(Epi-) genetic profiling of colorectal cancer : prognostic and biological relevance, with emphasis on tumor-hypoxia

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(Epi-) Genetic Profiling of Colorectal Cancer:

**Prognostic and Biological Relevance,
with emphasis on tumor-hypoxia**

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**(Epi-) Genetic Profiling
of Colorectal Cancer:
Prognostic and Biological Relevance,
with emphasis on tumor-hypoxia**

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 1 juni 2012 om 14.00 uur

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*“Ik weet dat je gaat promoveren, maar weet ook, dat als je niet promoveert,
ik je een fantastische zoon vind.”*

Ger Cleven, [†] 12 maart 2010

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List of abbreviations

AHEAD	Alliance for the Human Epigenome and Disease
AJCC	American Joint Committee on Cancer
ASCO	American Society of Clinical Oncology
CA9	carbonic anhydrase 9
CAN	cancer candidate genes
ChIP	chromatin immunoprecipitation
CIMP	CpG island methylator phenotype
CIN	chromosomal instability
COBRA	combined bisulfite restriction analysis
CRC	colorectal cancer
DAC	5-aza-2'-deoxycytidine
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
EPC's	endothelial progenitor cells
5-FU	5-fluorouracil
FAP	Familial Adenomatous Polyposis
GLUT1	glucose transporter 1
HDAC	histone deacetylases
HIF	hypoxia-inducible factor
HNPCC	Hereditary Non-Polyposis Colon Cancer
HR	hazard ratio
HRE	hypoxia responsive element
IHC	immunohistochemistry
LOH	loss of heterozygosity
MBD	methyl binding domain
MCA	methylated CpG island amplification
MeDIP	methylated DNA immunoprecipitation
MI	methylation index
MIN	microsatellite instable
MIRA	methylated-CpG island recovery assay
MLPA	multiplex ligation probe amplification
MMR	mismatch repair
MSI	microsatellite instability
MSP	methylation specific PCR
MSS	microsatellite stability
NSCLC	non-small cell lung cancer
PCR	polymerase chain reaction
sDNA	stool DNA test
SNP	single nucleotide polymorphism

TNM	tumor-node-metastasis
TS	thymidylate synthase
TSA	Trichostatin A
UICC	International Union against Cancer, together with the American Joint Committee on Cancer
WHO	World Health Organization

Chapter 1

General introduction

General introduction

Colorectal cancer (CRC) is one of the most common cancer types worldwide¹. In Europe 436.000 new CRC cases were diagnosed and 212.000 patients died of this disease in 2008². CRC occurs sporadically (70%), in families (25%) and as the inherited colon cancer syndromes Lynch syndrome/Hereditary Non-Polyposis Colon Cancer (HNPCC), Familial Adenomatous Polyposis (FAP) and *MUTYH*-associated polyposis in approximately 5% of cases³.

CRC can be treated and often cured by surgery when localized to the submucosa or muscularis propria of the bowel (stage I)¹. Importantly, stage II CRC patients (cancer progressed into subserosa or perforating the visceral peritoneum) undergo surgery alone, despite the recognition that a subgroup with a poor prognosis would probably benefit from adjuvant chemotherapy. Within stage III (lymph node metastases) and stage IV (distant organ metastases) CRC, adjuvant chemotherapeutic drugs such as fluoropyrimidines, irinotecan and oxaliplatin are now used as part of standard care and have been shown to improve survival significantly⁴. Although the anatomical based staging system predicts the survival accurately, variation in the course of the disease and response to treatment among individuals within the same stage exists. Emerging understanding of the underlying biology of CRC is expected to identify tumor specific molecular markers that improve risk assessment and therapeutic options within different stages of CRC⁵.

The last decades, comprehensive analysis of the CRC (epi)genome has provided novel information on the biology underlying CRC carcinogenesis. These studies revealed that cancer cells have acquired genomic instability, enabling limitless replicative potential, tissue invasion and metastasis, sustained angiogenesis, evasion of apoptosis, self-sufficiency in growth signals and insensitivity to anti-growth signaling⁶⁻⁸. The underlying cause of these so-called hallmarks of cancer are genetic and epigenetic alterations which can be subdivided in CRC into three categories: chromosomal instability (CIN), microsatellite instability (MSI) and CpG island methylator phenotype (CIMP)⁹⁻¹³. CIN is characterised by numerical and/or structural chromosomal abnormalities, increased rate of loss of heterozygosity (LOH) and mutations in the tumor suppressor genes *adenomatous polyposis coli* (*APC*), *v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog* (*KRAS*) and *tumor protein p53* (*TP53*)¹²⁻¹⁴. MSI results from inactivation of the mismatch repair (MMR) system, caused by germline mutations in MMR genes *mutS homolog 2, colon cancer, nonpolyposis type 1* (*E. coli*) (*MSH2*), *mutS homolog 6* (*E. coli*) (*MSH6*) and *PMS2 postmeiotic segregation increased 2* (*S. cerevisiae*) (*PMS2*) in Lynch syndrome/HNPCC¹⁵ and promoter CpG island methylation of *mutL homolog1, colon cancer, nonpolyposis type 2* (*E. coli*) (*MLH1*) in sporadic colorectal cancer¹⁶. CIMP CRCs are characterized by frequent promoter CpG island

methylation and subsequent silencing of tumor suppressor genes and DNA repair genes and are associated with MSI and *v-raf murine sarcoma viral oncogene homolog B1 (BRAF)* mutations¹⁷⁻¹⁹. CRCs are hypothesized to develop through one of the three routes: CIN, MSI or CIMP, eventually resulting in the inactivation of tumor suppressor genes and activation of oncogenes and thereby achieving the hallmarks of cancer^{16,20}. This illustrates that CRC is a heterogeneous disease consisting of subgroups with a different biology and each subgroup of CRC is associated with more or less specific morphological features. For example MSI, *BRAF* mutated and CIMP CRCs are associated with a serrated and/or mucinous morphology^{21,22}, whereas the presence of dirty necrosis in malignant glandular lumina is negatively correlated with MSI and thought to be primarily present in CRCs with a high frequency of *APC* and *TP53* mutations (CIN pathway)²³.

Increased proliferation, which is one of the hallmarks of cancer cells, especially at a stage that the tumor has grown beyond a size that can be fed by the surrounding micro-vessels, induces hypoxia^{8,24}. Hypoxia is defined as an oxygen tension below the physiological level, and occurs frequently in solid tumors²⁵. In CRC, the importance of hypoxia has been demonstrated by clinical studies in which hypoxia predicts for worse outcome and resistance to chemotherapy and radiotherapy^{26,27}. Understanding the underlying biology explaining how CRCs will adapt to a hypoxic environment will improve patient risk assessment and predict response to chemotherapy²⁸. The coordinated cellular response to hypoxia that influences the pattern of gene expression is mediated by hypoxia-inducible factors (HIFs) and other cellular pathways such as translation regulation, microRNA induction and chromatin remodelling^{29,30}. Furthermore, under chronic hypoxic conditions epigenetic changes occur, notably a genome-wide adjustment of promoter CpG island methylation, suggesting an adaptation to the altered environment thereby promoting and maintaining a hypoxia-adapted cellular phenotype, with a potential role in tumor development³¹.

Regarding HIF's, there are three homologues of the alpha subunit (HIF-1 α , HIF-2 α , HIF-3 α)³². HIF-1 α is the best understood isoform and together with HIF-2 α , both transcription factors are thought to play a significant role in tumor neovascularization, enhanced cellular proliferation, decreased apoptosis, and development of resistance to chemotherapeutic agents^{30,33-35}. Studying the physiology of tumor hypoxia also implies studying the downstream effects of HIF. These downstream effects are regulated amongst others by *vascular endothelial growth factor (VEGF)* involved in angiogenesis, *glucose transporter 1 (GLUT1)* involved in glycolysis, *carbonic anhydrase IX (CA9)* regulation of pH, *TP53* and *BCL2/adenovirus E1B 19kDa interacting protein 3 (BNIP3)* involved in regulation of apoptosis and autophagy^{36,37}.

In understanding the mechanisms through which tumor hypoxia can induce a more aggressive phenotype, attention should be directed to both the epithelial and stromal tumor compartments. This is illustrated by a recent study in breast cancer showing that not only the epithelial compartment of the tumor, but also the stromal compartment, consisting of (myo)-fibroblasts, endothelial cells, smooth muscle cells, adipocytes, inflammatory cells and nerve cells, has undergone changes in gene expression. The most prominently altered genes included hypoxia-associated genes and this gene expression profile was associated with clinical outcome³⁸. Hypoxia-driven cell biological and metabolic changes might alter the tumor stroma towards an environment that can facilitate cancer progression³⁹. Cancer development depends upon changes in the interactions between epithelial cells and their surrounding stroma, leading to biological effects along several important pathways so-called “hallmarks of cancer”, that collectively constitute malignant growth⁸. Studying these biological processes and correlate it with patient outcome, will besides a better understanding of the underlying tumor biology, identify molecular markers that could improve therapeutic options within CRC, additional to the traditional staging method.

Aim and outline of thesis

The identification of prognostic markers for CRC, to determine risk profile and to identify patients that need adjuvant therapy respectively, is urgently needed. The practice of medicine could benefit from integration of genomic and epigenomic information from both the tumor and stroma, to accurately determine prognosis and select the best therapies for the longest durable responses and the lowest likelihood of toxicity.

The aim of this thesis was to study genetic and epigenetic alterations in relation to morphological features and hypoxia in CRC, providing insight in the underlying biology of this cancer and possibly yielding relevant novel prognostic information. **Chapter 2** reviews the current knowledge on the epidemiology, diagnosis, treatment and the prognostic and predictive value of (epi)genetic markers in CRC. In **chapter 3** we classified CRC by computer assisted unsupervised hierarchical clustering, based on key genetic and epigenetic events in CRC including: CIMP, MSI, and APC-, KRAS-, TP53-, and BRAF mutation status, in a series of 160 CRC cases. Subsequently, we investigated whether these molecular clusters are associated with morphological features, patient characteristics and prognosis. In addition, we evaluated the prognostic significance of promoter CpG island methylation of a variety of tumor suppressor- and DNA repair genes in CRC patients treated with surgery alone, taking into account MSI, BRAF and KRAS mutations (**chapter 4**). **Chapter 5** summarizes the current evidence of altered CHFR

expression in cancer and discusses its promising role as a prognostic and predictive biomarker.

Promoter CpG island methylation and associated gene expression alterations, has been reported to play a role in hypoxia. In **chapter 6** we studied the baseline protein expression of four hypoxia induced markers (hypoxia-inducible factor (HIF)-1 α , HIF-2 α , carbonic anhydrase 9 (CA9) and glucose transporter 1 (GLUT1)), in both the epithelial and stromal compartment of CRC's, and evaluated their association with clinicopathological parameters. In **chapter 7**, we investigated whether hypoxia in CRC is related to promoter CpG island methylation of a series of genes frequently and functionally methylated hallmark of cancer genes. Understanding the mechanisms by which hypoxic tumors can overcome cell death signals (one of the hallmarks of cancer) and adapt is critical for our understanding of tumor progression and development of effective therapeutics in CRC patients with adverse prognostic profiles. Therefore in **chapter 8**, we attempt to elucidate whether changes in the epithelial cell compartment of CRC, such as apoptosis and concomitant (epi)genetic changes, are related to hypoxia-related changes in the stromal compartment. For this purpose, we correlated alterations of *TP53* and *BNIP3* in tumor cells with expression of hypoxia-related proteins HIF-2 α and CA9 in relation with patient outcome and apoptotic activity in CRCs. **Chapter 9** discusses the major findings and potential clinical applications of our research.

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Chapter 2

Pharmacoepigenomics in colorectal cancer;
a step forward in predicting prognosis
and treatment response

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Abstract

Despite therapeutic innovations and increasing education on lifestyle to prevent colorectal cancer (CRC), it is still one of the most common cancer types, and for men the second cause of cancer death. Lately, much attention has been given to identify molecular markers involved in CRC prognosis and treatment with the aim to develop a more accurate classification system based on (epi)genetic alterations and, in addition, find markers that could potentially enhance management of CRC by predicting treatment response in advance. Although many genetic markers have been claimed to have prognostic or predictive influence, results are often inconclusive and, with some exception, they are not used in standard practice. Epigenetic alterations have received less attention although they are probably even more interesting as they can potentially be reversed through drug treatment. This review describes the current knowledge on the prognostic and predictive value of genetic, and especially epigenetic markers in CRC.

Introduction

Colorectal cancer (CRC) is one of the most common cancer types worldwide, with annual incidence rates of 401,000 in men and 381,000 in women. Both environmental and (epi)genetic factors play important roles in CRC aetiology. Most CRCs are thought to develop from precursor lesions (adenomas) which can readily be detected and removed by use of endoscopic techniques. The majority of CRCs are sporadic carcinomas, although a small percentage (<5%) are due to well-defined inherited syndromes such as familial adenomatous polyposis (FAP) and hereditary non-polyposis colon cancer (HNPCC), currently designated as Lynch syndrome. CRC prognosis and treatment are strongly related to the extent of tumor spread, which makes accurate staging highly important. At this moment, the tumor-node-metastasis (TNM) system is the main tool to provide information on prognosis and to determine treatment protocols^{1,2}. However, there is considerable variation in the course of the disease and the treatment response among individuals within the same stage. In recent years, increasing attention has been given to the potential prognostic significance of (epi)genetic markers implicated in CRC, but results of studies are contradictory¹, mainly due to methodological differences and a lack of standardization². In addition to the impact of molecular markers on prognosis, studies have also focussed on the use of molecular markers to predict or influence treatment outcome. Although some markers seem promising, overall results are inconclusive and further studies are necessary before predictive markers can be used to influence therapeutic decisions in daily practice. A clear distinction should be made between molecular markers that act as independent prognostic markers and markers that can predict treatment outcome. A biomarker can only be considered as an independent prognostic marker if it adds additional information regarding the prognosis to clinical characteristics such as TNM-stage and differentiation grade and does not act as an intermediate factor between clinical prognostic factors and outcome³. In addition, to correctly assess whether a biomarker is an independent prognostic factor, it should preferably be studied in an untreated population because treatment is a strong prognostic factor that may bias study results.

Colorectal cancer & prognosis

Epidemiology

CRC is the fourth most common cancer type worldwide. It is estimated that 783,000 new cases are diagnosed each year; a number that has increased rapidly since 1975⁴. Fewer than 20% of cases occur before the age of 50, and the disease affects men and women almost equally⁵.

As many as 70% of CRCs are estimated to develop due to environmental or lifestyle factors. Theoretically, a large number of cases are therefore avoidable. A recent report on diet and cancer concluded that intake of red meat, processed meat, alcohol, body fatness (especially abdominal fat) adult attained height and low levels of physical activity are convincing risk factors for CRC. Intake of calcium and foods containing dietary fibre probably reduces CRC risk. There is only limited evidence for the protective effects of intake of fruits, non-starchy vegetables, and fish; foods containing folate, selenium and vitamin D; and for the detrimental effects of cheese and foods containing iron, animal fats and sugars⁶.

Large survival differences are observed between CRC patients, even between patients within a similar disease stage. For men it is the second most common cause of cancer death in the European Union⁵. Five year survival of the disease is 90% if it is diagnosed while still localized, but this drops to only 68% for regional disease with lymph node involvement and to only 10% if distant metastases are present⁷.

Clinical diagnosis

CRC can take many years to develop. Early detection of CRC greatly improves the prognosis, however, first symptoms are usually vague, like weight loss and fatigue. Local (bowel) symptoms including constipation or diarrhea and bloody stools or rectal bleeding are rare and only occur when the tumor has already grown to a considerable size.

Colonoscopy is the golden standard for routine detection of CRC⁸. It allows for the direct inspection of the colorectal mucosa and biopsy sampling or definitive treatment by polypectomy during the same session⁹. Several CRC screening options are available. These can be subdivided into two categories: (1) structural examinations, which include flexible sigmoidoscopy, colonoscopy, double-contrast barium enema, and computed tomographic colonography and (2) stool tests, which include tests for occult blood or exfoliated DNA.

Molecular knowledge provides a basis for new screening methods that test stool for the presence of DNA alterations that are common in CRC. At present, one commercially stool DNA test (sDNA) is available that includes 21 separate point mutations in genes commonly mutated in CRC and a marker of DNA integrity analysis¹⁰. However, additional research is necessary to further improve this method¹¹. Molecular testing could also be useful to determine CRC risk (e.g. methylation of the *SFRP2* gene)^{12,13}.

