp40*) *homolog, subfamily A, member 4* (*DNAJA4*), *transforming growth factor beta induced* (*TGFβi*) and *tripartite motif-containing 59* (*TRIM59*), showed the largest expression differences. Promoter CpG island methylation of *DNAJA4*, *TGFβi* and *TRIM59* was confirmed in sodium bisulfite sequencing and MSP analyses, indicating a gene transcriptional repressive mechanism involving Myc as well as promoter CpG island methylation (Licchesi *et al*, in preparation).

Figure 1: Experimental flow chart of identification of Myc repressed and promoter CpG island methylated genes in CRC.

In the present study we investigate whether *DNAJA4*, *TGFβi* and *TRIM59* are methylated in CRC as well. In order to identify additional putative Myc target genes which are involved in CRC, the H209^{MYC} hypermethylome was compared to the CRC hypermethylome, i.e. genes identified as being transcriptionally silenced in CRC and re-expressed after by treatment with Aza and not TSA in a genome-wide array-based expression study³⁰.

By this approach we identified *DNAJA4* and 52 additional putative Myc targeted- and hypermethylated genes in CRC. Genes which are re-expressed in more than 1 out of 6 CRC cell lines³⁰ (n=27/53) were considered to play a significant role in CRC. Based on known gene function or reported involvement in cancer, 5 genes, *B3GALT4*, *CLIC6*, *CXCL14*, *DKK3* and *KRM1* were selected for further analysis. An initial screen by MSP and quantitative reverse transcription PCR, showed that only 2 genes, *Chemokine (C-X-C motif) ligand 14 (CXCL14)* and *Dickkopf homologue 3 (DKK3)* display cancer specific promoter CpG island methylation and transcriptional repression (data not shown). Therefore, *B3GALT4*, *CLIC6* and *KRM1* were not further analyzed.

Putative Myc target genes show promoter CpG island methylation and transcriptional silencing in CRC cell lines

In order to verify putative Myc target gene promoter CpG island methylation in CRC, *CXCL14*, *DNAJA4*, *DKK3*, *TGFβi* and *TRIM59* promoter methylation was analyzed with methylation-specific PCR (MSP) in CRC cell lines HCT116, LOVO, RKO, SW480, HT29, LS174T and CACO2.

These analyses showed that *CXCL14*, *DNAJA4*, *DKK3*, *TGFβi* and *TRIM59* genes were indeed methylated in 6/7, 2/7, 3/7, 3/7 and 4/7 CRC cell lines respectively (Figure 2A). All genes were methylated in RKO cells, while none of the genes was methylated in LS174T cells.

These results were confirmed by sodium bisulfite sequencing of individual clones of CRC cell lines HCT116, RKO and LS174T (Figure 3). Since *DNAJA4* had monoallelic methylation in RKO cells, unmethylated as well as methylated strains were detected.

When we correlated promoter CpG island methylation status to gene expression, strong inverse relationships between DNA methylation and gene expression was observed for all of the genes, with the exception of cell line LS174T in which *CXCL14*, *DKK3* and *TGFβi* promoters were unmethylated and showed low to undetectable mRNA expression levels (Figure 2B).

Figure 2: A) Methylation-specific PCR analyses of *CXCL14*, *DNAJA4*, *DKK3*, *TGFβi* and *TRIM59* on CRC cell lines. Black box indicates methylated gene, white box indicates unmethylated gene, grey box indicates methylated- and unmethylated allele. B) Quantitative reverse transcription PCR of *CXCL14*, *DNAJA4*, *DKK3*, *TGFβi* and *TRIM59* on CRC cell lines. Gene expression levels are relative to the mean expression level of normal colon tissues (n=10), which is set to equal 1. Data represent mean and standard error of mean of 2 or 3 independent experiments.

Figure 3: Sodium bisulfite sequencing results of *CXCL14*, *DNAJA4*, *DKK3*, *TGFβi* and *TRIM59* gene promoters on cell lines HCT116, RKO and LS174T. Each row represents an individually cloned allele and each square indicates a CpG dinucleotide. Black square; methylated CpG site, white square; unmethylated CpG site, grey square; not determined. Due to technical difficulties, only a smaller region of the *DKK3* gene was sequenced for HCT116 cells. Line above sequencing result indicates region amplified by MSP.

Compared to expression levels in normal colon tissues, which was set to a level of 1, *CXCL14*, *DNAJA4* and *DKK3* expression levels were lower in CRC cell lines. To the contrary, *TGFβi* and *TRIM59* expression levels were, when detectable, comparable or higher to expression levels in normal colon. Together these results demonstrate that, except from cell line LS174T, promoter CpG island methylation of putative Myc target genes correlates to gene repression.

Demethylating agents, but not HDAC inhibitors, restore Myc target gene expression

In order to directly investigate the role of DNA promoter methylation on transcriptional silencing of putative Myc target genes, HCT116, RKO and HT29 cells were treated with demethylation agent 5-aza-2'-deoxycytidine (Aza). The role of histone deacetylation, which has been related to Myc induced transcriptional silencing previously²¹, was studied by treating cells with histone deacetylase (HDAC) inhibitor Trichostatin A (TSA). These analyses showed that expression of *CXCL14*, *DKK3* and *TRIM59* was restored in Aza-treated HCT116, HT29 and RKO cells when expression was absent in untreated conditions (Figure 4). The same was observed for *TGFβi* in HCT116 but not in RKO cells. *DNAJA4* increased gene expression was observed for all three cell lines, which was most profound in RKO and HT29 cells which showed monoallelic DNA promoter methylation. Treatment with TSA alone had no effect on gene expression which indicates that transcriptional silencing of the putative Myc target genes involves promoter CpG island methylation and is not primarily dependent on histone deacetylation.

Figure 4: Gene expression levels after treatment with 5 μ M 5-aza-2'-deoxycytidine (Aza) for 96 hours or 300 nM Trichostatin A (TSA) for 18h in HCT116, RKO and HT29 cells. C; control, A; treatment with Aza, T; treatment with TSA.

Putative Myc target gene promoter CpG island methylation is not restricted to high Myc expressing CRC cell lines

Next we investigated the association between Myc expression levels and *CXCL14*, *DNAJA4*, *DKK3*, *TGFβi* and *TRIM59* promoter CpG island methylation in CRCs.

We first studied this association in CRC cell lines. Myc expression was analyzed by reverse transcription reverse transcription PCR which showed that cell lines HCT116 and RKO showed up to 4-5 fold higher Myc mRNA expression levels compared to normal colon tissue (Figure 5A). LS174T cells showed a 3 times higher Myc expression, while HT29, LOVO, SW480 and CACO2 Myc expression levels were comparable to normal colon tissues.

Figure 5: Quantitative reverse transcription PCR of Myc on CRC cell lines. Gene expression levels are relative to the mean expression level of the normal colon tissues (n=17), which is set to equal 1. Data represent mean and standard error of mean of 2 or 3 independent experiments. B) Western blot analysis of Myc expression in CRC cell lines. β-actin was used as a loading control.

In order to study the relationship between mRNA and protein expression levels, a Western blot expression analysis was performed which showed high Myc expression levels for HCT116, LOVO, RKO, and SW480, while HT29 and CACO2 showed lower Myc expression levels (Figure 5B).

Next we correlated Myc expression to promoter CpG island methylation. High Myc expressing HCT116 and RKO cells, demonstrated the highest number of methylated genes of 3/5 and 5/5 (Figure 1A) respectively. However, also in lower Myc expressing cell lines HT29 and CACO2, in 2/5 and 4/5 putative Myc target genes were methylated respectively. These results indicate that *CXCL14*, *DNAJA4*, *DKK3*, *TGFβi* and *TRIM59* promoter CpG island methylation is not restricted to high Myc expressing CRC cell lines.

Myc overexpression and association with gene promoter methylation and mRNA expression in CRC tissues

Next we studied the promoter CpG island methylation status of *CXCL14*, *DNAJA4*, *DKK3*, *TGFβi* and *TRIM59* in a series of 25 patients of which normal and tumor tissue was available. These analyses showed that *TGFβi* and *TRIM59* promoter are unmethylated in this series (Table 1A). To the contrary, *CXCL14* was methylated in 54.2% (13/24) of CRCs and none of the normal colon mucosa adjacent to carcinoma tissues. *DKK3* promoter CpG island methylation was observed in 33.3% (8/24) of carcinoma tissues while absent in adjacent normal colon tissue. *DNAJA4* promoter methylation was observed in 8.3% (2/24) of normal tissues and only 12.5% (3/24) of carcinomas tissues.

Table 1A: Promoter CpG island methylation in primary CRC tissue

genes	N	methylation		expression		
			T	-	+	=
<i>CXCL14</i>	0/25 (0%)		13/24 (54.2%)	5/10	4/10	1/10
<i>DNAJA4</i>	2/24 (8.3%)		3/24 (12.5%)	7/10	1/10	2/10
<i>DKK3</i>	0/25 (0%)		8/24 (33.3%)	6/10	4/10	0/10
<i>TGFβi</i>	0/25 (0%)		0/24 (0%)	3/10	7/10	0/10
<i>TRIM59</i>	0/25 (0%)		0/25 (0%)	3/10	7/10	0/10

N: normal colon tissue, T; carcinoma tissue. -, expression N>T, +; expression N<T, =; expression N=T

Myc mRNA expression levels were assessed in 17 patients, which showed increased Myc mRNA levels in carcinoma tissues compared to normal tissue for 11/17 (64.7%) patients and a decrease in expression for 3/17 patients.

When we correlated Myc expression levels, which were classified as increased (+), decreased (-) or no difference in (=) expression in carcinoma relative to adjacent normal tissue, to promoter CpG island methylation status of the putative target genes, no significant association was observed (Table 1B).

CXCL14, *DNAJA4*, *DKK3*, *TGFβi* and *TRIM59* expression levels were assessed in carcinomas and adjacent normal tissue of 10 patients as well. *CXCL14*, *DNAJA4*, *DKK3* expression levels were most frequently reduced in carcinoma tissues compared to normal tissue (5/10, 7/10 and 6/10 patients respectively) (Table 1A). Increased gene expression was observed in 4/10, 1/10 and 4/10 patients respectively. For *TGFβi* and *TRIM59* the opposite was observed, both *TGFβi* and *TRIM59* showed increased expression in 7/10 CRCs and a reduction in only 3/10 CRCs.

No clear association between Myc overexpression and target gene expression patterns was observed.

Presence of Myc on target gene promoters and association to chromatin marks

In order to investigate if Myc binding to target promoters and promoter CpG island methylation occurs concomitantly, Myc occupancy at putative target gene promoters was studied by ChIP in CRC cell lines HCT116, RKO and LS174T. The association between Myc enrichment and/or promoter methylation and inactive histone marks H3K9me3 and/or H3K27me3 and active chromatin mark H3K4me3 were studied as well.

Since the putative Myc target gene promoters did not have an E-box in the promoter regions (which accounted for 52 of the 53 putative Myc target genes, P-match analysis for transcription factor binding places, data not shown), primers were designed for the region -500 to -100 bp relative to the transcription start site (TSS).

Table 1B: Promoter CpG island methylation in primary CRC tissue in relation to Myc expression

patient	Myc expr (+/-)	<i>CXCL14</i> methylation		<i>DNAJA4</i> methylation		<i>DKK3</i> methylation	
		N	T	N	T	N	T
1	+						
2	+						
3	+						
4	+						
5	+						
6	+						
7	+						
8	+						
9	+						
10	+						
11	+						
12	=						
13	=						
14	=						
15	-						
16	-						
17	-						

N: normal colon tissue, T; carcinoma tissue. Black indicates methylated gene, white indicates unmethylated gene.

-; expression N>T, +; expression N<T, =; expression N=T

First we correlated target gene expression status to inactive- (H3K9me3 and H3K27me3) and active (H3K4me3) chromatin marks and observed that active gene promoters were enriched for active chromatin mark H3K4me3 in HCT116 and LS174T cells (Figure 6). In RKO cells, all genes except *DNAJA4* are transcriptionally repressed, but for *DNAJA4*, no H3K4me3 and only low H3K9me3 enrichments were observed. Inactive gene promoters were enriched for inactive histone marks H3K9me3 and/or H3K27me3 in all cell lines. *TRIM59* promoter was enriched for both active H3K4me3 and inactive chromatin marks H3K9me3 in HCT116 and LS174T cells, also referred to as a bivalent chromatin state, which was associated with transcriptional activation. Thus, we showed a clear association of transcriptional activity and active chromatin marks and transcriptional inactivity and inactive chromatin marks. In this study, promoter CpG island methylation showed a clear positive correlation with transcriptional repression. However, in LS174T cells, *CXCL14* and *DKK3* were transcriptionally inactive in the absence of promoter CpG island methylation. ChIP analysis, however, showed enrichment of inactive histone marks H3K9me3 and H3K27me3 on *CXCL14* and *DKK3* gene promoters in LS174T cells, providing an explanation for transcriptional repression.

Figure 6: Chromatin immunoprecipitation (ChIP) studies in HCT116, RKO and LS174T cells using antibodies to Myc, repressive histone-tail marks H3K9me3 and H3K27me3 and active chromatin mark H3K4me3. Immunoprecipitated DNA was quantified by real-time PCR. Enrichment was calculated as percentage of input DNA. HA was used as a negative control and subtracted from enrichment values. ChIP results were compared to gene expression and promoter CpG island methylation results. Black box indicates expression or methylated gene. White box indicates repressed or unmethylated gene. Grey box indicates low gene expression or monoallelic gene methylation.

Next we studied the presence of Myc on the target gene promoters, which was observed for all 3 cell lines on positive control *CDKN1A*. Myc enrichment was observed on the promoter of *TRIM59* in HCT116 and LS174T cells. Although less profound, Myc was observed on *DNAJA4* and *TGFBI* promoters as well. Remarkably, Myc enrichment was not profoundly found at gene promoter sites when these genes were transcriptionally inactive and methylated, but rather associated with the presence of active chromatin mark H3K4me3. Also, on methylated control gene *WIF1*, which has previously been shown to be a Myc target gene (Licchesi *et al*, in preparation), no Myc enrichment was observed (data not shown).

Thus we show that in cell lines, promoter CpG island methylation of the *CXCL14*, *DNAJA4*, *DKK3*, *TGFBI* and *TRIM59* correlated to inactive histone marks, while unmethylated promoters correlated to active chromatin mark H3K4me3. While enrichment of Myc was observed on its target gene promo-

ters, this was mostly observed when the target genes were unmethylated and transcriptionally active.

Discussion

Since the publication of Brenner *et al.*¹, a role for Myc in the induction of promoter CpG island methylation has been hypothesized. As Myc overexpression and promoter CpG island methylation occur frequently in CRC, this study set out to identify genes repressed by Myc induced promoter methylation in CRCs.

In a previous study, genes repressed by a mechanism involving Myc and promoter CpG island methylation were identified in a small cell lung carcinoma (SCLC) cell line H209 (Licchesi *et al.*, in preparation). Based on these data we analyzed promoter CpG island methylation of putative Myc target genes *CXCL14*, *DNAJA4*, *DKK3*, *TGFβi* and *TRIM59* in CRCs.

With sodium bisulfite sequencing and MSP we showed that *CXCL14*, *DNAJA4*, *DKK3*, *TGFβi* and *TRIM59* are methylated in CRC cell lines as well. *CXCL14*, *DNAJA4* and *DKK3* promoter CpG island methylation was also observed in primary CRC tissues, of which *CXCL14* and *DKK3* showed the most cancer specific DNA hypermethylation.

CXCL14 is a chemokine that controls leukocyte migration³¹⁻³³ and angiogenesis³³. Loss of *CXCL14* expression in tumor cells correlates with a reduced capacity of immature dendritic cells to infiltrate³⁴ and thereby allows tumor cells to escape from normal immune restriction. It is known that *CXCL14* expression is frequently lost in a wide range of epithelial malignancies^{32,35}, but the underlying mechanism was not yet clear. Here we identified promoter CpG island methylation as silencing mechanism which occurs in 55% of CRCs while no methylation was observed in normal colon tissues.

In addition to *CXCL14*, WNT antagonist *Dickkopf homologue 3*, *DKK3*, was identified as putative Myc repressed and methylated target gene. *DKK3* promoter CpG island methylation has been described previously in gastrointestinal malignancies including colorectal cancer (CRC)^{36,37}. Interestingly, Myc induced repression of WNT antagonists, such as *DKK1* and *SFRP1*, has been demonstrated in breast cancer and is hypothesized to provide a positive regulatory loop³⁸. The WNT antagonists *DKK1*, *DKK3*, *SFRP1* and *WIF1* are frequently silenced by promoter CpG island methylation in multiple diseases among which CRC^{37,39}, but the role of Myc in these DNA methylation processes is not clear.