Pathological diagnosis

CRC is classified according to the WHO histological classification system which makes a subdivision into several histological subtypes: mucinous and signet ring cell adenocarcinomas, adenosquamous carcinomas, medullary carcinomas and undifferentiated carcinomas¹⁴.

The TNM system, as defined by the UICC (International Union against Cancer, together with the American Joint Committee on Cancer), is most commonly used for pathological staging in routine medical practice¹⁵ and is the most important prognostic factor at the moment. Increasing depth of bowel wall invasion is reflected in the T stage, a scale of T1 (invasion of submucosal layer) to T4 (serosal penetration/invasion of adjacent organs). Based on the combination of T stage, lymph nodes involved (N) and presence of distant metastasis (M), CRCs are grouped in stages from I to IV, with stage I having a 90% 5-year survival, compared to stage IV having a 5-8% 5-year survival.

Genetics of colorectal cancer

Apart from traditional histological classification of CRC, increased cancer biology knowledge has led to the idea of classifying CRCs according to (epi)genetic aberrations. CRC is a genetic disease in which accumulation of mutations in oncogenes and tumor suppressor genes plays an important role. In 1990, Fearon and Vogelstein proposed a model for CRC that describes the transition from early adenomas to carcinomas as a consequence of a growing number of somatic mutations in oncogenes and tumor suppressor genes^{16,17}. In the adenoma-carcinoma sequence, several well-described genetic aberrations are described, such as *APC*, *KRAS* and *TP53*¹⁸⁻²⁰. Most mutations in *APC* result in a truncated APC protein²¹ and are considered to occur early in CRC²². Loss-of-function mutations in *APC* result in nuclear β -catenin accumulation, a key component of the Wnt signaling transduction pathway²³, which modulates the expression of downstream Wnt target genes implicated in cell proliferation, migration, differentiation and apoptosis²². However, it is highly unlikely that these mutations alone are sufficient for the progression of adenomas²⁴. Activating *KRAS* mutations, found in 40-50% of CRCs, abolish GTPase activity and as a result, the protein is locked in the active, GTP-bound state. This leads to increased and unregulated cellular proliferation and malignant transformation. *TP53* can induce apoptosis or cell cycle arrest to correct occurring defects depending on the severity of the cell damage¹. Loss-of-function of *TP53* is seen in approximately 50% of CRCs and is considered a late event during the disease^{17,25}. Other common genetic defects implicated in colorectal carcinogenesis are *BRAF* mutations²⁶, allelic loss of chromosome 18q²⁷, and alterations in DNA mismatch repair genes (MMR). Approximately 10-15% of sporadic CRCs show defects in DNA MMR leading to an

accumulation of alterations in nucleotide repeat sequences; this is defined as microsatellite-instability (MSI)²⁸. Most MSI CRCs are believed to arise mainly by epigenetic silencing of the *MLH1* repair gene²⁹ and are associated with specific tumor characteristics such as proximal location, diploid DNA content, abundant mucin secretion and poor differentiation²⁸. Since the proposition of the adenoma-carcinoma sequence, other aberrations frequently seen in CRC have been described and the Fearon and Vogelstein model is constantly being modified to take into account these alternative pathways to CRC^{24,30}. However only a small proportion of genes has been analyzed and the number of genetic alterations that are responsible for the development of CRC is still unknown³¹.

Cancer Candidate Genes (CAN) in colorectal cancer

The recent determination of the human genome sequence provided the possibility to examine the cancer genome unbiased by systematically screening genetic alterations in cancer instead of focusing on single candidate genes as has been the approach until recently. This has led to the identification of a panel of cancer candidate genes (CAN genes)³¹. CAN genes are most likely to be involved in the neoplastic process and are therefore interesting for further study³². Strikingly, mutation frequencies in the CAN genes were relatively low, in the range of 5-15%³¹. The majority were heterozygous missense mutations (81%): 59% of which were C:G to G:C transitions. Moreover, a large part of the mutations (44%) were found at 5'-CpG-3'dinucleotides³¹. There was also considerable heterogeneity between mutations from different specimens derived from the same tissue. This suggests that single gene alterations reflect only a small part of the tumor mutational spectrum. This could perhaps explain why it has proven to be difficult to find associations between a single gene mutation and prognosis or therapy response³¹. It also confirms the idea that pathways rather than individually mutated genes are responsible for the course of tumorigenesis³³. Indeed, many mutated CAN genes are involved in phosphatidylinositol 3-kinase (PI3K) signaling or in pathways related to cell adhesion³².

Genetic markers and CRC prognosis

The majority of CRC, about 85%, are characterized by chromosomal instability (CIN) which is associated with microsatellite stability (MSS), aneuploidy and genetic alterations in genes such as *KRAS*, *APC* and *TP53*³⁴. A recent meta-analysis suggested a poorer prognosis for CIN tumors, irrespective of anatomical location or treatment with chemotherapy³⁵. Because of this, CIN could be useful for further stratification after standard pathological staging³⁵. Nevertheless, individual study results on the prognostic influence of CIN are inconsistent and the 2006 American Society of Clinical Oncology (ASCO)

guidelines state that measuring CIN in CRC is nothing more than an experimental tool^{35,36}. Indeed, the question remains whether it is really the effect of CIN that has been measured in previous studies. Individual genetic alterations could possibly act as markers for CIN³⁵. In a number of studies, *KRAS* has been associated with a poorer prognosis, independent of other prognostic characteristic such as tumor stage in a number of studies. However, other studies were not able to replicate this finding and therefore the clinical relevance of these findings is still unclear^{1,3,37,38}. Similarly, inactivation of *TP53* and mutations in *BRAF* have been associated with a poorer prognosis, but not consistently^{1,3,38-40}. Previous studies also suggested that loss of chromosome 18q is associated with a poor survival in stage II and stage III patients^{41,42}. Gain of chromosome 20q has also been associated with a worse patients survival⁴³. In addition, aneuploid or tetraploid DNA content has been associated with a higher tumor stage and distal tumor location⁴⁴, which are known to cause a poor prognosis.

Other genetic markers not associated with CIN have also been suggested to play a role in CRC prognosis. Single Nucleotide Polymorphisms (SNPs) in the gene that codes for thymidylate synthase (TS) (TSER polymorphisms) results in reduced TS expression and have been associated with a reduced CRC risk⁴⁵. Data on the prognostic role of these polymorphisms are inconsistent⁴⁵⁻⁴⁸ and independency from tumor stage is only reported in one study⁴⁶. Over expression of TGF- β in the primary tumor and in tumor associated-stroma has also been suggested as a prognostic factor in CRC, but results are scarce and for overexpression, independency from tumor stage was not reported^{3,49-51}. Similarly, epidermal growth factor receptor (EGFR) expression has been studied in relation to prognosis, but results are inconsistent and an independency of tumor stage has not been proven; a clear relationship between EGFR expression and CRC prognosis has therefore not been established^{3,52}.

Although there are many more claims of prognostic significance of other genetic markers or gene expression in CRC, the question of which marker is strong enough to be applied in daily clinical practice remains unanswered because finding consistent associations between genetic markers and CRC prognosis has proven to be difficult. In addition, questions have arisen as to whether the progression and prognosis of CRC could be attributed to mutations alone. Because the frequency of mutations in the CAN genes is often low and the majority are missense mutations, it is likely that additional mechanisms contribute to tumor progression and prognosis.

The role of epigenetics

Epigenetic silencing is a prevalent mechanism that could lead to abnormal gene inactivation in cancer⁵³. It has been suggested that aberrant promoter

CpG island hypermethylation leading to gene silencing also plays an important role in the neoplastic process, and molecular evidence of this concept has been accumulating since the late 1990s^{54,55}. Epigenetic alterations may cooperate with genetic abnormalities to affect gene function, eventually leading to cancer⁵⁶. Hypermethylation occurs when DNA methyltransferases (DNMTs) add a methyl group to cytosine to form methyl-cytosine. In humans, these epigenetic alterations include methylation of cytosine located in CpG-islands and may be tissue-specific^{55,57}. Methylation within or around the promoter region is associated with gene silencing⁵⁵. Methyl-cytosine-binding proteins and histone deacetylases are recruited to modify nucleosomes, forming transcriptionally repressive chromatin⁵³. Traditionally, epigenetic events have primarily been investigated in classic tumor suppressor genes. In this setting, methylation acts as a “second genetic hit” to inactivate the gene⁵³. However, more recently, Baylin and co-workers demonstrated that the majority of these genes show promoter CpG island hypermethylation in addition to the genetic alterations^{53,58}. Most mutations found in the CAN genes were heterozygous missense mutations suggesting that these result in haploinsufficiency which is augmented by DNA methylation. In many cases involving tumor suppressor genes with low mutation rates, hypermethylation is a much more frequent event than genetic alterations. This implies that pathway analysis would show that CRCs are not that heterogeneous when both genetic and epigenetic alterations are taken into account⁵³.

So far, CRCs have been genetically categorized as either CIN or microsatellite instable (MIN). In addition to these two categories, a subset of CRCs, 17%-29%⁵⁹⁻⁶¹, with a high frequency of CpG-island methylation has recently been described as the CpG-island methylator phenotype (CIMP)^{59,62-64}. However, the existence of CIMP and role of CIMP in CRC carcinogenesis has been controversial, mainly due to lack of a generally accepted standard definition and the confounding role of MSI in determining the CIMP phenotype^{60,63,65}. Tumors characterized by CIMP have been associated with *BRAF* mutations, older age, advanced tumor stage and proximal location²⁶. CRCs with less extensive promoter methylation are proposed to be characterized by CIMP-L (CIMP low) and it is hypothesized that these tumors are different compared to CIMP-H (CIMP high)^{26,63}. However, evidence is still scarce and this hypothesized distinction between CRCs based on the level of methylation needs further investigation and definition. The distinct molecular genotypes that have been described, CIMP, CIN and MIN, do not seem to be mutually exclusive and thus assessing a single molecular phenotype may not provide all relevant prognostic information³⁵.

Epigenetic markers and CRC prognosis

The CIMP panel according to Weisenberger et al.⁵⁹ has been associated with poor survival in metastatic MSS CRCs⁶⁶. However, the influence of this panel has not yet been assessed in other stages, and the use of this panel as a prognostic factor remains unresolved. One study reported an association between CIMP (although defined differently, namely using the panel *p16^{INK4A}*, *MINT2* or *MDR1*) and a worse prognosis among stage III patients treated with surgery alone⁶⁷. In addition, the CIMP panel according to Toyota et al.⁶² was associated with worse survival in stage I-IV CRC patients⁶⁸. This was not observed in another study⁶⁹ suggesting that other epigenetic markers may be responsible for the association. As stated by Ogino and colleagues, additional studies are necessary to further examine the prognostic value of methylation markers among CRC patients in the different stages⁶⁶.

Recently, Chan and colleagues combined information on the methylation of the CAN genes with information from gene expression databases to gain more insight into the prognostic roles of these genes⁵³. They analyzed eight genes that were predicted to have a decreased overall expression in CRC by the analysis of microarray data. Two of those genes (*PTPRD* and *RET*) were found to be associated with clinical characteristics such as metastasis and grade. However, a number of other CAN genes which were determined to not have altered expression levels related to DNA methylation, no associations with clinical characteristics were found⁵³. These results have not yet been validated in other, independent populations.

Despite the growing number of studies on molecular genotypes in CRC, many questions remain unresolved. For example, what is the prognostic relationship of the different genotypes? It is known that they are not mutually exclusive. MIN and CIMP appear to be associated, making the conclusion of whether either one is an independent prognostic factor even more difficult³⁵. MSI-H has consistently been associated with better prognosis and decreased metastasis risk⁷⁰⁻⁷², although this is often associated with high frequency of CpG island methylation. The presence and role of MSI-L (MSI low) remains controversial. Mutations often seen in MSI-H appear to be absent in MSI-L and until now, no large differences in clinical or molecular characteristics have been observed between MSI-L and MSS patients²⁶. However, reports suggest that MSI-L is indeed a unique subtype of CRC which is associated with loss of *O⁶-MGMT*⁷³ and related to a poor prognosis in stage III CRC⁷⁴. Ward et al. showed that DNA methylation in CIMP-associated genes was related to a poor outcome in CRC, but this adverse prognostic effect was lost within those methylated tumors showing MSI⁷⁵. There seems to be considerable overlap between CIMP and MSI and it has been suggested that MSI-H has a major effect on the expression of CIMP⁶⁰ although more recent results imply that MSI-H in

sporadic CRC can be explained by CIMP-H associated *MLH1* methylation⁵⁹. These results suggest that MMR deficiency in sporadic CRC is caused by a broad epigenetic control defect affecting *MLH1* in most CIMP tumors; an accurate definition of CIMP could therefore be essential in understanding the role of MSI-H⁵⁹.

At this moment, reports in the literature are too limited to understand the interplay between CIN and MIN in CRC prognosis. MSI-H is consistently associated with a better prognosis in CRC, but the underlying explanation for this finding is not yet clear. It has been suggested that this is due to the fact that allelic loss or mutation of 18q, *TP53*, or *KRAS*, which are thought to be associated with a poor prognosis, are rare in MSI tumors⁷⁰. However, a recent study on the relation between CIN and MIN concluded that the survival benefit in MSI-H patients was not dependent of CIN status^{35,44} thereby questioning this explanation. In addition, DNA diploidy seemed to be a stronger prognostic factor than MSI in patients treated with 5-FU, and the effect was similar in both MSI-H and MSS/MSI-L cases. This could indicate that diploidy may contribute to the often reported better survival in MSI-H patients⁴⁴. Nevertheless, diploidy did not seem to be related to treatment response, suggesting that the less aggressive behavior of diploid tumors is not the result of a better treatment response⁴⁴.

Colorectal cancer treatment

Due to therapeutic innovations, management of advanced CRC has changed considerably in recent years⁷⁶. This has led to an increase in survival, although not substantially. The mainstay of treatment is surgery, with primary tumor, adjacent bowel and lymph nodes removal⁷⁷. Although surgery may be performed for palliative symptom control in advanced cases, in most circumstances it is performed with curative intent. However, with an increasing tumor stage, the chance of cure by surgery decreases. For early-stage CRC (Stage I) only resection is required, without adjuvant therapy⁷⁷. Stage III and IV CRC patients additionally receive adjuvant or palliative chemotherapy. The benefit of adjuvant 5-fluorouracil (5-FU) and leucovorin (LV) has been well established in stage III colon cancers, and is the current standard of care⁷⁸. More recently, oxaliplatin-based chemotherapy, added to regimens containing 5-FU/LV has been shown to further improve disease-free and overall survival in these patients⁷⁹. Other cytostatic agents that have been introduced include DNA topoisomerase I inhibitors, such as irinotecan.

For stage II CRCs, no survival benefit from postoperative chemotherapy in patients with standard risk has been shown in randomised clinical trials, but, despite the initially good prognosis, 20-30% of these patients will die from CRC

within 5 years. Unfavourable tumor features in stage II CRCs, such as serosal tumor penetration (T4), tumor perforation, and (extramural) lymphovascular invasion appear to be correlated with an increased risk for recurrence. These features may identify high-risk disease patients, who might benefit from postoperative therapy⁸⁰. Previously, a survival benefit has been suggested for selected stage II tumors after adjuvant chemotherapy⁸¹.

Recently, therapeutic strategies have also involved the use of humanized monoclonal antibodies, such as bevacizumab and cetuximab. Bevacizumab is a monoclonal antibody directed against vascular endothelial growth factor (VEGF); it improves the outcome of first-line chemotherapy (5-FU/LV)⁸². Cetuximab is a monoclonal antibody directed at the EGFR, and is thought to inhibit signal transduction through this pathway. It is added to chemotherapy with irinotecan (DNA topoisomerase I inhibitor)⁸³. Recently, the European Medicine Agency licensed cetuximab and panitumumab, another EGFR-targeted monoclonal antibody, only for patients with metastatic CRC without *KRAS* mutations after several reports showed an association between *KRAS* or *BRAF* mutations and clinical resistance to treatment with EGFR antibodies in metastatic CRC^{52,84-88}. Nevertheless, even among wildtype *KRAS* CRC patients, response rates for EGFR antibodies are low, ranging from 17% (for EGFR antibody monotherapy) to 61% (for EGFR antibody therapy plus oxaliplatin-based chemotherapy). These observations suggest that other mechanisms are involved in therapy resistance. For example, TSER polymorphisms have been implicated as determinants of 5-FU treatment response, with several studies suggesting a poor response to chemotherapy with the 3R/3R genotype⁸⁹⁻⁹² although others reported a better response with this genotype^{46,93}. Other polymorphisms, such as those in *MTHFR* and *GSTP1* have also been related to treatment response in CRC, but evidence is still scarce and inconclusive⁹⁴.