In addition to *CXCL14* and *DKK3*, *DNAJA4*, *TGFβi* and *TRIM59* were identified as putative Myc target genes. Although the physiological function of *DNAJA4* and *TRIM59* remains to be elucidated, *TGFβi* is a downstream target of *TGF-β*-signaling and is involved in cell matrix interactions and cell migration⁴⁰. Myc and *TGF-β*-signaling are linked and function in an antagonistic way, as *TGFβ* is able to downregulate Myc expression and prevent its downstream effects⁴¹ and Myc can repress *TGFβ* induced genes *CDKN2A* and *CDKN2B*. Here we report another *TGFβ* induced gene, *TGFβi*, putatively repressed by Myc. Although *TGFβi* promoter CpG island methylation has been described in lung and prostate cancer previously⁴², in primary CRC tissues *TGFβi* promoter CpG island methylation was not observed and rather increased *TGFβi* expression levels were associated with CRC development. Since it was recently re-

ported that high *TGFβ1* expression levels enhances metastatic potential of CRC cells, the role of *TGFβ1* in CRC is controversial⁴³.

Furthermore, although Myc function might be independent of Myc expression levels, no association between Myc overexpression and promoter CpG island methylation of *CXCL14*, *DNAJA4*, *DKK3*, *TGFβ1* and *TRIM59* was observed in CRC cell lines and primary CRC tissues. With ChIP we were able to demonstrate Myc enrichment on target genes *DNAJA4*, *TGFβ1* and *TRIM59*. However, Myc enrichment was mostly observed in combination with active histone marks H3K4me3 and transcriptional activation, and showed an inverse correlation to promoter CpG island methylation. Myc enrichment was generally not observed when the promoters of *TGFβ1* and *TRIM59* were methylated, which correlated to enrichment of inactive marks H3K9me3 and/or H3K27me3. This was a rather unexpected finding, since *CXCL14*, *DNAJA4*, *DKK3*, *TGFβ1* and *TRIM59* were selected for their repressive state after Myc overexpression. While lung and colon cells might respond differently to Myc overexpression and these analyses need to be performed in a CRC cell line model as well, one might reconsider the involvement of Myc in DNA methylation processes. Also, genome-wide ChIP studies have found extensive Myc occupancy on promoters enriched for active chromatin marks⁴⁴, of which the strongest correlation was with H3K4me3⁴⁵. Myc occupancy showed an inverse relationship to H3K27me3. When Myc and H3K27me3 were enriched on the same promoters, which occurred in a minority of sites, this was in the presence of H3K4me3⁴⁵. Cells depleted for Myc exhibit global histone changes enriched for H3K9me2 and -me3 and substantial loss of H3K4me3 and histone acetylation, while the opposite is observed after Myc overexpression^{15,46}. This is in agreement with a recent study which showed that activation of Myc in epidermal stem cells induced a widespread reduction of H3K9me3 and increased H4 acetylation⁴⁷.

However, from the fact that Myc was not DNA associated when these genes were transcriptionally inactive, one can not simply deduce that Myc in general is not involved in the process of initiating or regulating the methylation process. Myc might be important for the establishment, but not the maintenance of CpG island methylation. Another possibility could be that Myc fulfills a more indirect role, by enhancing general, not gene-specific, DNA methylation processes.

Brenner *et al.*¹, however, have shown evidence pointing towards a direct involvement of Myc in the recruitment of DNMT3A, initiation of DNA methylation and transcriptional gene silencing of *CDKN1A*. Since *CDKN1A* promoter methylation is infrequently observed in CRC, future research on the identification of more prevalent Myc target genes that are repressed by a process involving Myc as well as promoter CpG island methylation in CRC is needed.

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CHAPTER 9|

General discussion

General discussion

Colorectal cancer genetics and epigenetics; a complex interplay

Over the past two decades it has become clear that CRC is a complex and heterogeneous disease in which numerous genetic and epigenetic abnormalities play an important role. Extensive research has led to the identification of different molecular pathways, driving the normal colon-, adenoma- to carcinoma progression. Their interaction, however, is less well understood. Understanding the timing and interaction of (epi)genetics in CRC will lead to a better understanding of the disease and might contribute to the ultimate goal of reducing colorectal cancer deaths. The main aim of this thesis was to study the interplay between genetic and epigenetic abnormalities in colorectal cancer (CRC). In this chapter, I will discuss the main findings of the studies performed and point out future research avenues and clinical applications.

Promoter CpG island methylation is an early event in CRC development

The first aim of this thesis was to study the timing of frequently occurring genetic and epigenetic abnormalities in colorectal cancer development. Therefore, we analyzed the genetic abnormalities chromosomal instability (CIN) and *TP53*-, *APC*- and *KRAS* mutation as well as promoter CpG island methylation of 9 DNA repair- and tumor suppressor genes known to be frequently and functionally methylated in CRC in a well characterized series of adenoma and carcinoma tissues. This unique series consisted of progressed and nonprogressed adenomas. The term progressed adenoma refers to adenomas in which a focus of malignant tissue is already present. In these malignant polyps, the adenoma and carcinoma parts were analyzed separately. Nonprogressed adenomas are adenomas without any sign of malignancy. In a previous study, Hermesen *et al.*¹ showed by comparative genomic hybridization (CGH) that accumulation of chromosomal deletions in 8p21-pter, 15q11-q21, 17p12-13 and 18q12-21 and amplifications in 8q23-qter, 13q14-31, and 20q13 mainly occur in the development of non-progressed- to progressed adenomas and thereby can be used as indicator of progression towards malignancy. We show in the same series that high frequencies of promoter CpG island methylation are already present in nonprogressed adenomas and associates with the transition of normal- to adenoma tissue. Also other studies have linked promoter CpG island methylation to the initiation of cancer development. Some studies have identified promoter methylation to occur in the transition of an early to an advanced adenoma², defined by a size >1 cm, containing at least 25% of villous component, or highgrade dysplasia, which is known to be associated with increased malignant potential.

Together these results demonstrate that promoter CpG island methylation can be considered as an early event in colorectal carcinogenesis. Although a causal relationship is difficult to address and remains to be elucidated, promoter CpG island methylation occurs prior to chromosomal instability. Given that aberrant DNA methylation can also be detected in stool DNA^{3,4}, studying methylation as common event in pre-malignant lesions is promising to provide novel specific biomarkers for early detection⁵. However, adenomatous polyps are common in adults over 50 years and only a low percentage will develop into a malignant lesion⁶. Therefore, one could argue whether CRC screening should be focused on the detection of premalignant lesions or on a

marker distinguishing adenomas with malignant potential from adenomas that will never progress into cancer would be more applicable. Nevertheless, since it has been shown that early detection and prevention through polypectomy results in a great incidence- and mortality reduction⁷, CRC guidelines nowadays recommend screening for premalignant lesions⁶. In these screenings tests, methylation markers are promising to play a powerful role.

CRC must be considered as multiple different diseases in which diverse genetic and epigenetic molecular changes occur

Although numerous studies have contributed to the identification of genetic and epigenetic abnormalities in CRC development, the question how these abnormalities interact is largely unanswered. In chapter 5 we studied (epi)genetic interrelations and subcategorized CRCs based on their genetic composition, microsatellite instable (MSI) or chromosomal instable (CIN) and/or epigenetic profile based on promoter CpG island methylation of 9 DNA-repair or tumor suppressor genes (TSGs). Integrative analysis showed that 16.4% of CRCs are MSI and 77.5% of CRCs are CIN, which occur in a mutually exclusive manner and overlap in only 3% of CRCs. A subgroup of CRCs (21.1%) could be characterized by a high number of methylated genes identified by unsupervised hierarchical cluster analysis of promoter CpG island methylation of 9 TSGs. This subgroup showed a great overlap with MSI CRCs while most CIN CRCs showed only low numbers of methylated genes and a rather inverse relationship. The association between extensive promoter methylation and MSI, mostly due to promoter CpG island methylation of mismatch repair gene *hMLH1*⁸⁻¹¹, and an inverse relationship to CIN is in agreement with other studies^{12,13}. However, by using comparative genomic hybridization (CGH) to analyze DNA copy number change, we were able to look at specific (epi)genetic associations and showed that amplification of chromosomal region 8q23-qter strongly related to promoter CpG island methylation of individual genes as well as a high number of methylated genes. Chromosomal loss at 15q11-21 and gain at 20q13 showed an inverse relationship to promoter CpG island methylation of individual genes. Furthermore, by integrating MSI, CIN and epigenetic instability (Figure 1) we show that from the perspective of CRCs with a high number of methylated genes, 50% was MSI and 50% was CIN. Taken into account that promoter methylation is an early event, half of CRCs with extensive promoter methylation becomes MSI, and half of them CIN. Therefore we conclude that extensive promoter methylation does not exclusively predispose to MSI, but might contribute to CIN as well. The actual mechanism involved is unknown but as epigenetic silencing of mismatch repair (MMR) genes causes MSI and a mutator phenotype¹⁴ one would hypothesize that promoter methylation of 'chromosomal stability genes' can initiate chromosomal instability. Our study, however, was not designed to identify stability genes but promoter CpG island methylation of other control genes needs to be evaluated to determine if epigenetic changes can initiate CIN.