Evidence for a role of MSI in predicting treatment outcome is also not conclusive. One study has shown that patients with MSI-H did not benefit from treatment with 5-FU but MSS or MSI-L tumors did seem to benefit from this treatment⁹⁵. Another study was not able to replicate this finding and even observed an opposite effect in MSI-H tumors that had a greater benefit from 5-FU⁹⁶. With the current evidence, it is not yet possible to draw any conclusions regarding the question of whether MSI-H could be used as a predictive marker to distinguish patients that would benefit from 5-FU treatment or not. Given the molecular heterogeneity of the MSI genotype, it may be very difficult to use MSI-H as a single predictive marker for CRC treatment²⁶.

In addition to clinical response, pharmacogenetic studies have also focussed on the impact of genetic alterations on drug toxicity^{94,97}. While these findings are interesting and have led to the first genetic test approved by the U.S. Food

and Drug Administration, the *UGT1A1* test for the prediction of irinotecan toxicity, this topic is beyond the scope of this review.

Implications of epigenetic markers in colorectal cancer treatment

A critical difference can be made between gene silencing as a result of mutations or aberrant promoter methylation. While mutations are irreversible, epigenetic changes are potentially reversible and therefore present potential new opportunities for cancer treatment. Because of this, studies on epigenetic markers and the prediction of treatment response are gaining increasing attention. For example, loss of *O*⁶-*MGMT* function through hypermethylation is thought to sensitize cells to the effects of chemotherapy based on alkylating mechanisms, since cells have a diminished capacity to repair alkylation damage⁹⁸. Early studies in brain tumors and lymphomas have indeed shown that patients with methylated *O*⁶-*MGMT* respond better to alkylation treatment⁵⁵. In addition, over the last few years, new molecular techniques have been developed which allow researchers to more easily determine DNA methylation. Because of this, DNA-methylation-based technologies have a promising future in both clinical diagnostics and therapeutics⁹⁹. However, results on pharmacoepigenomics are still scarce.

CpG Island methylator phenotype

The role of aberrant promoter methylation as a predictive marker for treatment response is inconsistent. Although not statistically significant, a trend towards resistance to chemotherapy among CIMP-H tumors has been observed⁶⁶. Furthermore, patients with advanced CRC and hypermethylation of multiple genes, including CIMP-specific genes, have a poor outcome following standard 5-FU-based chemotherapy¹⁰⁰. However, these results could not be replicated by another study that showed contrasting data; a survival benefit for CIMP patients after 5-FU treatment⁶⁷.

Even though the number of studies on the predictive effect of molecular genotypes is slowly increasing, a conclusive answer to the question of whether they are predictive of treatment effect cannot be given at the moment. Further research is needed to resolve this issue. However, although the influence of molecular genotypes on treatment response is one of the main focuses of current research, other markers are potentially important for the future treatment of CRC.

HDAC2

Disruption of histone acetylation patterns is common in cancer cells and histone modifications that affect chromatin structure are implicated in the inactivation of tumor suppressor genes^{101,102}. Recently, truncating mutations in

one of the human histone deacetylases, HDAC2, were described in cancer cell lines and CRCs with MSI¹⁰². Histone deacetylases maintain gene silencing through histone deacetylation⁵⁵ and are potentially clinically relevant as they might be responsible for the response to HDAC inhibitors; drugs that show promising anticancer activities in clinical trials^{102,103}. Indeed, a recent study reported that the presence of a truncating mutation in *HDAC2* leads to a loss of protein expression and consequently more resistance to the antiproliferative and proapoptotic effects of HDAC inhibitors¹⁰². This report also suggested that HDAC2-deficiency confers resistance to TSA-induced histone acetylation, but this could not be confirmed by others¹⁰⁴. Furthermore, it has been demonstrated that cells lacking HDAC2 are not able to induce APAF1, which appears to be the key to apoptosis induced by HDAC inhibitors¹⁰⁵. These recent findings support the use of *HDAC2* mutational status as a guide for the prescription of HDAC inhibitors in CRC. However, literature on this topic is still limited and more research on the potential use of *HDAC2* as a marker in CRC is needed.

Werner (WRN)

Other epigenetic markers, such as those associated with metastasis, have been suggested to act as prognostic or predictive markers¹⁰⁶. One of these is promoter CpG island methylation of the Werner syndrome gene (*WRN*), which has been associated with longer survival of patients treated with chemotherapy¹⁰⁷. The *WRN* gene and its protein are involved in the maintenance of telomere structure and initiation of DNA damage response after telomere disruption¹⁰⁸. As it was observed that patients with *WRN* germline mutations often develop epithelial or mesenchymal tumors, it was suggested that the *WRN* gene had a tumor-suppressor function¹⁰⁷. It has been demonstrated that *WRN* is inactivated due to hypermethylation in human cancer cells. Inactivation of WRN leads to hypersensitivity to topoisomerase inhibitors, such as irinotecan which is often used in the treatment of CRC, and DNA-damaging agents¹⁰⁷. Indeed, recent studies have observed an increased survival in patients with *WRN* methylation after treatment with a topoisomerase inhibitor¹⁰⁷. In addition, WRN methylation appears to be associated with mucinous differentiation in colorectal tumors independent of MSI or CIMP¹⁰⁸. Mucinous differentiation is also suggested to be associated with other molecular tumor characteristics, including MSI-H, *BRAF* mutations, *KRAS* mutations and loss of *TP53*¹⁰⁸. This raises questions of whether WRN, in addition to its possible role as predictive marker for treatment response, could also have a prognostic influence in CRC. Moreover, the observation that *WRN* methylation is associated with increased survival in CRC after treatment with irinotecan needs further exploration and confirmation in other larger populations before this marker can be used.

CHFR

Recently, *CHFR* was identified as a mitotic checkpoint gene, located on chromosome 12q24.33¹⁰⁹. *CHFR* functions early in the G2/M transition and mediates a delay of entry into metaphase in response to mitotic stress induced by nocodazole or paclitaxel. *CHFR* functions as a tumor suppressor and several groups have demonstrated that *CHFR* expression is lost or decreased in most human cancers, mostly due to DNA promoter hypermethylation. Epigenetic loss of *CHFR* expression is however rare in primary breast cancer^{110,111}. In endometrial and cervical cancer, it has been shown that DNA hypermethylation of *CHFR* predisposes cancer cells to a higher sensitivity to taxanes. Cell lines hypermethylated for *CHFR* with high sensitivity to taxanes became resistant after demethylation^{112,113}. It has been suggested that *CHFR* methylation status could act as a new molecular index, allowing for the design of personalized treatment in endometrial cancer. In advanced gastric cancer however, *CHFR* hypermethylation is not consistently associated with response to docetaxel and paclitaxel, both of which are taxanes^{114,115}.

While taxanes are not used in the adjuvant treatment setting of CRC, because they failed to demonstrate a significant clinical benefit in phase II trials in CRC¹¹⁶, frequent hypermethylation of *CHFR* is detected in CRC. Approximately 37% of all primary CRCs are hypermethylated for *CHFR*^{117,118}, raising the possibility that *CHFR* methylation could be used in CRC to identify those patients who might benefit from taxanes.

Although application of these molecular markers is not yet accepted in standard patient care, they serve as examples of a shifting paradigm, drawing on the notion that awareness of molecular alterations in tumors can be harnessed for more selective and effective treatment schedules. It is both conceivable and desirable that molecular profiling will more accurately identify those patients with a greater chance of benefitting from adjuvant treatment. These predictive molecular profiles will help define the best way to integrate chemotherapy with biology.

Future perspectives

Recently, increasing attention has been given to the role of genetic and epigenetic alterations in attempting to answer the question of which CRC patients should receive (adjuvant) treatment and which treatment is most suitable for an individual patient. Numerous studies have been published in the last decade discussing the influence of (epi)genetic markers on CRC prognosis and treatment outcome prediction.

Although epigenetic alterations are heritable in somatic cells, they could potentially be reversed by drug treatment; a characteristic that is not shared by genetic alterations. This could have significant implications for the treatment of

CRC. The recent publications on the use of HDAC inhibitors in clinical trials yielding promising results, suggest that the use of epigenetic therapy is promising. In addition, epigenetic alterations are frequent in CRC, even more frequent than genetic alterations, and recent new molecular techniques enable researchers to easily assess methylation status and identify novel epigenetic markers. Because of this, it is conceivable that epigenetic markers will eventually have a high impact, maybe even higher than to genetic markers, on the management of CRC.

Figure 2.1 provides an overview of the current knowledge on (epi)genetic markers discussed in this review and their role in prognosis and treatment prediction. A more extensive staging system based on (epi)genetic markers will have more impact on stage II and III patients, as it is often not clear which patients to treat or which treatment to prescribe to these patients. Although many studies have led to the overview in Figure 2.1, results are often inconsistent and many findings have not yet been replicated in other, independent populations. Because of this, it is difficult to draw definitive conclusions on the prognostic or predictive role of these (epi)genetic markers.

Figure 2.1 Current knowledge on (epi)genetic markers and prognosis / prediction

High-throughput technologies and novel approaches

In the last few years, an increasing number of molecular techniques to detect DNA methylation on a larger scale has been reported. Following the development of bisulfite sequencing¹¹⁹, rapid progress has been made in the characterization of the methylation state of individual cytosines. However, as this involves locus-specific amplification, it is challenging to use on a large scale¹²⁰. Bisulfite-conversion based microarrays and high-throughput PCR sequencing approaches, and more recently, next-generation bisulfite sequencing¹²¹ have been developed in an attempt to use this approach on a larger scale. In 2002, Suzuki and colleagues described a new microarray-approach¹²², a method which was recently adapted by Schuebel et al.^{53,58,122}. This approach has proven to be successful in identifying genes silenced by hypermethylation which can subsequently be studied for prognostic influence as described by Chan and colleagues⁵³. Methylated CpG island amplification (MCA) coupled with Representational Difference Analysis (RDA) has been described as a tool to identify methylation at multiple loci¹²³. Alternatively, immunoprecipitation-based methods (ChIP) can be used for distinguishing between methylated and unmethylated fractions^{120,124,125}. Other methods either use 5-methylcytosine-specific antibodies (methylated DNA immunoprecipitation, MeDIP or MDIP) or methyl-binding domain proteins to enrich for the methylated fraction of the genome¹²¹. Indirect approaches for DNA methylation profiling on array platforms have also been developed which are based on the high affinity of some proteins to methylated DNA. This technique, the methylated-CpG island recovery assay (MIRA), has already been used in a genome-wide screen to identify DNA methylation markers^{125,126}. Although many methylation-associated gene silencing events in human cancer have been studied, little is known about why some CpG islands are methylated in cancer whereas others seem resistant to this *de novo* methylation. In 2003, Feltus and colleagues described that not all CpG islands are equally affected¹²⁷. Using DNA pattern recognition and supervised learning techniques, they obtained a classification function that could be used to distinguish CpG islands sensitive to methylation¹²⁷. Other techniques also make use of a bioinformatics approach. In 2002, several tumor suppressor gene candidates in esophageal squamous cell carcinoma were identified using a technique based on functional reactivation of epigenetically silenced tumor suppressor genes by 5-aza-2'-deoxycytidine and TSA in combination with an intuitive algorithm¹²⁸. Recently, next generation sequencing technologies processing millions of sequence reads in parallel, have emerged as powerful tools for whole genome profiling of epigenetic modifications. Next generation sequencing technologies have successfully been combined with ChIP techniques (ChIP-seq)¹²⁹. These studies demonstrate that different genome-wide approaches can successfully

be combined in order to gain more insight and knowledge on cancer epigenomics.

Conclusion

Recent innovations suggest that the current technology could indeed lead to the knowledge necessary to enable the implementation of epigenetic therapies in everyday practice. However, from a scientific point of view, many questions remain unresolved and although epigenetic alterations seem to be promising prognostic and predictive markers for CRC, some issues should be taken into account when assessing the clinical use of pharmaco(epi)genomics. Despite numerous studies on the relevance of molecular markers in oncology, the number of clinically useful markers is limited¹³⁰. Research on molecular markers is susceptible to publication bias and false-positive results, due to small population sizes and small effect sizes¹³¹⁻¹³³. Often, an initial study shows promising results that cannot be replicated in later studies^{130,131}. Moreover, the majority of previous studies describe single molecular markers³, even though it is suggested that using an approach of combining several markers is more likely to identify relevant prognostic factors¹³⁴. The available evidence on the prognostic or predictive influence of molecular markers is limited, even though this question has received much attention. It is likely that epigenetic alterations play a role in CRC prognosis or treatment response, however, it is not clear to what extent, and if this role is independent of other known prognostic factors, such as tumor stage. To answer these questions and to maximize the potential of the novel technologies, 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 collaborate and join the Alliance for the Human Epigenome and Disease (AHEAD) in order to help solve the problem of cancer¹³⁵. AHEAD is aiming to define the patterns of epigenetic regulation occurring in different cell states and therefore complements other ongoing projects such as ENCODE that aim to define the functional sequences in the genome¹³⁵.

More research is necessary to assess the prognostic and predictive influence of epigenetic markers in CRC and consequently, to determine the clinical use of such findings. However, existing technologies provide us with the tools so that in time, we will be able to fill all the voids in our current understanding of pharmacoepiggenomics in CRC and we will be capable to accurately answer the questions of which CRC patients are in need of a more aggressive treatment, and which treatment is most suitable for an individual patient.

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Chapter 3

Computer assisted (epi-)genotypic
categorization of colorectal cancer identifies
CIMP and *TP53* mutations as leading
classification tools

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Abstract

The increasing knowledge of (epi)genetic alterations in CRC and the observed variation in the course of the disease within the same CRC stage, have led to the recognition that classifying CRCs on the basis of molecular events could improve traditional classification. In the current study we molecularly classified CRCs using computer assisted unsupervised hierarchical clustering of genetic and epigenetic alterations and correlated this with clinicopathological parameters.

Genetic (mutations in *APC*, *KRAS*, *TP53*, *BRAF*, and microsatellite instability), and epigenetic (CpG island methylator phenotype, CIMP) alterations were determined in 160 colorectal cancers. Morphologic features were refined by inventarisation of differentiation grade, mucinous differentiation, dirty necrosis, circumscribed tumour growth, tumour budding and lymphocyte infiltration. Unsupervised hierarchical clustering was used to cluster CRCs based on these molecular characteristics. Correlations between these clusters, morphology and outcome were investigated.

Unsupervised hierarchical clustering divided CRC's in CIMP+ (CL2, 22% (36/160)) and CIMP- tumors. The latter group could be further subdivided in *TP53* mutated tumors (CL3, 37% (59/160)), and a group of CIMP- and *TP53* wildtype tumors (CL1, 41% (65/160)). CL2 consisted of two subgroups of 29% (10/35) microsatellite instable (MSI) and 71% (25/35) microsatellite stable (MSS) tumors. There was no consistent relationship between these clusters and morphology, except for mucinous differentiation, which was related to right-sided CL2/MSI/*TP53* wildtype tumors ($p=0.023$). There was no impact of computerized clustering on survival of CRC patients. In rectum tumors on the other hand, a poorer patient survival was noted for CL2 tumors as compared to CL3 tumors ($p=0.01$).

Molecular clustering of colorectal adenocarcinomas shows that CIMP status is the principal classifier, and that both CIMP+ and CIMP- tumors are further classified on the basis of *TP53* mutational status. Hierarchical clustering is only modestly related to morphology and outcome, which appear to be dependent on additional factors such as microsatellite stability and localization in the intestine. Although current molecular clustering provides knowledge in the underlying biology of CRC, it did not improve traditional classification with respect to prognostic value.

Introduction

The increasing knowledge of molecular alterations in CRC and the observed variation in the course of the disease and treatment response within the same CRC stage, have led to the recognition that classifying CRCs on the basis of molecular events could improve traditional classification¹⁻⁴. CRCs are currently classified according to traditional clinical and pathological features². According to the WHO histological typing system, adenocarcinoma is by far the most prevalent diagnosis (>95%)⁵. Adenocarcinomas are graded predominantly on the basis of the extent of glandular formation, and are classified as low-grade (encompassing well and moderately differentiated adenocarcinomas) and high-grade (including poorly differentiated adenocarcinomas and undifferentiated carcinomas)^{5,6}. The tumor-node-metastasis (TNM) system⁷, as defined by the UICC (International Union against Cancer, together with the American Joint Committee on Cancer), is the most commonly used staging system, and constitutes the most important prognostic factor and adjuvant treatment indicator for patients with colorectal cancer in routine medical practice. However, this traditional mode of classifying CRC through typing, grading and staging does not account for tumor heterogeneity. Each CRC patient has a unique disease that has been caused by distinctive biology^{8,9}. The presence or absence of specific molecular alterations could predict the response to targeted individualised therapy and overall prognosis in contrast to the traditional classification.