Figure 1: Relationship between genetic and epigenetic phenotypes in CRC. Circles in Venn diagram represent all CRCs and show overlap between genetic instability phenotypes (microsatellite instability (MSI) and chromosomal instability (CIN)) and an epigenetic phenotype characterized by extensive promoter methylation (EPM) of 9 DNA repair- and tumor suppressor genes.

What becomes clear from our studies is that CRCs can not be subcategorized as being genetic or epigenetic unstable; both abnormalities occur concomitantly. The fact that some strong correlations are observed reflects a programmed collaboration which contributes to different CRC phenotypes¹⁵.

The CpG island methylation phenotype as prognostic marker

The term “epigenetic unstable” describes a cluster of CRCs characterized by a high number of methylated genes, also designated as the “CpG island methylation phenotype” (CIMP). Since the introduction of CIMP by Toyota in 1999¹⁶, the concept is under debate. CIMP generally refers to a phenotype characterized by an exceptionally high frequency of concordantly hypermethylated genes, which associates with clinicopathologic characteristics such as older age, female sex, proximal tumor location, poor differentiation, microsatellite instability (MSI) and *BRAF* mutation¹⁷⁻²¹. However, these associations are dependent on the panel of genes selected to define CIMP. The use of different ‘CIMP’ markers throughout the years has caused confusion by reporting conflicting associations. Different attempts have been made to standardize the gene panel, among which a study of Weisenberger *et al.*²⁰ who systematically screened 195 CpG islands in 295 CRCs for a high specificity to a CRC subgroup with a high number of methylated genes. Using different algorithms and bimodality assessment 5 gene promoters (*CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3* and *SOCS1*) were selected as markers for CIMP²⁰. Also this CIMP strongly correlate to features such as female sex, age, proximal tumor location, MSI and *BRAF* mutation status, suggesting a biological relationship of this phenotype to tumorigenesis. Weisenbergers marker panel has been validated in different large series and outperformed other definitions of CIMP in this respect²¹⁻²³. The biological significance of the marker panel, however, was not clear.

Therefore we performed a study, described in chapter 6, to investigate the prognostic value of CIMP in CRC. In this study CIMP was analyzed in a series of 173 patient with primary CRC and complete data on follow-up. At the time this trial was conducted, only surgical removal of the tumors was performed. These patients did not receive adjuvant chemotherapy, as is nowadays recommended for all stage III and some stage II CRC patients. Therefore we were able to avoid an adjuvant treatment bias.

After stratifying for tumor stage, MSI and *BRAF* mutation status, which influence prognosis themselves²⁴, we showed that within microsatellite stable, *BRAF* wild type tumors, CIMP did not correlate with patient survival.

However, we were interested in the prognostic value of CpG island methylation of individual genes as well. Most interestingly, these analysis showed (chapter 6) that promoter CpG island methylation of *checkpoint with fork-head and ring finger domains CHFR* was associated with a poor prognosis.

Based on these reports one might argue whether CIMP as a phenotype or promoter CpG island methylation of individual genes accounts for biological significance. Since promoter CpG island methylation and subsequent transcriptional silencing targets a spectrum of different genes, i.e. DNA mismatch repair genes *hMLH1*, *O⁶MGMT*, cell cycle related genes as *p14^{ARF}* and *p16^{INK4A}* and chemotaxis gene *CXCL14*, the effect on disease progression might be diverse as well.

Furthermore, not all methylated genes relate to a subgroup of CRCs characterized by high numbers of methylated genes. In our studies (chapter 5 and 6) this was observed for *APC* and *O⁶MGMT*, which has also been demonstrated by others²⁵. It has been suggested in the literature that different types of CIMP exist²⁰; CIMP-negative, CIMP-low and CIMP-high. CIMP-low refers to a group of CRCs with low numbers of methylated genes but frequent *O⁶MGMT* promoter methylation and mutant *KRAS*^{26,27}. Recently the prognostic significance of the three groups was studied in a large series of 582 patients which showed that CIMP-high as well as CIMP-low was an independent prognostic factor in MSS CRCs²⁸. However, reports on the contribution of CIMP-low to colorectal carcinogenesis are conflicting, mainly due to persistent use of different marker panels.

Genetics and epigenetics collaborate in targeting DCC on chromosomal region 18q21

In addition to the association between genetic and epigenetic phenotypes, we were interested in the relationship between genetic and epigenetic abnormalities in targeting TSGs in CRC. In theory, genetic and epigenetic abnormalities can join forces in any possible combination to inactivate TSGs. Knudsons two-hit hypothesis²⁹ describes how two alleles in a diploid cell need biallelic gene dysfunction for full inactivation of a TSG. In a polyploid model a third hit hypothesis is assumed in which multiple inactivating processes can occur in the same cell³⁰. The choice underlying the mechanism of inactivation is not entirely clear but genes affected by different mechanisms are thought to play a significant role in carcinogenesis. However, different studies have shown that promoter CpG island methylation does not frequently occur simultaneously with gene mutation or chromosomal copy number changes^{31,32}. In general, mutations are heterozygous and of low frequency, with an average of 9 cancer related gene mutations per tumor. In contrast, a much higher number of approximately 200-400 genes is hypermethylated per tumor,

which can occur in a homozygous fashion^{32,33}. Genes affected by both mechanisms, are more frequently affected by hypermethylation than mutation, and within one tumor cell mutation and hypermethylation usually are mutually exclusive³². This has also been observed for *APC* promoter methylation and *APC* mutations, which typically occur separately of each other, as shown in chapter 4 and by others³⁴. The same inverse relationship has been observed for promoter CpG island methylation and DNA copy number changes: only a very small number (1,8%) of TSGs inactivated by promoter CpG island methylation is located on chromosomal regions targeted by deletion or copy number change^{33,35,36}. However, in chapter 4 we show that *GATA4* promoter CpG island methylation and deletion of its chromosomal region, 8p21-pter, can occur concomitantly. *GATA4* methylation occurred prior to loss of 8p21-pter and the number of cases in which both events were present increased during tumor progression.

In chapter 7 we studied genetic and epigenetic targeting of TSGs located on another chromosomal region, 18q21, a chromosomal region frequently lost in colorectal carcinogenesis¹. Several candidate TSGs, among which *MBD1*, *CXXC1*, *SMAD4*, *DCC* and *MBD2*, are closely linked on a 4-MB DNA region on 18q21. Mutations of these genes have only been found at low frequencies. Our study shows that *DCC* and not *MBD1*, *CXXC1*, *SMAD4* and *MBD2* is targeted by promoter CpG island methylation- and H3K9me3 and H3K27me3-mediated epigenetic silencing in CRC. *DCC* promoter methylation occurred in 80% of adenoma and carcinoma tissues and 20% of normal colon tissues and can be considered as early event. Deletion of chromosomal region 18q21 occurred in 40% of CRCs. Thus, this study showed how genetic and epigenetic alterations can collaborate in transcriptional silencing of *DCC*, amounting to 90% of CRCs being affected by promoter methylation and/or loss of the *DCC* locus. While chromosomal deletion frequently involves the entire chromosomal region, a more subtle role was observed for promoter CpG island methylation and H3K9me3- and H3K27me3 mediated epigenetic silencing. This in contrast to chromosomal regions 2q14³⁷, 3p22³⁸ and 5q32.2³⁹, for which long-range H3K9me3-mediated epigenetic silencing has previously been reported in CRC.