The precise incidence and clinical presentation of molecularly defined profiles in CRC are subject to ongoing research. With respect to this, genetic instability and DNA methylation play a pivotal role. Well characterized forms of genetic instability are chromosomal instability (CIN) and microsatellite instability (MSI)^{10,11}. CIN includes genetic events occurring through accumulation of numerical or structural chromosomal abnormalities (aneuploidy)¹². The CIN pathway is associated with mutations in key genes involved in the adenoma-carcinoma sequence of CRC: *APC* (85%), *KRAS* (40%) and the tumor suppressor gene *TP53* (50%)¹². MSI results from failure of the mismatch repair (MMR) system. In cases of sporadic colorectal cancer, MMR dysfunction is strongly associated with bi-allelic DNA promoter methylation of *hMLH1*¹³. A third main pathway in the carcinogenesis of CRC is the CpG island methylator phenotype (CIMP) which refers to a subset of tumors with an exceptionally high frequency of promoter CpG island methylation of tumor suppressor-and DNA repair genes. CIMP+ tumors represent a distinct group of tumors, including the majority of cases of sporadic colorectal cancer with MSI and tumors with mutations in the *BRAF* oncogene¹⁴.

A more sophisticated theoretical categorization of CRC in five distinct molecular subtypes was proposed by Jass², based on previous publications of

underlying genetic instability and the presence of promoter CpG island methylation within CRC. A group of sporadic CRCs was suggested consisting of CIMP-high, MSI-high and *BRAF* mutated tumors, presumed to originate in serrated polyps and approximately being present in approximately 12% of all sporadic CRCs. A second group representing approximately 8% of sporadic CRCs, consisted of CIMP-high, *BRAF* mutated and MSI-low tumors, also originating from serrated polyps. CRC tumors with CIMP-low status, MSS or MSI-low with a *KRAS* mutation, originating from either adenomas or serrated polyps formed a third group, with a presumed 20% overall frequency within sporadic CRCs. A large group of almost 57% CRCs, named sporadic or FAP-associated CRCs were designated as a fourth group, containing CIMP- and MSS tumors, supposedly being derived from traditional adenomas. The last minor group (3% frequency) of CRCs, named Lynch Syndrome associated or familial MSI-H tumors, contained CIMP- and MSI-H tumors, also derived from traditional adenomas². More or less characteristic clinicopathological and morphologic features have been attributed to each of these suggested CRCs subgroups².

Studying the association between molecularly defined clusters in CRC and their clinicopathological features is important for the improvement of therapeutic options within CRC. Therefore, in the light of numerous previous efforts that have been undertaken to unravel the complex underlying molecular changes in CRC, we classified CRC by computer assisted unsupervised hierarchical clustering, based upon well described markers in the genesis of CRC including: CIMP, MSI, *APC*-, *KRAS*-, *TP53*-, and *BRAF* mutation status, in a series of 160 CRC cases. Subsequently, we investigated in which way this classification was related to patient data, including clinicopathological parameters, morphological features (differentiation grade, mucinous differentiation, dirty necrosis, circumscribed tumour growth, tumour budding and lymphocyte infiltration) and biological behavior in terms of patient prognosis.

Materials and methods

Patient population

Patients were 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 colonic cancer by conventional surgery or the no-touch isolation technique¹⁵. 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 undergo 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. Hereby a major advantage is achieved since no bias of different adjuvant therapy protocols was introduced. After surgery, tumor tissues and lymph nodes were fixed in buffered formalin, sectioned, and embedded in paraffin. Experienced pathologists documented the histopathological characteristics of the tumors, 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 analysis, tumor tissues from 160 patients with primary colorectal cancer were available. The distribution of age, gender, tumor stage, location and type of tumor, frequency of events and mean follow-up time of the patients in this study are representative for the patients in the trial and are provided in Table 3.1.

Table 3.1 Clinicopathological characteristics of CRC series.

	Total
Age	
Mean age (SD)	67.7 (11.6)
Gender	
Male	75/160 (47%)
Female	85/160 (53%)
Tumor location	
Right-sided colon	59/160 (37%)
Left-sided colon	45/160 (28%)
Rectum	56/160 (35%)
Tumor Grade	
Well/Moderately	118/141 (84%)
Poor	23/141 (16%)
CRC Stage	
I	3/160 (2%)
II	93/160 (58%)
III	47/160 (29%)
IV	17/160 (11%)
Event frequency**	58 (37%)
Median follow up time	4,7 years

SD: Standard Deviation, ** colorectal cancer specific death

Genomic DNA Isolation

Genomic DNA was extracted from CRC tissues using PureGene™ Genomic DNA Isolation Kit (Gentra Systems) according to the manufacturer's protocol.

Methylation-specific PCR

Promoter CpG island methylation of the following genes: *calcium channel, voltage-dependent, T type, alpha-1G subunit (CACNA1G)*, *insulin-like growth factor 2 (somatomedin A) (IGF2)*, *neurogenin 1 (NEUROG1)*, *runt-related transcription factor 3 (RUNX3)* and *suppressor of cytokine signaling 1 (SOCS1)*, was determined using sodium bisulfite modification of genomic DNA using the EZ DNA methylation kit (ZYMO research Co., Orange, CA). Methylation Specific PCR (MSP) was performed as described in detail elsewhere^{16,17}. 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 3.S1.

Microsatellite instability

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 described¹⁸. MSI was defined to be present if ≥ 3 of 5 markers (BAT-26, BAT-25, NR-21, NR-22 and NR-24) showed allelic size variants.

BRAF, *KRAS*, *APC* and *TP53* mutation analysis

The common V600E *BRAF* mutation in exon 15 was analyzed by a semi-nested PCR and subsequent RFLP analyses as previously described¹⁹.

For *KRAS* mutation analysis a flanking 179 bp PCR product was amplified including codons 12 and 13 as described previously²⁰.

Since the majority of somatic mutations in *APC* occur within the MCR, we amplified the MCR as four overlapping fragments (codons 1286-1520) in a nested PCR strategy. Flank PCR was performed to generate two fragments A and B. Fragment A was used as starting material for the amplification of nested fragments S1 and S2, and fragment B was used for nested fragments S3 and

S4 as previously described²¹. Mutation analyses of *TP53* exons 5-8 was performed using a semi-nested PCR approach, (see supplemental Table 3.S2 for primer sequences). CaCo2 (exon 6, codon 204 non-sense mutation) was included as a positive control. Direct sequencing of PCR products was performed using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) and analysed on the ABI 3730 DNA Analyzer (Applied Biosystems). Mutation detection was performed using Mutation Surveyor DNA Variant Analysis Software v3.0 (SoftGenetics LLC, USA). Tumors with silent mutations or a common polymorphism were classified as having wild-type *TP53*.

Figure 3.1 Illustrations of CRCs with: (A) serrated morphology; (B) mucinous morphology; (C) locations of “dirty necrosis”; (D) well differentiated tumour morphology; (E) poorly differentiated tumour morphology; (F) circumscribed tumour growth pattern; (G) area of tumour budding (black arrows); (H) tumour infiltrating lymphocytes; (I) localisation of Crohn-like lymphoid aggregates (black arrows). (A,B,D-G, I) Original magnifications $\times 20$. (C,H) Original magnifications $\times 40$.

Morphological characteristics

Figure 3.1 A-I illustrates the morphological characteristics we determined in our study population. Initially we scored several morphological features including:

serrated morphology, mucinous differentiation, dirty necrosis, differentiation grade, circumscribed tumor growth, tumor budding and lymphocytic infiltration, including Crohn-like host response or diffuse tumor infiltrating lymphocytes. Serrated morphology was left out of our analyses due to the low observed frequency (n=1) in our study-population. Poor differentiation grade was present in 16% of cases, mucinous differentiation was present in 16%, dirty tumor necrosis in 59%, circumscribed invasion pattern in 35%, 21% showed tumor budding at the invasive margins and 23% of all cases showed a clear lymphocytic infiltration in the tumor. For details on scoring methods of morphologic characteristics see below.

Differentiation grade

Tumors were graded by pattern of poorest differentiation, grading was based on the retention of glandular differentiation and given a single grade of differentiation (well, moderately, poor). The worst grade of tumor seen was used for the overall grade. For analysis well and moderately differentiated tumors were grouped together.

Mucinous differentiation

Tumors with greater than 50% area showing extracellular mucin were classified as mucinous. Tumors with less than 50% area showing extracellular mucin were classified as having focal mucinous differentiation. Tumours with no extracellular mucin were classified as negative for mucinous differentiation.

Tumor necrosis

Tumors were assessed for the presence or absence of “dirty necrosis” defined by intra-tumoral necrotic debris in tumor glands as well as tumor necrosis, often considered a characteristic of colorectal carcinomas. If only a rare focus of necrosis was present (<10%) then the tumor was considered negative.

Tumor budding

When there was a diffuse growth pattern with a transition from glandular structures to single cells or clusters of up to four cells at the invasive margin, tumor budding was present²².

Circumscribed

Tumors with a so-called “pushing” invasive margin were classified as well circumscribed. When the tumor invaded in a diffuse manner with widespread penetration of normal tissues, tumors were named non-circumscribed²³.

Lymphocytic infiltration

If peritumoral chronic inflammation was present with or without Crohn's like lymphocytic nodules or if tumor infiltrating lymphocytes were present in the tumor epithelium, lymphocytic infiltration was scored positive⁶.

Data analysis

CIMP was determined using the panel as suggested by Weisenberger *et al.*¹⁴ CRCs are defined as CIMP+ 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 CIMP, MSI, *BRAF*, *APC*, *KRAS*, *TP53* status was performed by using half square Euclidian distance (Wards method linkage rule)^{24,25}. Correlations between our computed clusters, morphology data and clinicopathological parameters were determined by the Pearson Chi-Square and Fisher's exact test as appropriate. To evaluate the relationship between the calculated clusters and patient survival, Kaplan-Meier survival curves were calculated. The endpoint for analyses was overall survival starting from the day of surgery. Independent variables predicting survival were evaluated by regression analyses using Cox Regression. The Cox-regression model included the variables: CL1, CL2, CL3, age, gender, tumor location, differentiation grade and Stage. All p-values are two sided and p-values <0.05 were considered statistically significant. SPSS 12.0.1 software was used for data analyses.

Results

CIMP, MSI, *BRAF*-, *APC*-, *KRAS*- and *TP53* mutation status and association with tumor location

A summary of the baseline (epi-)genetic aberrations determined in our study population is provided in Table 3.2. Using the Weisenberger criteria to define CIMP, 25% (39/156) of CRCs could be classified as CIMP+. This frequency is in line with previous publications where the reported frequency of CIMP+ ranged between 18% and 25%^{14,26-29}. MSI was detected in 12% (19/158) of our study population, which is in accordance with previous reports³⁰. CIMP+ tumors were positively correlated with MSI status, $p < 0.001$ (data not shown). *BRAF* mutations were detected in 9% (14/157) of cases, which is in line with the reported frequency within CRC by others^{31,32}. CIMP as well as MSI were positively correlated with *BRAF* mutations ($p = 0.027$ and $p = 0.012$, data not shown), which is in agreement with current understanding of CIMP+ CRC.

Table 3.2 Correlations between frequencies of CIMP, MSI, *BRAF/APC/KRAS/TP53* mutations and molecular clusters (CL) versus clinicopathological variables.

Total (%)	Age		Sex		Tumor location			Differentiation		TNM				
	<69 years	>69 years	Male	Female	Right-sided colon	Left-sided colon	Rectum	Well/ Moderate	Poor	1	2	3	4	
CIMP+														
39/156 (25%)	17/85 (20%)	22/83 (26%)	15/78 (20%)	24/90 (27%)	20/56 (36%)	5/45 (11%)	14/55 (26%)	19/93 (20%)	20/75 (27%)	0/4 (0%)	18/97 (19%)	17/48 (35%)	4/19 (21%)	.381
MSI														
19/158 (12%)	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%)	.926
BRAF mut														
14/157 (9%)	8/79 (10%)	6/82 (7%)	7/74 (10%)	7/87 (8%)	6/59 (10%)	3/48 (6%)	5/54 (9%)	7/88 (8%)	7/73 (10%)	0/3 (0%)	10/93 (11%)	3/47 (6%)	1/18 (6%)	.724
APC mut														
75/134 (56%)	39/66 (59%)	36/68 (53%)	35/61 (57%)	40/73 (55%)	28/48 (58%)	20/34 (59%)	27/52 (52%)	36/73 (49%)	39/61 (64%)	0/2 (0%)	45/74 (61%)	21/41 (51%)	9/17 (53%)	.298
KRAS mut														
40/152 (26%)	14/77 (18%)	27/80 (34%)	18/73 (25%)	23/84 (27%)	17/58 (29%)	11/45 (24%)	13/54 (24%)	22/85 (26%)	19/72 (26%)	0/2 (0%)	25/93 (27%)	11/45 (24%)	5/17 (29%)	.826
TP53 mut														
73/146 (50%)	39/74 (53%)	34/72 (47%)	39/79 (49%)	34/67 (51%)	23/54 (43%)	22/38 (58%)	28/54 (52%)	40/82 (49%)	33/64 (52%)	2/3 (67%)	45/82 (55%)	17/44 (39%)	9/17 (53%)	.328
CL1														
65/160 (41%)	32/79 (40%)	33/81 (41%)	35/75 (47%)	30/85 (35%)	20/59 (34%)	19/45 (42%)	26/56 (47%)	40/89 (45%)	25/71 (35%)	1/3 (33%)	38/93 (41%)	21/47 (45%)	5/17 (30%)	
CL2														
36/160 (22%)	14/79 (18%)	22/81 (27%)	13/75 (17%)	23/85 (27%)	18/59 (30%)	5/45 (11%)	13/56 (23%)	18/89 (20%)	18/71 (25%)	0/3 (0%)	16/93 (17%)	16/47 (34%)	4/17 (23%)	
CL3														
59/160 (37%)	33/79 (42%)	26/81 (32%)	27/75 (36%)	32/85 (38%)	21/59 (36%)	21/45 (47%)	17/56 (30%)	31/89 (35%)	28/71 (40%)	2/3 (67%)	39/93 (42%)	10/47 (21%)	8/17 (47%)	.111

50% (73/134) of the tumors had a mutation of the *APC* gene in the mutation cluster of exon 15. Point mutations in exon 1 of the *KRAS* gene were found in 26% (40/152) of all cases: 20% (30/152) in codon 12 and 6% (10/152) in codon 13, respectively. Mutations in exons 5 to 8 of the *TP53* gene were present in 53% (77/146) of all cases. *TP53* mutations were positively correlated with *APC* mutations, $p=0.021$ (data not shown) and not with MSI, CIMP or *BRAF* mutations, likely reflecting tumors within the CIN category. The frequency of *APC*, *KRAS* and *TP53* mutations in our study population are in accordance with the frequencies reported by others in CRC^{3,31-33}.

Regarding baseline clinicopathological features, CIMP+ tumors were more often located in the right sided colon (located proximal to the splenic flexure) 36% (20/56), and rectal tumors 26% (14/55), compared to left sided colon tumors (located distal to the splenic flexure) 11% (5/45), $p=0.01$ (Table 3.2). Furthermore MSI was positively correlated with right sided tumor location, 26% (16/61) of right-sided colon tumors were MSI tumors, compared to 4% (2/51) in the left sided colon and 2% (1/57) in the rectum ($p<0.001$). *BRAF*-, *APC*-, *KRAS*- and *TP53* mutations did not correlate with tumor location (Table 3.2).