Gene specific hypermethylation targeting mechanisms

The observation that *DCC* is specifically targeted by promoter CpG island methylation and H3K9me3- and H3K27me3-mediated epigenetic silencing, while neighboring genes remain unaffected, raises the question which underlying mechanism causes this gene specificity. Recent studies have shown that genes which become *de novo* methylated in cancer are often bivalently marked by both active (H3K4me3) and repressive (H3K27me3) histone marks and Polycomb Group proteins in human embryonal stem cells⁴⁰⁻⁴². Interestingly, in these studies the *DCC* locus was found enriched for H3K27me3 and Polycomb group protein SUZ12 in its promoter region, whereas *MBD1*, *CXXC1*, *SMAD4* and *MBD2* were not. As is clear for other cancer associated hypermethylated genes⁴¹, these findings seem to suggest an inherent susceptibility of *DCC* for aberrant methylation during cancer progression.

Also other explanations for gene specific promoter methylation have been proposed among which a role for transcription factor c-Myc. *c-MYC* is a proto-oncogene, which is overexpressed in 70% of CRCs⁴³, mostly by upregulated

WNT signaling and by amplification of its chromosomal region 8q23-qter, due to chromosomal instability (CIN)⁴⁴. c-Myc has been linked to DNA promoter methylation by Brenner *et al.*⁴⁵ which have shown a role for c-Myc in recruiting DNA methyltransferases (DNMTs) to target promoter *CDKN1A*, which resulted in DNA methylation. However, apart from the association between 8q23-qter amplification and an increased number of methylated genes as observed in chapter 5, there is no evidence that c-Myc can induce widespread DNA methylation. Therefore we studied the role of c-Myc in the induction of promoter methylation, and attempted to identify genes that are repressed by c-Myc induced promoter methylation in chapter 8. Using microarray technology we were able to identify genes that were repressed in conditions of over-expression of c-Myc. Treating these cells with demethylating agent 5-Aza-2'deoxyctidine, we were able to select for genes that were specifically repressed by processes involving DNA methylation. By this approach we identified novel candidate genes affected by promoter CpG island methylation in CRC. Chemokine (C-X-C motif) ligand 14 (*CXCL14*) and WNT antagonist *Dickkopf homologue 3*, *DKK3*, showed the most profound cancer specific promoter methylation. *CXCL14* is a chemokine that controls leukocyte migration⁴⁶⁻⁴⁸ and angiogenesis⁴⁸. Loss of *CXCL14* expression in tumor cells correlates with a reduced capacity of immature dendritic cells to infiltrate⁴⁹ and thereby allows tumor cells to escape from normal immune surveillance. Future research will show if *CXCL14* and *DKK3* can be used as marker of disease progression or tumor detection. However, the role of c-Myc in transcriptional silencing and methylation of these genes was not clear and remains to be elucidated. No association between increased c-Myc expression and promoter CpG island methylation of c-Myc target genes was observed. To the contrary, ChIP analysis demonstrated c-Myc enrichment at target gene promoters showing transcriptional activation and enrichment of the active chromatin mark H3K4me3. Thus, while c-Myc target genes can be methylated in CRC, promoter CpG island methylation and c-Myc enrichment are not observed concomitantly. Furthermore, *CDKN1A* is not methylated in CRC⁵⁰. Future research focusing on the identification of genes repressed by a mechanism involving c-Myc as well as promoter CpG island methylation is needed to unravel the role of c-Myc induced promoter methylation in CRC.

Future avenues and clinical applications

This thesis provides further insight into how genetics and epigenetics interact and lead to different phenotypes in CRC development.

The observation that promoter CpG island methylation is prevalent and occurs early in colorectal carcinogenesis is highly relevant for biomarker based CRC screening. Several studies have shown proof-of-principle for the detection of epigenetic biomarkers in colorectal adenoma- or carcinoma-derived DNA in stool⁵¹⁻⁵⁵ or blood^{56,57} using sensitive methylation-specific PCR (MSP)-based assays.

To apply current results to clinical oncology, technical aspects as the best technique to be used to determine the presence of the promoter CpG island methylation, must be considered. As stated in chapter 3, methylation-specific PCR and its nested variant are very suitable for the detection of methylated tumor DNA in a variety of body fluids and/or excretions. Although primer design and PCR optimization is a critical step which will influence sensitivity and specificity of a particular marker, MSP is a well manageable and cost

effective technique which does not require special equipment¹². This in contrast to quantitative approaches which are nowadays more fashionable⁵⁸. A combination between sensitive detection of methylation markers and a more specific method as colonoscopy will be an ideal screening method. However, the number of markers which meet these criteria are still limited.

Besides markers for early detection of CRC, it is imperative to identify prognostic- and predictive biomarkers to improve personalized cancer treatment. It is clear that some patients with stage II cancer might benefit from adjuvant chemotherapy, and biomarkers to assist in selecting these patients are needed. We and others show that CIMP CRCs, as well as CRCs with promoter CpG island methylation of individual markers⁵⁹⁻⁶² such as *CHFR*, are associated with a poorer prognosis⁶³. In contrast, MSI is associated with a more favorable outcome²⁴, while chromosomal deletion of 18q is associated with a shortened survival in stage II and III CRCs⁶⁴. However, response to adjuvant chemotherapy greatly differs between phenotypical subgroups. Different studies have suggested that CIMP CRCs show a significant survival benefit from anticancer agent 5-Fluorouracil (5-FU)⁶⁵, while MSI CRCs show reduced sensitivity⁶⁶. *CHFR* promoter methylation has been associated with sensitivity to chemotherapeutic agents in endometrial-⁶⁷, cervical-⁶⁸ and gastric cancer⁶⁹, but no such correlation has been described in CRC yet. However, other studies have reported conflicting results and a poorer outcome of CIMP CRCs after treatment with 5-FU^{70,71}. This difference might be explained by the use of different marker sets in defining CIMP, but also by the strong association between MSI and promoter CpG island methylation, which may have opposing effects on prognosis on the one hand, and treatment response on the other.

Recently it has become clear that response to treatment with chemotherapeutic agents cetuximab and panitumumab is confined to patients with wild-type *KRAS*^{72,73} and *BRAF*^{74,75} CRCs. Whether promoter CpG island methylation of Ras effectors *NORE1* and *RASSF1A* can be used as predictive marker as well, remains to be elucidated.

Although validation of these biomarkers within the context of randomized clinical trials is required before adoption into routine clinical practice, these findings emphasize the importance of integrating results of genetic and epigenetic analyses in treatment protocols. Defining CRCs based on their (epi)genetic profile will be highly relevant and increase the possibilities to individualized cancer treatment.

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SUMMARY|

Colorectal cancer (CRC) is a complex and heterogeneous disease, characterized by the growth of precursor lesions of which a small percentage will eventually progress into carcinomas. Extensive research has led to the identification of numerous genetic and epigenetic abnormalities underlying colorectal carcinogenesis. Pioneering research of Vogelstein and coworkers has proposed a progression model in which genetic alterations as *APC*, *KRAS* and *TP53* mutations and allelic loss of 5q, 17q and 18q play an important role. Later genetic instability, microsatellite instability (MSI) and chromosomal instability (CIN) which occur in approximately 10-15% and 85% of CRCs respectively, have been added to the model. Seven chromosomal alterations, losses in 8p21-pter, 15q11-q21, 17p12-13 and 18q12-21 and gains in 8q23-qter, 13q14-31, and 20q13 are strongly associated with the progression of colorectal adenomas towards carcinomas and can therefore be considered as cancer associated events (CAE). Furthermore, it has become clear that initiation and progression of cancer also involves epigenetic alterations such as promoter CpG island methylation of pivotal tumor suppressor- and DNA repair genes. A subgroup of CRCs can be characterized by an extensive number of methylated genes, a phenotype also referred to as the CpG island methylation phenotype (CIMP).

The main aim of this thesis was to study the interplay between genetic and epigenetic abnormalities in CRC.

In chapter 1 we provide an historic overview of the identification of genetic- and epigenetic alterations in colorectal carcinogenesis. CRC has long been considered to be a genetic disease, but in the last decade a revolution in epigenetic cancer research has marked the place for epigenetics in CRC.

In chapter 2, we review recent evidence regarding the complexity of epigenetic regulation of gene expression in CRC and discuss the clinical use of these new insights. Furthermore, we conclude that only by considering the complex interactions of CRC genetics and epigenetics we will be able to obtain insight in CRC carcinogenesis and translate these findings to clinical practice.

Chapter 3 provides an overview of critical parameters of the methylation specific PCR (MSP) technique as well as the available MSP variants and (clinical) applications.