Molecular clusters and associations with clinicopathological characteristics

Unsupervised hierarchical clustering, based on (epi)genetic changes including CIMP, MSI, *BRAF*-, *APC*-, *KRAS*, and *TP53* mutations, resulted in three main clusters (CL1, CL2 and CL3, Figure 3.2, Table 3.2), which were reproducible with a similar linkage distance, independent of the sequence of input variables and stage of disease (data not shown). CL2 was characterized by CIMP+, and accounted for 22% (36/160) of CRC's. The remaining 78% of CIMP- tumors were subdivided into *TP53* mutated tumors in CL3, comprising 37% (59/160) of cases, and a remaining subset of *TP53* wildtype tumors representing CL1, containing 41% (65/160) of cases, respectively $p<0.001$ and $p<0.001$ (Figure 3.3A). Overall, MSI was more prevalent in CL2 tumors 29% (10/35), than in CL1 6% (4/64) or CL3 9% (5/59), $p<0.01$ (Figure 3.3A). CL3 tumors more often harbored *APC* mutations (71%, 35/49), as compared to CL1 (45%, 23/51) and CL2 (50%, 17/34), $p=0.021$, (Figure 3.3A). To the contrary, *BRAF* and *KRAS* mutations were more or less equally distributed among the three clusters and thus did not give rise to separate clusters (Figure 3.3A). Furthermore, there was no consistent relationship between computerized clustering, morphology and other clinicopathological variables (Figure 3.3B, Table 3.2). Figure 3.3B illustrates the distribution of mucinous differentiation, dirty necrosis, differentiation grade, circumscribed tumor growth, tumor budding and lymphocyte infiltration among the three (epi)-genetic clusters.

Figure 3.2 CRCs clusters CL1 (41%, 65/160), CL2 (22%, 36/160), CL3 (37%, 59/160) obtained by unsupervised hierarchical clustering of CIMP, MSI, *BRAF/KRAS/APC* and *TP53* mutation status. (Black box indicates positive, white box indicates negative, grey box indicates missing value).

Interestingly, CIMP+ tumors of CL2 could be subcategorized on the basis of MSI status and *TP53* mutation status as follows. CL2 consisted of 29% (10/35) being MSI, a larger group of 71% (25/35) being MSS. Within CL2, MSI tumors more often were localized in the right hemicolon 90% (9/10) than in the rectum 10% (1/10) or left-sided colon 0%, $p < 0.01$ (Figure 3.3C). There was no consistent relationship between computerized clustering and morphology, except for right-sided MSI tumors in CL2 which more often showed mucinous differentiation 44% (4/9), compared to 0% (0/9) in rectum tumors, $p = 0.023$ (data not shown). Although not significant 20% (3/15) of *TP53* mutated tumors were MSI and 80% (12/15) MSS, $p = 0.46$ (data not shown). Furthermore, within CL2 tumors, *TP53* status seems to be related with tumor localization in the bowel: 33% (5/15) of *TP53* mutated cases were right-sided colon tumors compared to 54% (8/15) of *TP53* mutated cases being rectum tumors (borderline significance $p = 0.06$, Figure 3.3C).

Figure 3.3 (A) Percentages of CIMP+, MSI, *BRAF*/*KRAS*/*APC*/*TP53* mutated tumors within the three clusters CL1, CL2 and CL3 (bars indicate percentage of positive cases within each cluster). (B) Percentages of positive scoring of morphology markers: differentiation grade, mucinous differentiation, dirty necrosis, circumscribed tumour growth, tumour budding and lymphocyte infiltration within CL1, CL2 and CL3 (bars indicate percentage of positive cases within each cluster). (C) Within CL2 tumors distribution of MSI and *TP53* mutations, according to site of origin, respectively right-sided colon, left-sided colon and rectum (bars indicate percentage of positive cases). * indicates significant p-values

CIMP- tumors were further subdivided depending on *TP53* mutational status, into CL1 *TP53* wildtype tumors and CL3 *TP53* mutated tumors respectively. Overall, within CL3, *TP53* mutated tumors were equally distributed among right-sided colon 32% (17/54), left-sided colon 37% (20/54) and rectum 32% (17/54) (data not shown). Within CL3 we could not identify distinct patterns of morphology between (epi)genetic aberrations. As opposed to CL3 and CL2, CL1 represented a remaining group (41%), of CIMP- and *TP53* wild type tumors, which were not characterized by a single main (epi)-genetic aberration, as illustrated in Figure 3.3A. Within CL1, *APC* mutations (45%) and *KRAS* mutations (30%) were frequently present, in contrast to MSI, CIMP+ and *TP53* mutations, which showed an overall low frequency (<10%). Similar to cluster CL3, within CL1 we could not identify differences between (epi)-genetic aberrations and clinicopathological variables.

Subsequently we verified whether a particular cluster did relate to patient survival. Overall and after stratification for tumor stage, there was no impact of computerized clustering on survival in colon tumors (Figure 3.4A-D), $p=0.24$, $p=0.35$, $p=0.45$, $p=0.39$, respectively. Furthermore if we stratified for tumor location, no survival differences between the clusters were observed within proximal and distal colon tumors (Figure 3.4E,F, $p=0.82$ and $p=0.98$, respectively). In rectal tumors ($n=56$) on the other hand, a poorer prognosis was noted for CL2 tumors as compared to CL3 and CL1 tumors (Figure 3.4G, $p=0.01$). CL2 tumors, being CIMP+, showed lower patient survival rates compared to CL3 lesions, containing *TP53* mutations. This observation could not be confirmed in a multivariate Cox regression model (Table 3.3). Although CL2 tumors showed a HR of 2.35 (CI: 0.77-8.32), no statistical significance was reached, $p=0.13$. Probably the effect of CL2 is overruled by tumor stage IV, showing a HR=12.62 (CI: 2.75-58.0), $p<0.001$.

Table 3.3 Cox proportional hazard model in rectum tumors ($n=49$).

	Event: CRC mortality HR (95%CI)	p-value
Clusters		
CL2*	2.53 (0.77-8.31)	0.13
CL3*	0.79 (0.20-3.16)	0.74
Age (years)	1.03 (0.98-1.09)	0.26
Gender (female ™)	1.59 (0.50-4.98)	0.43
Differentiation grade (poor [#])	1.92 (0.54-6.78)	0.31
Stage		
III ⁼	1.17 (0.38-3.63)	0.78
IV ⁼	12.62 (2.75-58.0)	<0.001

HR: Hazard Ratio=Relative Risk. *Reference group = CL1; ™Reference group = Man, [#]Reference group = Well/moderate, ⁼Reference group = Stage II.

Figure 3.4 (A) Survival curves overall population of CL1 ($n=65$) versus CL2 ($n=36$) and CL3 ($n=59$), $p=0.24$; (B) Survival curves within Stage II of CL1 ($n=38$) versus CL2 ($n=16$) and CL3 ($n=39$), $p=0.35$; (C) Survival curves within Stage III of CL1 ($n=21$) versus CL2 ($n=16$) and CL3 ($n=9$), $p=0.45$; (D) Survival curves within Stage IV of CL1 ($n=5$) versus CL2 ($n=4$) and CL3 ($n=8$), $p=0.39$; (E) Survival curves within proximal colon tumors of CL1 ($n=20$) versus CL2 ($n=18$) and CL3 ($n=21$), $p=0.82$; (F) Survival curves within distal colon tumors of CL1 ($n=19$) versus CL2 ($n=5$) and CL3 ($n=21$), $p=0.98$; (G) Survival curves within rectal tumors of CL1 ($n=26$) versus CL2 ($n=13$) and CL3 ($n=17$), $p=0.012$.

Discussion

Studying the association between molecular clusters in CRC and their clinicopathological features may lead to a better understanding of the disease. Therefore, in the light of numerous previous efforts that have been undertaken to unravel the complex underlying molecular changes in CRC, we molecularly classified CRC using computer assisted unsupervised hierarchical clustering of genetic and epigenetic alterations and correlated this with clinicopathological parameters.

Computer assisted clustering divided CRC's in CIMP+ (22%) and CIMP- (78%) tumors. The latter group could be further subdivided in *TP53* mutated tumors (37%), and *TP53* wildtype tumors (41%). The cluster of CIMP+ tumors consisted of two subgroups: 29% microsatellite instable (MSI) and 71% microsatellite stable (MSS) tumors. A subdivision of CIMP+ tumors, has already been suggested by Jass et al. and others: CIMP1 (MSI associated) and CIMP2 (non-MSI associated)^{2,34,35}. Recently it was suggested that in CRC, DNA methylation epigenotypes do exist, a high-methylation epigenotype which significantly correlates with MSI, right-sided tumor location and a high frequency of mucinous differentiation, and an intermediate-epigenotype which significantly correlates with distal tumor location, high *TP53* expression and a low frequency of mucinous differentiation^{36,37}. This subdivision is in concordance with CIMP+, right-sided colon tumors in our population, being MSI and more often displaying mucinous differentiation, and CIMP+ tumors in the rectum, which were more often *TP53* mutated, compared to those located in the right-sided colon. It has been shown that right-sided colon and distal colorectal cancers show distinct gene-specific methylation profiles and clinical and molecular characteristics³⁸. Although there is scarce literature specifically on the subject of (epi)-genetic profiles in the rectum compared to those in the right-sided colon, it seems that CIMP exists in both MSI, right-sided tumors and MSS distal (rectum) tumors. CIMP has been postulated to explain silencing of the *MLH1* gene in right-sided CRC with MSI-High³⁹. The precise role or target genes/-pathways of CIMP in distal MSS tumors is unclear. Probably distal CIMP+ tumors originate via different pathways compared to proximal CIMP+ tumors. As already stated, CIMP+ tumors in the rectum seem to originate in a background of CIN since they more often contain *TP53* mutations and are MSS³⁶. Recently it was shown that 71% of MSS tumors with CIMP+ status have *APC* mutations, suggesting an interrelation with the CIN pathway⁴⁰.

Our study suggests that *TP53* is the second most important classifier for CRCs after CIMP. Within CL3, 98% of tumors harboured a *TP53* mutation ($p < 0.001$), and 71% an *APC* mutation ($p = 0.021$) all being CIMP-. Sweeney et al showed in 917 CRCs, that *TP53* and *APC* mutated tumors formed a distinct cluster within CIMP- CRCs⁴⁰. Jass et al., also suggested the existence of a distinct CRC

group consisting of CIMP-/MSS, *APC/TP53* mutated tumors, which overlaps with CL3 tumors in our study population. Moreover, Jass et al suggested that these tumors have characteristic morphological features, namely dirty tumor necrosis and tumor budding. We did not find a correlation with any specific morphological feature, and in line with our findings the impact of *APC* and *TP53* mutations on clinical outcome has been shown to be far from conclusive in other studies^{41,42}.

Overall there was a poor relation between our molecular clusters and morphological features. Only mucinous differentiation was associated with a subgroup of CIMP+, MSI, right-sided tumors. On the other hand, dirty necrosis, circumscribed tumor growth, tumor budding and lymphocyte infiltration did not show a relation with our molecular clusters. Several explanations are possible for this poor correlation. In our population, 43% of well differentiated tumors showed mucinous differentiation compared to 15% in moderately and 12% in poorly differentiated tumors ($p=0.016$, data not shown). Furthermore, none of well differentiated tumors showed tumor budding compared to 22% in moderately and 24% in poorly differentiated tumors ($p=0.13$, data not shown). These results indicate that some morphological parameters are tightly associated with the differentiation grade of a tumor. The poor correlation between our molecularly defined clusters and morphological features (other than mucinous differentiation) can be explained in part by differentiation grade: in a tumor with a higher level of differentiation, probably a single dominant gene is still able to dictate its morphology. To the contrary, in moderately and poorly differentiated tumors, a cumulative increase in epi-genetic aberrations makes it difficult for a single gene to dominate and dictate tumor morphology. Probably this explains why mucinous differentiation, strongly coupled to a better differentiation grade, can be related to a single genetic aberration like MSI. Along similar lines, this explains why morphological features as tumor budding, circumscribed tumor growth and dirty tumor necrosis, which are closely connected to a poorer differentiation grade, are not related to single epi-genetic aberrations.

We could not demonstrate an impact of computerized clustering on survival of CRC patients. Only in rectum tumors, a poorer patient survival was noted for CL2 tumors as compared to CL3 tumors. This was observed in a relatively small number of rectal tumors and could therefore not be confirmed in multivariate analysis taking into account other established prognostic factors such as tumor localization, age, gender and tumor stage. Few studies have evaluated CIMP+ as a prognostic marker in distal tumors, in particular rectal cancer⁴³. De Maat and colleagues reported the quantitative MINT locus methylation profile (=surrogate marker for CIMP) and its association with local recurrence in rectal cancers⁴⁴. Based on the methylation profile of *MINT3* and

MINT17 they were able to subdivide patients into groups with an increased risk for local recurrence.

Although current molecular clustering provides knowledge in the underlying biology of CRC and partly confirms the molecular classification proposed by Jass et al. and others, our computed molecular clusters did not have strong clinicopathological correlations, and therefore could not improve traditional classification with respect to prognostic power. Maybe this was due to the relatively small population size, or the fact that we included a lot of single genetic aberrations compared to a few epigenetic aberrations. The overall frequency of genetic aberrations like mutations in *APC*, *KRAS* and *TP53* in CRC is relatively low, and additional mechanism such as epigenetic aberrations contribute to tumor progression and prognosis⁴⁵. Another explanation for the lack of prognostic power for our molecular clusters, could be the fact that we did not include chromosomal aberrations, which are associated with a poorer patient survival in CRC^{46,47}. Significantly, recent advances in technology enable researchers to determine the genetic and epigenetic alterations in cancers on a genome-wide scale^{9,48-50}. It reveals true biomarkers that enable monitoring of individual tumor progression, tailoring response to therapeutic treatment, and identification of residual disease at a level previously undetectable by current methods^{51,52}. Future research should point out whether these genome wide screening methods are able to identify new molecular markers that improve traditional classification.

In summary, computer assisted classification of CRCs shows that CIMP status is the principal classifier, and that both CIMP+ and CIMP- tumors are further classified on the basis of *TP53* mutational status. Hierarchical molecular clustering is only modestly related to morphology and outcome. However, there was evidence that some clusters represent biologically distinct subgroups, which appear to be dependent on additional factors such as microsatellite stability and localization in the intestine. CIMP+ CRC appears to comprise two subsets; one localized in the right-sided colon, with MSI and mucinous differentiation, the other occurring in the rectum, being MSS.

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Table 3.S1 Primer sequences and PCR conditions.

Gene	Primer	Sense primer	Antisense primer	Amplicon size (bp)	Annealing temp (°C)	PCR cycles
CANA1G	flank	5'-GGT YGG TTT TTT TTT ATT TTG TT-3'	5'-CRC CRC TAC CCC CCT TTT C-3'	122	56	35
CANA1G	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
CANA1G	M	5'-TCG TTT CGC GTT TAG GTT TC-3'	5'-CTC GAA ACG ACT TCG CCG-3'	62	64	35
IGF2	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
IGF2	U	5'-GGA GTG GTT TTG GTG TTG TTA TT-3'	5'-CCC AAC TCA ATT TAA ACC AAC A-3'	90	66	35
IGF2	M	5'-GCG GTT TCG GTG TCG TTA TC-3'	5'-CCA ACT CGA TTT AAA CCG ACG-3'	86	68	30
NEUROG1	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
NEUROG1	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
NEUROG1	M	5'-ATT TAT GTT CGC GGG AGG TC-3'	5'-GAC GAT AAT TAC GAA CAC ACT CCG-3'	65	66	35
RUNX3	flank	5'-GTT YGA TGG TGG AYG TGT TGG-3'	5'-AAT CCC RCA CTC ACC TTA AAA AC-3'	139	56	35
RUNX3	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
RUNX3	M	5'-GCG GAT TAC GTA GGC GAG TTC-3'	5'-ACC TTA AAA ACG ACG AAC AAC G-3'	107	66	35
SOCS1	flank	5'-GTT YGT GGG TAT TTT TTT GGT G-3'	5'-TAA ATC CCR AAA CCA TCT TCA C-3'	83	56	35
SOCS1	U	5'-GGG TAT TTT TTT GGT GTG TAG TT-3'	5'-CCA AAA CCA TCT TCA CAC TAA AAA CA-3'	71	66	35
SOCS1	M	5'-TTT TTT TGG TGC GCG ATA GTC-3'	5'-GAA ACC ATC TTC ACG CTA AAA ACG-3'	64	66	35

Table 3.S2 Primer sequences

<i>TP53</i>	Primer	Sequence	Product size
5 exon	Flank sense	5' CTG TTC ACT TGT GCC CTG AC 3'	268 bp
	Flank antisense	5' AGC CCT GTC GTC TCT CCA G 3'	
	Inside sense	5' TGC CCT GAC TTT CAA CTC TG 3'	
6 exon	Flank sense	5' GGC CTC TGA TTC CTC ACT G 3'	257 bp
	Flank antisense	5' ATA AGC AGC AGG AGA AAG CC 3'	240 bp
	Inside antisense	5' GAC CCC AGT TGC AAA CCA G 3'	164 bp
7 exon	Flank sense	5' CAT CTT GGG CCT GTG TTA TC 3'	261 bp
	Flank antisense	5' GAT GTG ATG AGA GGT GGA TGG 3'	230 bp
	Inside antisense	5' GGA AGA AAT CGG TAA GAG GTG 3'	
8 exon	Flank sense	5' GAT TTC CTT ACT GCC TCT TGC 3'	
	Flank antisense	5' CAT AAC TGC ACC CTT GGT CTC 3'	231 bp
	Inside antisense	5' TCC TCC ACC GCT TCT TGT C 3'	212 bp

Chapter 4

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

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Abstract

Data on the prognostic significance of promoter CpG island methylation in colorectal cancer (CRC) are conflicting, possibly due to associations between methylation and other factors affecting survival such as genetic alterations and use of adjuvant therapy. Here we examine the prognostic impact of promoter methylation in CRC patients treated with surgery alone in the context of microsatellite instability (MSI), *BRAF*- and *KRAS* mutations.