The first experimental study (Chapter 4) describes the timing of genetic- and epigenetic abnormalities in CRC development. The frequently occurring genetic abnormalities CIN, *TP53*-, *APC*- and *KRAS* mutation and promoter CpG island methylation of *hMLH1*, *O⁶MGMT*, *APC*, *p14^{ARF}*, *p16^{INK4A}*, *RASSF1A*, *GATA4*, *GATA5* and *CHFR* were studied in a well characterized series of normal colon, adenoma and carcinoma tissues. These analyses showed that while P53 immunopositivity and chromosomal abnormalities occur predominantly in carcinomas, nonprogressed adenomas, progressed adenomas and carcinomas show similar frequencies of promoter methylation for the majority of the genes. Since promoter CpG island methylation mainly occurred in the transition of normal- to adenoma tissue, we conclude that promoter methyla-

tion can be regarded as an early event preceding *TP53* mutation and chromosomal abnormalities in colorectal cancer development.

In chapter 5 we investigated (epi)genetic interrelationships at the level of CRC phenotypes and at the level of individual (epi)genetic events. We classify CRCs based on the presence of CIN, MSI and a high number of CpG island promoter methylated genes and investigate whether genetic and epigenetic abnormalities collaborate or form different subgroups in colorectal carcinogenesis.

These integrative analyses showed that CIN (77.5% of CRCs) and MSI (16.4% of CRCs) occur in different CRCs. A subgroup of CRCs with extensive promoter methylation (EPM) (21.2% of CRCs) showed a great overlap with MSI and an inverse relationship CIN. However, from the perspective of EPM CRCs, 50% is MSI and 50% is CIN. Furthermore, while promoter CpG island methylation of *GATA4* and *p16^{INK4A}* was inversely related to CAEs chromosomal loss at 15q11-21 and gain at 20q13, promoter CpG island methylation of *RASSF1A*, *GATA4*, *GATA5* and *CHFR* as well as a high methylation index was positively related to chromosomal gain at 8q23-qter in CIN CRCs. Thereby we showed that promoter CpG island methylation of pivotal tumor suppressor and DNA repair genes is associated with specific patterns of chromosomal changes in colorectal cancer, which is different from methylation patterns in MSI tumors.

In chapter 6 we aimed to unravel the prognostic significance of promoter CpG island methylation in colorectal cancer (CRC). Such studies are complicated by factors affecting the course of the disease, such as genetic alterations and adjuvant therapy. Therefore, we evaluated the prognostic value of promoter CpG island methylation of 5 "CIMP" genes, as defined by Weisenberger *et al.*, and 19 additional tumor suppressor and DNA repair genes in CRC patients independent of MSI- and *BRAF* mutation status in a retrospective study of patients treated with surgery alone. We first observed that CIMP (>3/5 CIMP genes methylated) had no prognostic significance. Interestingly, also promoter CpG island methylation of an individual gene, *CHFR*, was associated with a poorer prognosis in stage II, MSS, *BRAF* wild-type CRCs. This finding could be confirmed in an independent population-based series of CRCs.

In chapter 7 we studied the (epi)genetic interrelation by focusing on the mechanistic interplay between genetics and epigenetics in tumor suppressor gene silencing. Therefore, we analyzed CpG island promoter methylation as well as histone-tail modifications of 5 genes, *MBD1*, *CXXC1*, *SMAD4*, *DCC* and *MBD2*, on a chromosomal region frequently lost in CRC, chromosome 18q21, and evaluated if these abnormalities occurred concomitantly or had a mutually exclusive relationship. In this chapter we showed that *DCC* had promoter CpG island methylation in CRC cell lines and carcinoma tissues, while *MBD1*, *CXXC1*, *SMAD4* and *MBD2* promoter CpG islands were unmethylated. *DCC* promoter CpG island methylation was associated with reduced *DCC* expression, which was independent of 18q21 loss analyzed by multiplex ligation-dependent probe amplification. Reduced gene expression of *CXXC1*, *SMAD4* and *MBD2* correlated with 18q21 loss in CRC cell lines. Treatment with the demethylating agent 5-aza-2'-deoxycytidine (Aza), but not with the histone deacetylase inhibitor Trichostatin A (TSA) exclusively restored *DCC* expression in CRC cell lines. Chromatin immunoprecipitation (ChIP) studies revealed

that the *DCC* promoter is marked with repressive histone-tail marks H3K9me3 and H3K27me3, whereas activity related H3K4me3 was absent. Only active epigenetic marks were detected for *MBD1*, *CXXC1*, *SMAD4* and *MBD2*. Thereby this study demonstrates that while chromosomal loss of 18q21 frequently involved the entire chromosomal region tested, H3K9me3-, H3K27me3- and promoter CpG island DNA methylation mediated epigenetic silencing specifically targets *DCC* without affecting neighboring genes on chromosomal region 18q21.

Finally, we investigated the relationship of amplification of the oncogene *c-MYC* and promoter methylation of c-Myc target genes in chapter 8. c-Myc induced promoter methylation has been hypothesized, but the contribution to colorectal carcinogenesis remained to be elucidated. In a previous *c-MYC* transfection study, genes repressed by c-Myc overexpression and re-expressed after treatment with demethylation agent Aza were identified among which *CXCL14*, *DNAJA4*, *DKK3*, *TGFβi* and *TRIM59*. Promoter CpG island methylation analysis showed that all 5 of these genes were methylated in CRC cell lines. Quantitative reverse transcription PCR showed an inverse relation to the presence of DNA promoter methylation. However, no relationship between promoter CpG island methylation and high c-Myc transcript levels was observed. Although ChIP studies showed the presence of c-Myc on the promoter region of *DNAJA4*, *TGFβi* and *TRIM59*, c-Myc enrichment was associated with gene transcription and enrichment of active chromatin mark H3K4me3. In conclusion, we were indeed able to find genes that were repressed by promoter CpG island methylation in conditions of c-Myc overexpression, however, the presence of c-Myc and CpG islands promoter methylation could not be observed concomitantly. This suggests that c-Myc might be important for the establishment, but not the maintenance of CpG island methylation.

In conclusion, this thesis provides new insight into how genetics and epigenetics interact in CRC development. We have demonstrated that CpG island promoter methylation is frequently associated with the initiation of colorectal carcinogenesis, while chromosomal abnormalities are indicators of disease progression. In CRC genetic and epigenetic alterations, at the level of single events and at the level of phenotypes, show specific interrelations. These associations can be mutually exclusive, complementary or causal indicating a programmed collaboration leading to different CRC subgroups which might differ in prognosis and response to therapy. Thereby, CRC provide an excellent model of the (epi)genetic interplay in carcinogenesis. Future studies will unravel the importance of (epi)genetics for individualized cancer treatment.

SAMENVATTING|

Kanker van de dikke darm en endeldarm, ook wel colorectaal kanker genoemd, is een complexe ziekte, die via verschillende moleculair biologische routes kan ontstaan.

De ziekte begint vaak als een goedaardig gezwel, ook wel adenoom genoemd, waarvan een klein percentage zich zal ontwikkelen tot een maligne tumor, een carcinoom.

De afgelopen 20 jaar is er veel onderzoek gedaan naar genetische veranderingen (veranderingen in de basen volgorde van het DNA) die een rol spelen in het ontstaan van colorectaal kanker. De onderzoeksgroep van Vogelstein heeft baanbrekend werk verricht door het opstellen van een model over het ontstaan van colorectaal kanker waarin genetische veranderingen zoals *APC*, *KRAS* and *TP53* mutaties alsook chromosomale veranderingen 5q-, 17q- en 18q deletie een belangrijke rol spelen. Later is ook genetische instabiliteit, in de vorm van microsatelliet instabiliteit (MSI) of chromosomale instabiliteit (CIN), aan het model toegevoegd. Zeven chromosomale veranderingen, verlies van regio's 8p21-pter, 15q11-q21, 17p12-13 en 18q12-21 en amplificatie van regio's 8q23-qter, 13q14-31 en 20q13, zijn sterk geassocieerd met de progressie van adenomen naar carcinomen, en kunnen om die reden beschouwd worden als colorectaal kanker gerelateerde afwijkingen.

Verder is ontdekt dat ook epigenetische afwijkingen een belangrijke rol spelen bij het ontstaan en de progressie van colorectaal kanker. Epigenetische processen als promoter CpG eiland methylering en histon modificaties wijzigen niet de basenvolgorde van het DNA, maar zijn in staat via andere wegen de expressie van genen te beïnvloeden. Vooral promoter CpG eiland methylering is een uitvoerig bestudeerd mechanisme resulterend in de inactivatie van belangrijke tumor suppressor- en DNA reparatie genen. Een deel van alle colorectaal carcinomen wordt gekenmerkt door een uitzonderlijk hoog aantal gemethyleerde genen, ook wel het CpG eiland methylerings fenotype (CIMP) genoemd.