173 CRCs were analyzed for promoter methylation of 19 tumor suppressor- and DNA repair genes, the CpG island methylator phenotype (CIMP), MSI, the exon 15 V600E *BRAF* mutation and *KRAS* codon 12 and 13 mutations.

Unsupervised hierarchical clustering based on methylation status of 19 genes revealed three 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). CL3 had the highest methylation index (0.25, 0.49 and 0.69 respectively, $p < 0.01$) and was strongly associated with CIMP ($p < 0.01$). After stratification for tumor stage, MSI and *BRAF* status, no statistically significant differences in survival between CL1, CL2 and CL3 nor between CIMP and non-CIMP CRCs were detected. Analyzing genes separately revealed that *CHFR* promoter methylation was associated with a poor prognosis in stage II, MSS, *BRAF* wild-type CRCs: HR=3.89 (95% CI =1.58-9.60, $p=0.003$) and HR=2.21 (95% CI=1.09-4.48, $p=0.03$) in a second population-based study ($n=151$).

CHFR promoter CpG island methylation, which is associated with MSI, also occurs frequently in MSS CRCs and is a promising prognostic marker in stage II, MSS, *BRAF* wild-type CRCs.

Introduction

Accurate staging of colorectal cancer (CRC) is essential for optimal disease management. Although patients with the same stage can demonstrate considerable variation in outcome, the TNM staging system remains the gold standard for predicting prognosis and guiding clinical management of CRC¹.

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 might aid in selecting CRC patients who could benefit from adjuvant therapy.

CRC is characterized by (epi)genetic alterations of genes controlling the hallmarks of cancer^{2,3-5}. 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. A distinct subset of CRCs, characterized a greater degree of promoter CpG island methylation, is associated with proximal location, poor differentiation, MSI and *BRAF* mutations⁶⁻¹¹ and is referred to as the CpG island methylator phenotype (CIMP)^{10,12}.

CIMP has previously been associated with CRC prognosis. However, published reports are inconsistent, perhaps due to confounding factors such as MSI, *BRAF* and *KRAS* mutations, variations in use of adjuvant chemotherapy¹³⁻¹⁶, and methodological differences, such as different CIMP definitions.

The aim of this study was to evaluate the role of CIMP and frequently methylated promoter CpG islands on prognosis of CRC, eliminating the influence of genetic alterations and adjuvant chemotherapy.

Materials and methods

Study population

CRC patients were 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 technique¹⁷. The second trial was conducted to compare survival in rectal cancer patients with or without preoperative radiotherapy, of which we included only the patients in the no preoperative radiotherapy arm. At the time the trial was conducted, adjuvant chemotherapy was not yet standard practice. Tumor stage was defined according to the UICC-TNM staging system and American Joint Committee on Cancer classifications (AJCC), Cancer Staging Sixth Edition^{18,19}. 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 recurrence of disease and disease-related death. After five years of follow up, only time and cause of death were registered. Follow-up was complete for all patients. Failure was defined as death due to recurrent disease, excluding postoperative mortality within 30 days and non-disease related death. For molecular analyses, tumor tissues from 173 patients with primary CRC were available.

Independent, validation population of CRCs

A second, independent population of 734 CRC cases, derived from the prospective Netherlands Cohort Study on diet and cancer which started in 1986 with the enrolment of 120,852 healthy individuals between 55 and 69 years old from 204 municipalities throughout the Netherlands, was used to validate survival data. From 1989 until 1994, 925 incident CRC cases (ICD-O:153.0-154.1) were identified by computerized linkage with the Netherlands Cancer Registry and PALGA, a nationwide network and registry of histopathology and cytopathology²⁰. Information on tumor localization, tumor staging, differentiation grade and incidence date was available through the Netherlands Cancer Registry. Vital status until May 2005 was retrieved from the Central Bureau of Genealogy and the municipal population registries and could be obtained for all cases. Causes of death were retrieved through linkage with Statistics Netherlands. Paraffin-embedded tumor tissue was collected from 54 pathology registries; tissue blocks for 734 (90%) of the CRC cases contained sufficient DNA for analyses. Details of this cohort have been described previously²¹.

Clinical pathological characteristics are provided for both populations (Table 4.1). In the validation study more left-sided tumors (43%) ($p < 0.01$) and stage I tumors (27%) ($p < 0.01$) were diagnosed and the median follow up time was longer in the validation population: 7.6 years compared to 4.8 years. $p < 0.01$). However, event frequencies were comparable between both studies, making the validation population suitable for validation of prognostic markers.

Table 4.1 Clinicopathological characteristics of the CRC populations

	Study population (n=173)	Validation population (n=734)	p-value
Age			
Mean age (SD)	67.80 (11.8)	62.90 (4.1)	NS
Gender			
Male	82 (47%)	408 (56%)	NS
Female	91 (53%)	326 (44%)	
Tumor location			
Right-sided colon	62 (36%)	239 (33%)	<0.01
Left-sided colon	52 (30%)	310 (43%)	
Rectum	59 (34%)	176 (24%)	
CRC Stage			
I	4 (2%)	181 (27%)	<0.01
II	100 (58%)	236 (35%)	
III	50 (29%)	185 (28%)	
IV	19 (11%)	69 (10%)	
Event frequency**	64 (38%)	302 (41%)	NS
Median follow up time	4.80 years	7.60 years	<0.01

SD: Standard Deviation, ** colorectal cancer specific death

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 genes reported to be methylated in colorectal cancer: *mutL homolog1, colon cancer, nonpolyposis type 2 (E.Coli) (MLH1)*, *cyclin dependent kinase inhibitor 2A (p16INK4 and p14ARF)*, *O-6-methylguanine-DNA methyltransferase (MGMT)*, *Ras association (RalGDS/AF-6) domain family member 1 (RASSF1A)*, *adenomatous polyposis coli (APC)*, *helicase-like transcription factor (HLTF)*, *GATA binding protein 4 (GATA4)*, *GATA binding protein 5 (GATA5)*, *checkpoint with forkhead and ring finger domains (CHFR)*, *ADAM metalloproteinase domain 23 (ADAM23)*, *Rab32, member RAS oncogene family (RAB32)*, *junctional protein 3 (JPH3)*, *forkhead box L2 (FOXL2)*, *BCL2-adenovirus E1B 19kDa interacting protein 3 (BNIP3)*, *neuronalized homolog (Drosophila) (NEURL)*, *calcium channel, voltage dependent, alpha2-delta subunit 1 (CACNA2)*, *thrombospondin 1 (THBS1)*, *tissue factor pathway inhibitor 2 (TFPI2)* and the CIMP genes *calcium channel, voltage-dependent, T type, alpha-1G subunit (CACNA1G)*, *insulin-like growth factor 2 (somatomedin A) (IGF2)*, *neurogenin 1 (NEUROG1)*, *runt-related transcription factor 3 (RUNX3)* and *suppressor of cytokine signaling 1 (SOCS1)* was determined using sodium bisulfite modification of genomic DNA (EZ DNA methylation kit, ZYMO research Co., Orange, CA). To facilitate MSP analysis on DNA retrieved from formalin-fixed, paraffin-embedded tissue, nested

Methylation Specific PCR (MSP) was performed as described elsewhere^{22,23}. Primers and PCR conditions are provided in Supplementary Table 4.S3.

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 described²⁴. 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 semi-nested PCR and subsequent RFLP analysis, *KRAS* mutations were analyzed as described previously^{25,26}.

Data analysis

CIMP was analysed using the marker panel proposed by Weisenberger et al.¹¹. CRCs were defined as CIMP when >3/5 analyzed markers (*CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3*, *SOCS1*) were methylated. Unsupervised clustering (Spotfire DecisionSite® for Functional Genomics), based on the similarity of methylation of 19 CpG islands that have been reported to be methylated in colorectal cancer (CIMP genes excluded), was performed by using half square euclidian distance (Wards method linkage rule)^{27,28}. 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. Standard Error of Skewness was used as a test of symmetry for the distribution of CRC cases according to the number of methylated CIMP genes and number of methylated cluster genes, symmetry was rejected if the ratio was less than -2 or greater than +2. Differences between methylation-, clinicopathological- and molecular characteristics were determined by the Pearson Chi-Square and Fisher's exact test where appropriate. Kaplan-Meier curves were used to evaluate the relationship between promoter CpG island methylation and patient survival in the overall population and stratified for tumor stage, MSI and *BRAF* mutation status. Statistical differences between groups were assessed by use of the log-rank test. The endpoint for analyses was overall survival starting from the day of surgery until the time of death due to CRC. Independent variables predicting survival were evaluated in a multivariate model using Cox Regression analyses. The Cox-regression model including CIMP, CL1, CL2, CL3, *CHFR* promoter CpG island methylation, age, gender, tumor location, differentiation grade and TNM Stage was used to assess the prognostic influence of these variables. All p values (two sided) <0.05 were considered statistically significant. SPSS 15.0 and Stata 10.0 were used for data analyses.

Results

(Epi)genetic characterization of CRCs

MSI, *BRAF* and *KRAS* mutations were detected in 11% (19/169), 9% (14/161) and 26% (41/157) of CRCs of the study population respectively (Table 4.S1), which is in accordance with previously reported frequencies^{4,5,29}.

The methylation frequency for CIMP genes ranged from 13% to 61%: *IGF2* (13% (20/154)), *CACNA1G* 18% (30/170)), *RUNX3* (32% (52/163)), *NEUROG1* 42% (70/165) and *SOCS1* (61% (103/170)) (Figure 4.1A). 23% (39/168) of CRCs were classified as CIMP (Table 4.S2). This is consistent with previous publications with reported frequencies of CIMP between 18-25%^{11,30,31}. The distribution of the number of CIMP markers methylated for each tumor resembles a normal distribution (Figure 4.1C, standard error of skewness 0.187).

Figure 4.1 (A) Methylation frequencies of the five CIMP genes among the study-population (black bars indicate percentages). (B) Methylation frequencies of 19 cluster genes among the study-population (black bars indicate percentages). (C) Distribution of colon cancer cases according to the number of methylated CIMP genes (black bars indicate number of cases). (D) Distribution of colon cancer cases according to the number of methylated cluster genes (black bars indicate number of cases CIMP⁻, white bars indicate number of cases CIMP⁺).

We sought to more broadly examine the methylation of CpG islands in CRC, to determine whether the 5 genes used to define CIMP optimally separated phenotypic differences in colon tumors. The frequency of promoter CpG island

methylation in 19 additional tumor suppressor genes ranged from 5% (*THBS1*) to 87% (*TFPI2*) (Figure 4.1B and Table 4.S1). We compared the distribution of tumors according to the methylation status of the 19 additional genes (Figure 4.1D). This resembles a normal distribution with a standard error of skewness 0.185, and reveals that CRCs with a high number of methylated genes are predominantly characterized by CIMP (white bars). This suggests that the Weisenberger CIMP markers are sensitive but not completely specific in identifying CRCs with a high frequency of promoter CpG island methylation.

We assessed whether subgroups of CRCs could be identified using methylation patterns of the 19 non-CIMP genes. Unsupervised hierarchical cluster analysis identified three clusters of CRCs, CL1 (57% (98/173)), CL2 (25% (43/173)) and CL3 (18% (32/173)) (Figure 4.2, Table 4.S2). CL3 showed the highest number of methylated genes (MI) (mean MI, CL1=0.25, CL2=0.49, CL3=0.69, $p<0.001$).

Figure 4.2 CRCs clusters CL1 (57%), CL2 (25%), CL3 (18%) obtained by unsupervised hierarchical clustering of promoter CpG island methylation of *MLH1*, *p16INK4A*, *p14ARF*, *MGMT*, *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 individual CIMP markers (*CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3* and *SOCS1*), CIMP, MSI, *BRAF*- and *KRAS* mutations as visualized. Black box indicates positive, white box indicates negative, grey box indicates missing value.

Associations between genetic, epigenetic and clinicopathological characteristics

CIMP was highly associated with promoter CpG island methylation of 16/19 additional genes. Furthermore, CRCs grouped in CL3 were most often classified as CIMP ($p<0.01$) (Figure 4.2, Table 4.2). CIMP was associated with MSI, *BRAF* mutations and CL3 ($p<0.01$, $p=0.05$ and $p<0.01$ respectively) (Table 4.2). 69% (26/38) of CIMP CRCs and 60% (18/30) of CL3 CRCs are MSS (Table 4.2). Mutations in *KRAS* were neither associated with CIMP nor with the identified clusters (data not shown).

Table 4.2 Correlations between CIMP, CL1, CL2 and CL3 and MSI and *BRAF* mutation in the study population.

	MSI	MSS	<i>BRAF</i> M	<i>BRAF</i> WT	CIMP
CIMP	12/19 (63%)	26/145 (18%) <0.01	7/14 (50%)	32/143 (22%) <0.05	
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%) <0.01	6/14 (43%)	25/147 (17%) 0.06	25/39 (64%) <0.01

CIMP: CpG island methylation phenotype using the markers of Weisenberger *et al.*¹, CL1, CL2 and CL3: 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, M: Mutant, WT: Wild-type.

As expected, CIMP and MSI were associated with right-sided tumor location, as were CL3 CRCs ($p=<0.01$, $p=<0.01$ and $p=<0.01$ respectively, Tables 4.S1 and S2). We did not observe previously reported association between sex, age and CIMP or with our newly defined CL3 tumors.

The primary aim of this study was to evaluate the prognostic influence of promoter CpG island methylation using CIMP- and to extend this analysis to include 19 additional tumor suppressor- and DNA repair genes. As tumor stage, MSI, *BRAF*- and *KRAS* mutations have in some reports influenced survival, we analyzed whether these alterations were of prognostic significance in the study population. As expected, disease outcome was significantly influenced by tumor stage, with stage I having an improved overall survival ($p<0.01$) compared to other tumor stages. We also observed that wild-type *BRAF* tumors showed an improved overall survival compared to *BRAF* mutated tumors ($p=0.04$). MSI seems associated with improved overall survival as compared to MSS within CRC Stage II, although this association is not

statistically significant ($p=0.07$). *KRAS* mutations were of no prognostic value, neither in the overall population, nor in the separate stages (data not shown). Therefore, survival analyses were also stratified for stage, MSI status and *BRAF* mutation status.

No statistically significant survival differences between CIMP and non-CIMP CRCs were observed in the overall study population (Figure 4.3A) or when specifically examining Stage II (Figure 4.3C,) or Stage III (data not shown) MSS, *BRAF* wild type CRCs. The same was observed for CL1, CL2 and CL3 CRCs (data not shown). Examining the validation population showed that overall CIMP was borderline statistically significantly associated with prognosis (Figure 4.3B, $p=0.02$). This finding was lost within Stage II (Figure 4.3D) and Stage III (data not shown) MSS, *BRAF* wild type CRCs. We next sought to determine whether one possible explanation for additional variation in previous studies was that specific CpG island methylation, underlying any prognostic importance, was imperfectly associated with CIMP. When analyzing all 19 (or 24 if including CIMP) genes, only *APC* and *CHFR* methylation, although the latter was positively associated with MSI ($p=0.02$) and CIMP ($p<0.01$, Table 4.S4), were associated with a worse prognosis in stage II MSS, *BRAF* wild-type CRCs (HR= 2.63 (95% CI=1.21-5.68, $p=0.01$) and HR=2.59 (95% CI=1.16-5.76, $p= 0.02$) respectively) (Table 4.S5 and Figure 4.3G). For stage III MSS *BRAF* wildtype CRCs, associations with worse prognosis were found for *RASSF1A*, *THBS1* and *CACNA1G* (HR=3.89 (95% CI 1.23-12.3, $p=0.02$; HR=26.5 (95% CI=1.66-423, $p=0.02$) and HR=5.62 (95% CI=1.49-21.3, $p=0.01$)) (Table 4.S5). These effects were not observed in the overall study population (Table 4.S5). Since the small numbers of cases and the number of comparisons examined to find significance raises concerns, we validated these results in an independent validation population. This only confirmed a statistically significant association with prognosis for *CHFR* methylation in stage II, MSS, *BRAF* wild-type CRCs (Figure 4.3F-3H). The Cox-regression multivariate model within the study population of MSS, *BRAF* wild-type, stage II CRCs shows that *CHFR* methylation was associated with a poor prognosis (HR=3.89 (95% CI =1.58-9.60), $p<0.01$ and was, in this study, a better predictor of survival than differentiation grade (HR 1.52 (95% CI =0.69-3.36), $p=0.30$) (Table 4.3). Multivariate analysis in the independent, population-based validation cohort of 151 stage II, MSS, *BRAF* wildtype CRCs confirmed that *CHFR* methylation is associated with a worse prognosis in this specific subgroup of CRCs ($n=151$, HR=2.21 (95% CI =1.09-4.48, $p=0.03$) (Table 4.3).

Figure 4.3 Survival curves overall population of (A) CIMP- ($n=124$) versus CIMP+ ($n=38$), $p=0.381$; of validation series (B) CIMP- ($n=436$) versus CIMP+ ($n=167$), $p=0.016$. Survival curves in stage II, MSS and *BRAF* wild-type tumors of (C) CIMP+ ($n=12$) and CIMP- ($n=61$), $p=0.158$; of validation series (D) CIMP+ ($n=23$) and CIMP- ($n=113$), $p=0.701$. Survival curves overall population of (E) *CHFR* unmethylated ($n=89$) versus *CHFR* methylated ($n=71$), $p=0.259$; of validation series (F) *CHFR* unmethylated ($n=319$) versus *CHFR* methylated ($n=280$), $p=0.078$. Survival curves in stage II, MSS and *BRAF* wild-type tumors of (G) *CHFR* unmethylated ($n=43$) and *CHFR* methylated ($n=30$) CRCs, $p=0.016$; of validation series (H) *CHFR* unmethylated ($n=85$) and *CHFR* methylated ($n=50$) CRCs, $p=0.049$.

Table 4.3 Cox proportional hazard models for MSS, *BRAF* wild-type, stage II CRCs

	Study population (n=66)		Validation population (n=151)	
	CRC mortality HR (95% CI)	p value	CRC mortality HR (95% CI)	p-value
CHFR M*	3.89 (1.58-9.60)	<0.01	2.21 (1.09-4.48)	0.03
Age (years)	1.02 (0.97-1.07)	0.41	1.02 (0.94-1.11)	0.43
Gender (female ^{**})	0.53 (0.23-1.27)	0.16	1.32 (0.66-2.63)	0.62
Tumor location				
Left-sided Colon ⁺	0.52 (0.20-1.38)	0.19	1.19 (0.51-2.78)	0.69
Rectum ⁺	0.29 (0.09-0.93)	0.04	1.89 (0.64-5.52)	0.25
Differentiation grade (poor [#])	1.52 (0.69-3.36)	0.30	0.70 (0.21-2.36)	0.56

*Reference group = *CHFR* unmethylated cases; **Reference group = male, +Reference group = Right-sided colon, #Reference group = Well/moderate. HR: Hazard Ratio=Relative Risk, M : Methylated.

Discussion

Data on the prognostic significance of promoter CpG island methylation, and CIMP in particular, are conflicting in CRC¹³⁻¹⁶. These inconsistencies might be caused by factors affecting the course of the disease, such as genetic alterations, adjuvant therapy and differences in methodological study approaches. Here we analysed the prognostic value of promoter CpG island methylation in CRCs from patients not treated with (neo)adjuvant therapy taking into account the confounding role of clinicopathological and genetic (MSI and *BRAF*) characteristics. Previously reported associations between CIMP and proximal tumor location, MSI and *BRAF* mutations^{10,11,16} as well as the identification of 3 subgroups of CRCs^{9,32} based on promoter methylation profiles could be confirmed. In addition, the prognostic role of tumor stage, MSI and *BRAF*^{33,34} could be confirmed in the study and the validation series.

In the study series, neither CIMP nor CL3 classification were statistically significantly associated with prognosis, neither overall nor when stratified for stage or microsatellite status. These conclusions are valid for the subgroups of CRC defined by analyzing CIMP with the markers proposed by Weisenberger et al.¹¹ and also for unsupervised clustering of the methylation data for the 19 CpG islands that we added to the analysis. Since also other CIMP definitions have been used, caution with generalizing these conclusions is warranted. However, these data confirm the concept that based on CpG island methylation, 3 subgroups of CRC can be identified, independently of the specific markers used. The subgroup characterized by extensive promoter CpG island methylation is strongly, although not perfectly, associated with CIMP and its reported clinicopathological characteristics. The borderline overall statistically significant prognostic effect of CIMP in the validation series indicates that the prognostic role of CIMP in CRC is still unclear and that large,

independent studies are needed to answer this question. In addition, until the underlying biological cause for CIMP is being identified, an accurate definition remains hard to establish. Our evidence for imperfect correlation of highly methylated tumors using the independent 19 genes with CIMP illustrates this.

After stratification for established confounders including age, gender, tumor location, differentiation grade, TNM Stage, MSI and *BRAF* mutation status, promoter methylation of only 1 of 24 genes, namely *CHFR*, was statistically significantly associated with a poor prognosis in stage II MSS CRCs in the study population (HR=3.89 (95% CI =1.58-9.602), $p=0.003$) and the validation series (HR=2.21 (95% CI=1.09-4.48, $p=0.03$). Because of the assumption that many biomarker data are false³⁵ and the fact that we studied many variables, our validation of the effect of *CHFR* promoter CpG island methylation in an independent prospective cohort study as well as pilot data recently published by an independent team pointing to the same conclusion³⁶, underscores the potential of *CHFR* promoter CpG island methylation as a prognostic marker in colorectal cancer.

A dominant effect of MSI over DNA methylation regarding prognosis has been shown previously in CRC by Ward et al., for a 'CIMP'-like phenotype³⁷. The mechanisms underlying this paradox are still not clear. An explanation could be the increased lymphocytic infiltrate that is observed in MSI CRCs³⁸ or the extend of instability making MSI tumor cells less fit to metastasize^{39,40}. The prognostic effect of *CHFR* in MSS stage II CRCs is surprising, as we previously reported a strong association between *CHFR* promoter CpG island methylation, MSI and *MLH1* promoter methylation in CRC⁴¹. However, since a significant subset of MSS CRCs also have *CHFR* promoter methylation, this could be of clinical relevance.

CHFR is a tumor suppressor gene which is inactivated by promoter CpG island methylation in a variety of solid tumors⁴¹⁻⁴⁵. It encodes an ubiquitin ligase that regulates both entry into metaphase and chromosome segregation later in mitosis to maintain genomic stability^{46,47}. *CHFR* inactivation has been hypothesized to be associated with chromosomal instability, although conflicting data have been reported⁴⁸⁻⁵⁰. Although both *CHFR* and *MLH1* contribute to genomic integrity, they function through different mechanisms. *CHFR* deficiency triggers mild CIN and *hMLH1* deficiency leading to MSI⁵¹. Recent data from Oh et al 2009⁵² indicate that, *in vitro*, CHFR binds and downregulates HDAC1 thereby downregulating the Cdk inhibitor p21^{CIP/WAF1} and the metastasis suppressors KAI1 and E-cadherin. This eventually results in cell cycle arrest and a less invasive phenotype. In addition to a potential role for *CHFR* as prognostic biomarker, *CHFR* promoter methylation has been proposed as biomarker for response to microtubule inhibitor taxanes in endometrial-⁵³ cervical-⁵³, oral-⁵⁴ and gastric cancer⁵⁵. Although taxanes are not implemented in CRC treatment because they failed to demonstrate a significant

clinical benefit in Phase II trials^{56,57}, CRCs with *CHFR* promoter methylation might benefit from taxanes.

In summary, although our study failed to demonstrate a consistent prognostic effect of CIMP, we identified promoter CpG island methylation of *CHFR* as a prognostic biomarker in stage II, MSS, *BRAF* wild-type CRCs in two independent populations.

Future large-scale validation studies and randomized clinical trials are needed to study the clinical value *CHFR* methylation in CRC.

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Table 4.S1 (Epigenetic alterations in relation to clinicopathological characteristics of the study population.

	Age			Gender		Tumor location			Differentiation			CRC Stage		
	Total (%)	Mean	Age (SD)	Male	Female	Right-sided colon	Left-sided colon	Rectum	Well/Moderate	Poor	I	II	III	IV
MS-status	MSI 19/169 (11%)	MSS 67.7 (10.8)	MSI 67.3 (16.5)	MSI 9/80 (11%)	MSI 10/89 (11%)	MSI 16/61 (26%)	MSI 2/51 (4%)	MSI 1/57 (2%)	MSI 10/93 (11%)	MSI 9/76 (12%)	MSI 0/3 (0%)	MSI 11/99 (11%)	MSI 6/48 (13%)	MSI 2/19 (11%)
BRAF-status	Mut 14/161 (9%)	Wild-type 67.8 (11.5)	Mut 67.5 (12.4)	Mut 7/74 (10%)	Mut 7/87 (8%)	Mut 6/59 (10%)	Mut 3/48 (6%)	Mut 5/54 (9%)	Mut 7/88 (8%)	Mut 7/73 (10%)	Mut 0/3 (0%)	Mut 10/93 (11%)	Mut 3/47 (6%)	Mut 1/18 (6%)
KRAS-status	Mut 41/157 (26%)	Wild-type 66.7 (11.3)	Mut 70.7 (12.5)	Mut 18/73 (25%)	Mut 23/84 (27%)	Mut 17/58 (29%)	Mut 11/45 (24%)	Mut 13/54 (24%)	Mut 22/85 (26%)	Mut 19/72 (26%)	Mut 0/2 (0%)	Mut 25/93 (27%)	Mut 11/45 (24%)	Mut 5/17 (29%)
MLH1	M 52/173 (30%)	U 68.4 (11.1)	M 65.6 (12.8)	M 23/82 (28%)	M 29/91 (32%)	M 26/62 (42%)	M 8/52 (15%)	M 18/59 (31%)	M 26/96 (27%)	M 26/77 (34%)	M 1/4 (25%)	M 30/100 (30%)	M 13/50 (26%)	M 8/19 (42%)
p16INK4A	72/166 (43%)	66.4 (12.5)	68.6 (10.6)	31/77 (40%)	41/89 (46%)	30/60 (50%)	13/50 (26%)	29/56 (52%)	38/92 (41%)	34/74 (46%)	0/4 (0%)	39/95 (41%)	26/49 (53%)	7/18 (39%)
p14ARF	76/172 (44%)	68.8 (10.3)	65.9 (13.1)	33/81 (41%)	43/91 (47%)	29/62 (47%)	19/51 (37%)	28/59 (48%)	42/96 (44%)	34/76 (45%)	1/4 (25%)	43/99 (43%)	23/50 (46%)	9/19 (47%)
MGMT	55/173 (32%)	67 (11.5)	69 (12.1)	24/82 (29%)	31/91 (34%)	21/62 (34%)	17/52 (33%)	17/59 (29%)	29/96 (30%)	26/77 (34%)	1/4 (25%)	32/100 (32%)	16/50 (32%)	6/19 (32%)

	Total		Age		Gender		Tumor location			Differentiation		CRC Stage			
	(%)	Mean	Age (SD	Male	Female	Right-sided colon	Left-sided colon	Rectum	Well/Moderate	Poor	I	II	III	IV	
RASSF1A	34/172 (20%)	67.5 (12.2)	67.9 (10.0)	18/82 (22%)	16/90 (18%)	7/62 (11%)	17/52 (33%)	10/58 (17%)	15/95 (16%)	19/77 (25%)	0/4 (0%)	22/99 (22%)	10/50 (20%)	2/19 (11%)	
					0.49			0.01		0.15				0.50	
APC	60/173 (35%)	67.4 (11.4)	67.8 (12.2)	26/82 (32%)	34/91 (37%)	19/62 (31%)	21/52 (40%)	20/59 (34%)	34/96 (35%)	26/77 (34%)	1/4 (25%)	36/100 (36%)	17/50 (34%)	6/19 (32%)	
					0.43			0.55		0.82				0.95	
HLTF	68/168 (41%)	68.0 (11)	66.3 (12.7)	31/81 (38%)	37/87 (43%)	24/61 (39%)	18/50 (36%)	26/57 (46%)	29/93 (31%)	39/75 (52%)	2/4 (50%)	40/96 (42%)	20/49 (41%)	6/19 (32%)	
					0.57			0.59		<0.01				0.84	
GATA4	90/162 (56%)	67.4 (11.8)	67.3 (12.6)	41/75 (55%)	49/87 (56%)	34/59 (58%)	22/48 (46%)	34/55 (62%)	50/91 (54%)	40/71 (56%)	3/4 (75%)	51/91 (56%)	26/49 (53%)	10/18 (56%)	
					0.83			0.25		0.86				0.86	
GATA5	93/161 (58%)	67.2 (10.5)	67.8 (12.6)	38/76 (50%)	55/85 (65%)	35/59 (59%)	24/47 (51%)	34/55 (62%)	48/88 (55%)	45/73 (62%)	2/4 (50%)	51/92 (55%)	32/48 (67%)	8/17 (47%)	
					0.06			0.52		0.36				0.45	
CHFR	71/166 (43%)	67.8 (12.3)	67.2 (11)	31/78 (40%)	40/88 (46%)	31/62 (50%)	13/49 (27%)	27/55 (49%)	36/91 (40%)	35/75 (47%)	0/3 (0%)	39/97 (40%)	26/48 (54%)	6/18 (33%)	
					0.46			0.02		0.36				0.13	
ADAM23	64/152 (42%)	66.5 (11)	68.0 (12.9)	26/72 (36%)	38/80 (48%)	26/53 (49%)	11/44 (25%)	27/55 (49%)	37/83 (45%)	27/69 (39%)	0/4 (0%)	33/83 (40%)	27/48 (56%)	4/17 (24%)	
					0.16			0.02		0.50				0.02	
RAB32	21/170 (12%)	67.2 (11.5)	69.8 (12.7)	9/80 (11%)	12/90 (13%)	12/61 (20%)	4/51 (8%)	5/58 (9%)	11/94 (12%)	10/76 (13%)	1/4 (25%)	8/98 (8%)	9/49 (18%)	3/19 (16%)	
					0.68			0.09		0.77				0.26	

	Age			Gender		Tumor location			Differentiation			CRC Stage		
	Total (%)	Mean	Age (SD)	Male	Female	Right-sided colon	Left-sided colon	Rectum	Well/Moderate	Poor	I	II	III	IV
JPH3	115/171 (67%)	66.1 (9.8)	68.5 (12.4)	53/81 (65%)	62/90 (69%) 0.63	43/61 (71%)	35/51 (69%)	37/59 (63%) 0.64	60/94 (64%)	55/77 (71%) 0.29	2/4 (50%)	69/99 (70%)	34/49 (69%)	10/19 (11%) 0.43
FOXL2	56/165 (34%)	67.7 (11.2)	67.6 (12.2)	25/78 (32%)	31/87 (36%) 0.62	25/60 (42%)	13/48 (27%)	18/57 (32%) 0.25	27/91 (30%)	29/74 (39%) 0.20	0/3 (0%)	30/96 (31%)	20/47 (43%)	6/19 (32%) 0.33
BNIP3	94/171 (55%)	66.5 (11)	68.2 (12.4)	46/81 (57%)	48/90 (53%) 0.65	40/61 (66%)	21/51 (41%)	33/59 (56%) 0.04	51/95 (54%)	43/76 (57%) 0.71	1/4 (25%)	49/99 (50%)	37/50 (74%)	7/18 (39%) <0.01
NEURL	33/170 (19%)	68.3 (11.2)	64.9 (12.8)	17/80 (21%)	16/90 (18%) 0.57	19/61 (31%)	5/51 (10%)	9/58 (16%) 0.01	16/93 (17%)	17/77 (22%) 0.42	0/4 (0%)	18/99 (18%)	15/48 (31%)	0/19 (0%) 0.02
CACNA2	45/156 (29%)	66.9 (11)	69.0 (12.4)	21/75 (28%)	24 (30%) 0.82	22/52 (42%)	5/45 (11%)	18/59 (31%) <0.01	22/86 (26%)	23/70 (33%) 0.32	0/3 (0%)	26/91 (29%)	14/45 (31%)	5/17 (29%) 0.72
THBS1	8/147 (5%)	66.8 (11.8)	77.5 (9)	2/69 (3%)	6/78 (8%) 0.20	5/53 (9%)	2/43 (5%)	1/51 (2%) 0.24	5/80 (6%)	3/67 (5%) 0.64	0/4 (0%)	3/84 (4%)	3/43 (7%)	2/16 (13%) 0.47
TFPI2	125/144 (87%)	65.7 (13.2)	67.7 (11.8)	61/73 (84%)	64/71 (90%) 0.24	45/53 (85%)	35/39 (90%)	45/52 (87%) 0.79	70/79 (89%)	55/65 (85%) 0.48	3/4 (75%)	68/80 (85%)	38/43 (88%)	16/17 (94%) 0.66

SD: standard deviation, U: Unmethylated, M: Methylated, MS: Microsatellite, MSS: Microsatellite Stable, MSI: Microsatellite Instable, Mut: Mutation

Table 4.S2 CIMP and CL1, CL2 and CL3 in relation to clinicopathological characteristics of the study population.