Het doel van dit proefschrift was het bestuderen van de interactie tussen genetische en epigenetische afwijkingen in colorectaal kanker.

In hoofdstuk 1 wordt een historisch overzicht gegeven van de ontdekking van genetische- en epigenetische veranderingen in het ontstaan van colorectaal kanker. Gedurende lange tijd werd colorectaal kanker gezien als een genetische ziekte. In het afgelopen decennium echter, heeft een revolutie in epigenetisch kankeronderzoek de betrokkenheid van epigenetica in de biologie van colorectaal kanker aangetoond.

Hoofdstuk 2 geeft een overzicht van de complexiteit van epigenetische regulatie van genexpressie in colorectaal kanker en bespreekt de klinische toepasbaarheid van deze nieuwe inzichten. We concluderen dat het bestuderen van de complexe interactie tussen genetica en epigenetica inzicht zal geven in het ontstaan van colorectaal kanker, wat essentieel is om uiteindelijk een vertaalslag naar de kliniek te maken.

Hoofdstuk 3 betreft een overzicht van de kritische parameters van de methylerings-specifieke PCR techniek, samen met mogelijke varianten en de klinische toepasbaarheid.

De eerste experimentele studie (hoofdstuk 4) beschrijft op welk moment in de ontwikkeling van colorectaal kanker genetische en epigenetische veranderingen optreden. Hiervoor werden frequent optredende genetische afwijkingen als CIN, *TP53*-, *APC*- en *KRAS* mutatie, en promoter CpG eiland methylering van *hMLH1*, *O⁶MGMT*, *APC*, *p14^{ARF}*, *p16^{INK4A}*, *RASSF1A*, *GATA4*, *GATA5* and *CHFR* geanalyseerd in een uitvoerig gekarakteriseerde serie van zowel normaal darmweefsel alsook adenoom en carcinoom weefsel. Dit onderzoek toonde aan dat *TP53* mutaties en chromosomale afwijkingen voornamelijk optreden in adenomen met tekenen van kankerprogressie en carcinomen. Promoter CpG eiland methylering werd, voor de meerderheid van de bestudeerde genen, in ieder stadium van colorectaal kanker ontwikkeling gezien, ook in adenomen zonder kenmerken van progressie. Omdat promoter CpG eiland methylering dus voornamelijk optreedt in de overgang van normaal colon naar adenomateus weefsel, concluderen we dat promoter methylering een vroege afwijking is in het ontstaan van colorectaal kanker, die eerder optreedt dan *TP53* mutatie en chromosomale afwijkingen.

In hoofdstuk 5 werd ingegaan op de relaties tussen genetische- en epigenetische afwijkingen in het vormen van verschillende colorectaal kanker subgroepen. We deelden carcinomen in op basis van de aanwezigheid van CIN, MSI en een uitzonderlijk hoog aantal gemethyleerde genen, en bestudeerden de relatie tussen deze 3 fenotypes. Daarmee toonden we aan dat CIN (aanwezig in 77.5% van de carcinomen) en MSI (aanwezig in 16.4% van de carcinomen) nauwelijks samen voorkomen. Een subgroep van carcinomen, gekenmerkt door extensieve promoter methylering (EPM), toonde een grote overlap met MSI maar een inverse relatie met CIN. Echter, vanuit het perspectief van EPM carcinomen was de helft MSI en de helft CIN. Terwijl promoter methylering van *GATA4* and *p16^{INK4A}* een inverse relatie toonde met chromosomaal verlies van regio 15q11-21 en amplificatie van regio 20q13, bleek amplificatie van chromosomale regio 8q23-qter in CIN carcinomen juist gerelateerd te zijn aan promoter CpG eiland methylering van *RASSF1A*, *GATA4*, *GATA5* en *CHFR* en een hoog totaal aantal gemethyleerde genen. Hiermee hebben we aangetoond dat promoter CpG eiland methylering van belangrijke tumor suppressor- en DNA reparatie genen geassocieerd is met een specifiek patroon van chromosomale veranderingen, welke verschillend zijn in carcinomen met MSI.

Hoofdstuk 6 richt zich op het bestuderen van de prognostische waarde van promoter CpG eiland methylering in colorectaal kanker. Dit soort studies worden vaak verstoord door factoren als genetische afwijkingen en therapie, die ook hun invloed op het ziekte beloop hebben. Om deze reden hebben we ervoor gekozen de prognostische waarde van promoter CpG eiland methylering onafhankelijk van het tumor stadium, MSI, *BRAF*- en *KRAS* mutatie status te bestuderen, in een retrospectieve studie van patiënten met colorectaal kanker, die enkel behandeld werden middels chirurgie. Naast het bestuderen van de prognostische waarde van het CpG eiland methylerings genotype (CIMP), zoals dat gedefinieerd is door Weisenberger *et al.*, is ook de prognostische waarde van promoter methylering van 19 andere tumor suppressor- en DNA reparatie genen geanalyseerd. Uit deze analyse is gebleken dat hoewel CIMP geen relatie tot prognose vertoonde, promoter CpG eiland methylering van het *CHFR* gen dat wel deed in stadium II, microsatelliet stabiele,

BRAF wildtype carcinomen. Deze bevindingen werden bevestigd in een onafhankelijke colorectaal kanker populatie.

In hoofdstuk 7 werd de relatie tussen genetische en epigenetische afwijkingen bij het inactiveren van tumor suppressor genen bestudeerd. In dit hoofdstuk wordt gekeken naar CpG eiland methylering en histon modificaties in de promotor regio van 5 genen, *MBD1*, *CXXC1*, *SMAD4*, *DCC* and *MBD2*, die gelegen zijn op een chromosomale regio die frequent verloren gaat in colorectaal kanker, te weten chromosoom 18q21. Uit deze studie bleek dat enkel de promotor van *DCC* gemethyleerd was en dat *MBD1*, *CXXC1*, *SMAD4* en *MBD2* promotor CpG eilanden ongemethyleerd waren in colorectaal kanker cellijnen en carcinomen. *DCC* promotor CpG eiland methylering was gerelateerd aan gereduceerde *DCC* genexpressie, onafhankelijk van de afwezigheid van chromosoom 18q21. Gereduceerde genexpressie van *CXXC1*, *SMAD4* en *MBD2* was wel geassocieerd met verlies van chromosoom 18q21 in colorectaal kanker cellijnen. Behandeling met demethylerende agentia 5-aza-2'-deoxycytidine (Aza) leidde tot herstel van *DCC* expressie in colorectaal kanker cellijnen. Behandeling met histon deacetylering remmer Trichostatin A (TSA) in cellijnen had echter geen effect. Chromatine immunoprecipitatie (ChIP) studies toonden aan dat de *DCC* promotor verrijkt is met de aan gen inactiviteit gerelateerde histon modificaties H3K9me3 and H3K27me3, terwijl de aan gen activiteit gerelateerde histon modificatie H3K4me3 afwezig was. H3K4me3 kon wel waargenomen worden in de promotor regio's van *MBD1*, *CXXC1*, *SMAD4* en *MBD2*. Daarbij laat deze studie zien dat terwijl chromosomaal verlies van regio 18q21 meestal de gehele regio betreft, H3K9me3-, H3K27me3- en promotor CpG eiland methylerings- gemedieerde epigenetische repressie specifiek *DCC* treft.

Tenslotte hebben we de relatie tussen amplificatie van het oncogen *c-MYC* en promotor CpG eiland methylering van *c-Myc* doelgenen bestudeerd in hoofdstuk 8. Hoewel *c-Myc* geïnduceerde promotor methylering als mechanisme al eerder is beschreven, is de rol hiervan in de ontwikkeling van het colorectaal carcinoom onbekend. Een eerdere *C-MYC* transfectie studie heeft aangetoond dat na *c-Myc* overexpressie een aantal genen transcriptieel onderdrukt raken, waaronder de genen *CXCL14*, *DNAJA4*, *DKK3*, *TGFβi* en *TRIM59*. Na behandeling met de demethylerende agens Aza kwamen deze genen weer tot expressie, wat duidt op een transcriptie remmend proces met betrokkenheid van *c-Myc* en DNA methylering. Promotor CpG eiland methylering analyses toonde aan dat deze vijf genen inderdaad gemethyleerd zijn in colorectaal kanker cellijnen. De aanwezigheid van promotor methylering bleek echter niet specifiek voor te komen in cellijnen of carcinomen met een hoge *c-Myc* expressie. ChIP experimenten toonden de aanwezigheid van *c-Myc* in de promotor regio's van *DNAJA4*, *TGFβi* and *TRIM59* aan. Dit bleek echter geassocieerd te zijn met transcriptionele activatie en de aanwezigheid van histon H3K4me3. Door middel van deze studie hebben we nieuwe gemethyleerde genen in colorectaal kanker ontdekt, alhoewel de bijdrage van *c-Myc* in het methyleringsproces nog niet geheel duidelijk is. DNA methylering en *c-Myc* werden niet tegelijkertijd in promotor regio's aangetoond wat er op zou kunnen wijzen dat *c-Myc* betrokken is bij het ontstaan van promotor methylering, maar niet het onderhoud hiervan.

Concluderend levert dit onderzoek nieuwe inzichten op in het (epi)genetisch samenspel in het ontstaan van colorectaal kanker. We hebben aangetoond dat promoter CpG eiland methylering geassocieerd is met de initiatie van colorectaal kanker, terwijl chromosomale afwijkingen betrokken zijn bij de progressie van colorectaal kanker. Genetische en epigenetische afwijkingen vertonen specifieke interrelaties; ze kunnen elkaar uitsluiten, complementeren en elkaar induceren. Dit alles duidt op een geprogrammeerde samenwerking, leidend tot de vorming van verschillende colorectaal kanker subgroepen die verschillen in gedrag en respons op therapie. Toekomstige studies zullen de bijdrage van (epi)genetica aan geïndividualiseerde behandeling van colorectaal kanker ophelderen.

DANKWOORD |

Dit proefschrift is tot stand gekomen dankzij de medewerking en inspanningen van vele mensen, die ik allen graag zou willen bedanken. Een aantal mensen wil ik bij naam noemen.

Beste Prof. de Bruïne, beste Adriaan, Charlie, je nam me onder je hoede toen ik mijn interesse voor wetenschappelijk onderzoek in jouw onderwijsgroep kenbaar maakte. Je opperde het plan te promoveren, wat ik in eerste instantie niet echt serieus nam, maar later werkelijkheid werd. Ik ben blij dat ik het volbracht heb, en daarbij hebben jouw menselijkheid, kritische blik en creativiteit een groot aandeel geleverd.

Beste Prof. Meijer, beste Gerrit, ook jou wil ik bedanken. Je hebt me keer op keer verbaasd met het tempo waarin mijn stukken kritisch bekeken werden. Je hebt me veel kunnen leren over de genetische kant van het verhaal tijdens bezoeken aan de VU, Hattem, maar ook op terrasjes in Maastricht!

Beste Manon, ik wil je graag bedanken voor alle kansen die je me gegeven hebt. Zonder jouw hulp had ik het niet op deze manier kunnen doen. Jouw grenzeloze enthousiasme is aanstekelijk en heb ik als erg stimulerend ervaren. Ik hoop dat er in de toekomst ruimte blijft bestaan voor gemeenschappelijke onderzoeksprojecten; ik kijk er naar uit!

Dear Prof. Herman, dear Jim. Thank you for the inspiring discussions we have had during your visits to Maastricht, my stay in Baltimore and by phone. You have taught me much about epigenetics, science, writing an article and many other things. I greatly appreciate the opportunity to work on the MYC-project in your laboratory. It has been of great value!

Alle analisten en AIO's van het lab, "Ingelkes", Marjolein, Sandra, Veerle, Peter, Arjen, Iris, Kathleen, Kim, Angela, Sarah, Guido, Debby, Ingrid, Hanneke, Edith en Kim; bedankt voor de gezellige tijd. Ik heb me erg thuis gevoeld in onze groep en ben blij dat jullie begrip hadden voor mijn chaotische schema, mijn directheid en absolute onwetendheid toen ik begon (Guido ;-)). Het 'angels-shirt' is nog steeds een collectors-item!

Kamergenoten, Arjen en Marjolein, later ook Iris; je moest een stukje lopen, maar dan had je ook wat. Het was fijn de chaos van het lab achter te kunnen laten en je terug te trekken op ons kamertje (al was de waterkoker krakemikkig, het printernetwerk werkelijk onbegrijpelijk en de kerstboom te afzichtelijk). Peter, bedankt voor alle hulp. Ik vond het erg leuk om samen te werken en hoop dat je me ook in de komende jaren kan voorzien van adviezen voor wandelroutes, reisbestemmingen en films. Veerle, je ben naast collega een hele lieve vriendin. Het reisje Madrid en onze talrijke koffiepauzes tekenen mijn AIO-tijd!

Linda, Beatriz, Sandra, Cindy, Begona en Anne van de VU: bedankt voor de gezelligheid en prettige samenwerking!

Buiten het werk zijn er veel mensen die mij op meer en minder cruciale momenten terzijde hebben gestaan en er voor gezorgd hebben dat ik er plezier in bleef houden. Lieve Femke, Eveline, Els, Marlous, Ellen en Mirthe; ik ben erg blij dat we elkaar uit alle hoeken van het land blijven opzoeken. Jullie hebben me altijd gestimuleerd, maar ook weten af te remmen als ik te hard

van stapel liep. Mijn hardlooptmaatje, Eva, ik hoop nog steeds dat je je bedenkt en terugkomt naar Maastricht. Lieve Eva, Miriam, Maarten, Marije en Edith (in chronologische volgorde): we kennen elkaar al van de basisschool, middelbare school en het eerste jaar psychologie en ben blij met alles wat we samen meegemaakt hebben en blijven beleven.

Lieve Abel, bedankt voor je creatieve bijdrage!

Ik wil ook graag mijn paranimfen, Belle en Arjen bedanken. Ik ben blij dat jullie me vergezellen. Belle, ik ken je al mijn hele leven en ben blij dat we op dit soort gebeurtenissen zo dicht bij elkaar staan. Arjen, we hebben elkaar op het lab leren kennen en hadden hetzelfde traject. Bedankt voor de gesprekken, de pittige discussies en bijstand in mijn oorlog met statistiek. Ook al hebben we nu voor een verschillende richting gekozen, ik hoop dat we elkaar nog vaak blijven zien en op de hoogte blijven van elkaars perikelen.

Lieve Floris en Rie Hilje, bedankt voor jullie grenzeloze steun en vertrouwen. Jullie zijn er altijd en hebben me alle mogelijkheden geboden me te ontwikkelen tot wie ik ben en daarin mijn eigen weg te gaan. Ik voel me trots en gezegend met zoveel zussen en broers: Belle, Elin, Jona, Olga en Menzo, het feit altijd terug te kunnen keren naar ons warme groepje is van onmetelijk belang in mijn bestaan.

En tenslotte, lieve Joep, ik zou je tekort doen wanneer ik hier zou proberen te beschrijven hoeveel je voor me betekent; jij maakt het verschil!

Sarah

CURRICULUM VITAE|

About the author

Sarah Derks was born on June 20th 1981 in 's Gravenhage, the Netherlands. She grew up in Zeist and Bunnik where she attended elementary school. After completing secondary school (VWO) at the St. Bonifatius College in Utrecht in 1999, she went to university in Maastricht. There she spent a year studying Psychology, followed by a year of Health Sciences, before she was admitted to Medical School in 2001.

During the first years of Medical School she developed an interest in science and fascination with cancer genetics and consequently started as research assistant in the cancer biology laboratory of Dr. M. van Engeland and Prof. de Bruijne from the Department of Pathology, Maastricht University Medical Center. In 2004, Sarah was awarded with "Kootstra Talent Fellowship", which allowed her to continue her research in a Ph.D. program. During her Ph.D. studies, which she combined with the last 3 years of Medical School, she worked on a project entitled "The identification of novel colorectal tumor suppressor genes: role in tumor biology and application as biomarkers" in collaboration with Prof. Dr. G.A. Meijer of the VU Medical Center in Amsterdam, which resulted in this dissertation.

Spring 2008 she completed her Medical School cum laude and had the opportunity to work in the laboratory of Prof. J.G. Herman of the Johns Hopkins University Baltimore, USA, on a collaborative project.

In April 2009 Sarah started her residency training Internal Medicine at the Maastricht University Medical Center, Maastricht.

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