Overall (%)	Age		Gender		Tumor location		Differentiation		CRC Stage				
	Mean	Age (SD)	Male	Female	Right-sided Colon	Left-sided Colon	Rectum	Well/ Moderate	Poor	I	II	III	IV
CIMP 39/168 (23%)	-	+	+	+	+	+	+	+	+	+	+	+	+
	67 (11.6)	69.5 (11.6)	15/78 (20%)	24/90 (27%) 0.26	20/58 (35%)	5/52 (10%)	14/58 (24%) <0.01	19/93 (20%)	20/75 (27%) 0.34	0/4 (0%)	18/97 (19%)	17/48 (35%)	4/19 (21%) 0.38
CL1 98/173 (57%)		67.3 (10.7)	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%)	67.4 (13.4)	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%)		68.7 (12.4)	13/82 (16%)	19/91 (21%) 0.53	17/62 (27%)	2/52 (4%)	13/59 (22%) <0.01	16/96 (17%)	16/77 (21%) 0.11	0/4 (0%)	16/100 (16%)	11/50 (22%)	5/19 (26%) 0.59

CIMP: CpG island methylation phenotype using the markers of Weisenberger et al. 1, CL1, CL2 and CL3: subgroups of CRCs identified by unsupervised hierarchical clustering based on methylation patterns of 19 tumor suppressor and DNA repair genes.

Table 4.S3 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 ACC 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 CAA 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>SOCs1</i>	flank	5'-GTT YGT GGG TAT TTT TTT GGT G-3'	5'-TAA ATC CCR AAA CCA TCT TCA C-3'	83	56	35
<i>SOCs1</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>SOCs1</i>	M	5'-TTT TGA YGT AGA YGT TTT ATT AGG GT-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 TTT 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>p16INK4A</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>p16INK4A</i>	U	5'-GTT GGT TAT TAG AGG GTG GGG TGG ATT GT-3'	5'-AAC CAA AAA CTC CAT ACT ACT CCC CAC CA-3'	124	62	25
<i>p16INK4A</i>	M	5'-TTA TTA GAG GGT GGG GCG GAT CGC-3'	5'-GAA AAC TCG ATA CTA CTC CCC GCG G-3'	115	62	25
<i>p14ARF</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>p14ARF</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>p14ARF</i>	M	5'-GTG TTA AAG GGC 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 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
<i>RASSF1A</i>	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
<i>RASSF1A</i>	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
<i>RASSF1A</i>	M	5'-GGG TTC GTT TTG TGG TTT CGT TC-3'	5'-TAA CCC GAT TAA ACC CGT ACT TCG-3'	76	64	30
<i>APC</i>	flank	5'-TGG GYG GGG TTT TGT GTT TTA TT-3'	5'-TAC ROC CAC ACC CAA CCA ATC-3'	136	56	35
<i>APC</i>	U	5'-GTG TTT TAT TGT GGA GTG TGG GTT-3'	5'-CCA ACT AAC AAA CTC CCA ACA A-3'	108	60	25
<i>APC</i>	M	5'-TAT TGC GGA GTG CCG GTG-3'	5'-TCG ACG AAC TCC CGA CGA-3'	98	60	25
<i>HLTF</i>	flank	5'-GTA GGT ATY GTA GTY TTA TTT TTG GG-3'	5'-CAA AAC ACA AAA AAA ACA ACT CC-3'	155	56	35
<i>HLTF</i>	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
<i>HLTF</i>	M	5'-GTG GTT TTT TCG CGC GTT C-3'	5'-CGG TAC CAT TCA AAA ACG ACG-3'	96	65	30

Gene	Primer	Sense primer	Antisense primer	Amplicon size (bp)	Annealing temp (°C)	PCR cycles
GATA4	flank	5'-GGG AGT TTT TYG TAT AGT TTY GTA G-3'	5'-COR ACC RCC TOC AAA TOC CCA AC-3'	167	56	35
GATA4	M	5'-TTT GTA TAG TTT TGT AGT TTG TGT TTA GT-3'	5'-CCC AAC TCA CAA CTC AAA TOC CCA-3'	140	64	30
GATA4	U	5'-GTA TAG TTT CGT AGT TTG CGT TTA GC-3'	5'-AAC TCG CGA CTC GAA TOC CCG-3'	136	68	25
GATA5	flank	5'-TAG ATA YGG AGT TYG TTT TTA GGT TAG-3'	5'-CRA AAC CCR AAC CAA TAC AAC TAA AC-3'	160	56	35
GATA5	M	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	U	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'-CHC TCA CCA AAA ACR ACA ACT AAA AC-3'	167	56	35
CHFR	M	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	U	5'-GTT ATT TTC GTG ATT CGT AGG CGA C-3'	5'-CGA AAC CGA AAA TAA CCC CGC-3'	100	66	30
ADAM23	flank	5'-GGG GGG TTG TTG YGT TTA G-3'	5'-CAA AAC 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'-CCG AAC CGA AAA CAA ACC CG-3'	91	66	30
RAB32	flank	5'-GGA GAG GAA AGT TTA GTT GGG TT-3'	5'-CCG 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 CGC-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
JPH3	U	5'-GGT TTT TAA TAT GGT GTA GTT GTT GTT-3'	5'-CCC CCC ACC TAC AAC TAC CAC A-3'	103	64	35
JPH3	M	5'-TTT AAT ATG GTG TAG TCG TTA GCG TC-3'	5'-CCG ACC TAC GAC TAC CGC G-3'	103	64	35
FOX L2	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
FOX L2	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
FOX L2	M	5'-TTG GAG ATG AAT TCG TTC GTG C-3'	5'-CGT ATA AAC TCG ACC GAA TTC CG-3'	89	65	20
BNIP3	flank	5'-TTT TTG TTG TTT TGT GAG TTT TTT-3'	5'-COR AAC TAC AAA ATA TAC TTC AAC TAC-3'	129	56	35
BNIP3	U	5'-GGT TTT GAT TAG TTT GGG AGT G-3'	5'-CCT CAA CTA CAA ACA ATA AAA AAA CA-3'	74	61	35
BNIP3	M	5'-TTT CGT TTA GTT CGG GAG CG-3'	5'-CAA CTA CGA ACG ATA AAA AAA CG-3'	69	61	35
NEURL	flank	5'-CGT TTA GGG TTT TGT TCG GAG-3'	5'-CCT CAA CTT CCT TTC TCT AAA CTT-3'	132	56	35
NEURL	U	5'-TTG TTT GTG GTT TTT GTT TTT GTT AT-3'	5'-CTT TCT CTA AAC TTC ACA ATC CTT AAC A-3'	111	65	35
NEURL	M	5'-GTT TGT GGT TTT CGT TTT CGT TAC-3'	5'-TCT AAA CTT CGC GAT CTT TAA CG-3'	104	65	35
CACNA2	flank	5'-GYG GAG YGG AGT AGG TAG TTT-3'	5'-AAA ACR CCR AAA CCC CC-3'	159	56	35
CACNA2	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
CACNA2	M	5'-CGC GTA GTT TTT GCG GGT C-3'	5'-CCG CCC GAA ACG CG-3'	102	62	35
THBS1	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
THBS1	U	5'-TAT TTT TTT TTT TTT TTT TGG TTG TT-3'	5'-ACT AAC AAA ACA AAA AAA CCA CAC A-3'	99	64	30
THBS1	M	5'-TTC GTT TTT TGT TCG GTC GTC-3'	5'-ACC GAA AAA ACC GCG CG-3'	86	64	30
TFPI2	flank	5'-GTG TAT GAA TTA GTT ATT TTT TAG GTT T-3'	5'-CTA AAC AAA ACR TOC RAA AAA AC-3'	144	56	35
TFPI2	U	5'-TTA GTT ATT TTT TAG GTT TTG TTT TGG T-3'	5'-AAA AAC ACC TAA CAA AAA ATA CAC A-3'	118	64	35
TFPI2	M	5'-ATT TTT TAG GTT TCG TTT CGG C-3'	5'-GCC TAA CGA AAA ATA CGC G-3'	106	66	35

Table 4.S4 Correlations between promoter CpG island methylation of 19 tumor suppressor/DNA repair genes, MSI, BRAF, KRAS and CIMP in the study population.

	MSI	MSS	BRAF M	BRAF WT	KRAS M	KRAS WT	CIMP	No-CIMP
<i>MLH1</i>	13/19 (68%)	37/150 (25%) <0.01	8/14 (57%)	41/147 (28%) 0.03	11/41 (27%)	38/116 (33%) 0.48	20/39 (51%)	32/129 (25%) <0.01
<i>p16INK4A</i>	12/18 (67%)	59/144 (41%) 0.04	7/14 (50%)	63/141 (45%) 0.70	19/39 (49%)	49/111 (44%) 0.62	30/37 (81%)	42/125 (34%) <0.01
<i>p14ARF</i>	14/19 (74%)	59/149 (40%) <0.01	7/14 (50%)	64/146 (44%) 0.66	22/40 (55%)	48/116 (41%) 0.14	27/39 (69%)	49/128 (38%) <0.01
<i>MGMT</i>	5/19 (26%)	49/150 (33%) 0.58	3/14 (21%)	50/147 (34%) 0.34	14/41 (34%)	40/116 (35%) 0.97	19/39 (49%)	35/129 (27%) 0.01
<i>RASSF1A</i>	2/19 (11%)	31/149 (21%) 0.29	1/14 (7%)	32/146 (22%) 0.19	9/40 (23%)	22/116 (19%) 0.63	10/38 (26%)	24/129 (19%) 0.30
<i>APC</i>	8/19 (42%)	51/150 (34%) 0.49	3/14 (21%)	54/147 (37%) 0.25	17/41 (42%)	38/116 (33%) 0.32	13/39 (33%)	46/129 (36%) 0.79
<i>HLTF</i>	11/19 (58%)	55/145 (38%) 0.10	6/14 (43%)	60/142 (42%) 0.97	15/37 (41%)	48/115 (42%) 0.90	24/38 (63%)	44/125 (35%) <0.01
<i>GATA4</i>	13/18 (72%)	75/140 (54%) 0.13	11/14 (79%)	76/139 (55%) 0.09	24/38 (63%)	58/110 (53%) 0.27	30/38 (79%)	60/120 (50%) <0.01
<i>GATA5</i>	15/19 (79%)	75/138 (54%) 0.04	9/14 (64%)	81/138 (59%) 0.69	23/39 (59%)	64/107 (60%) 0.93	33/38 (87%)	60/120 (50%) <0.01
<i>CHFR</i>	13/19 (68%)	57/143 (40%) 0.02	9/13 (69%)	60/142 (42%) 0.06	17/39 (44%)	50/112 (45%) 0.91	29/39 (74%)	42/122 (34%) <0.01
<i>ADAM23</i>	10/16 (63%)	51/132 (39%) 0.07	8/12 (67%)	54/131 (41%) 0.09	21/36 (58%)	39/103 (38%) 0.03	25/38 (66%)	38/111 (34%) <0.01

	MSI	MSS	BRAF M	BRAF WT	KRAS M	KRAS WT	CIMP	No-CIMP
RAB32	9/19 (47%)	11/147 (8%) <0.01	6/13 (46%)	15/145 (10%) <0.01	4/41 (10%)	17/115 (15%) 0.42	17/39 (44%)	4/126 (3%) <0.01
JPH3	16/19 (84%)	95/148 (64%) 0.08	10/13 (77%)	100/146 (69%) 0.53	33/41 (81%)	75/115 (65%) 0.07	32/39 (82%)	82/127 (65%) 0.04
FOX L2	13/19 (68%)	41/142 (29%) 0.01	9/13 (69%)	46/141 (33%) 0.01	17/40 (43%)	35/111 (32%) 0.21	21/39 (54%)	34/122 (28%) <0.01
BNIP3	15/19 (79%)	76/148 (51%) 0.02	9/14 (64%)	79/145 (55%) 0.48	28/40 (70%)	58/115 (50%) 0.03	30/39 (77%)	62/127 (49%) <0.01
NEURL	9/19 (47%)	23/147 (16%) <0.01	7/13 (54%)	25/145 (17%) <0.01	7/39 (18%)	23/116 (20%) 0.80	20/39 (51%)	12/126 (10%) <0.01
CACNA2	14/17 (82%)	29/135 (22%) <0.01	8/12 (67%)	36/133 (27%) <0.01	14/38 (37%)	27/104 (26%) 0.21	26/38 (68%)	19/115 (17%) <0.01
THBS1	4/17 (24%)	3/126 (2%) <0.01	2/12 (17%)	6/129 (5%) 0.08	3/35 (9%)	5/99 (5%) 0.45	7/38 (18%)	1/108 (1%) <0.01
TFPI2	15/17 (88%)	106/123 (86%) 0.82	12/14 (86%)	106/122 (87%) 1.00	28/35 (80%)	87/97 (90%) 0.15	35/39 (90%)	89/104 (86%) 0.51

CIMP: CpG island methylation phenotype as defined by the markers of Weisenberger et al. 1, MSI: microsatellite instability, MSS: microsatellite stability, M: Mutant, WT: Wild-type

Table 4.S5 HR's of 24 single genes overall and after stratification for stage 2, MSS, BRAF-wild type and stage 3, MSS, BRAF-wild type within the study population.

	Overall			Stage 2, MSS, BRAF-wildtypes			Stage 3, MSS, BRAF-wildtypes		
	HR (95%CI)	P value		HR (95%CI)	P value		HR (95%CI)	P value	
<i>MLH1</i>	0.92 (0.53-1.59)	0.77		0.65 (0.25-1.73)	0.39		0.91 (0.26-3.21)	0.89	
<i>p16INK4A</i>	1.17 (0.71-1.92)	0.54		0.88 (0.40-1.91)	0.75		1.84 (0.68-4.90)	0.23	
<i>p14ARF</i>	1.28 (0.78-2.08)	0.33		1.40 (0.65-3.03)	0.39		1.49 (0.55-4.0)	0.43	
<i>MGMT</i>	1.62 (0.99-2.68)	0.57		1.66 (0.77-3.58)	0.20		1.56 (0.58-4.2)	0.38	
<i>RASSF1A</i>	1.20 (0.65-2.21)	0.55		0.76 (0.30-1.88)	0.55		3.89 (1.23-12.3)	0.02	
<i>APC</i>	1.30 (0.78-2.15)	0.32		2.63 (1.21-5.68)	0.01		0.59 (0.19-1.84)	0.37	
<i>HLTF</i>	1.05 (0.64-1.73)	0.85		1.43 (0.65-3.15)	0.37		2.16 (0.78-5.96)	0.14	
<i>GATA4</i>	0.92 (0.56-1.50)	0.73		0.89 (0.41-1.93)	0.77		1.22 (0.46-3.25)	0.70	
<i>GATA5</i>	1.20 (0.71-2.02)	0.50		1.40 (0.60-3.28)	0.44		1.46 (0.53-4.02)	0.47	
<i>CHFR</i>	1.33 (0.81-2.20)	0.26		2.59 (1.16-5.76)	0.02		1.33 (0.50-3.57)	0.57	
<i>ADAM23</i>	0.99 (0.59-1.69)	0.98		1.49 (0.64-3.49)	0.36		1.11 (0.42-2.99)	0.83	
<i>RAB32</i>	0.56 (0.20-1.53)	0.26		0.05 (0.00-443)	0.51		0.45 (0.06-3.40)	0.44	
<i>JPH3</i>	1.14 (0.67-1.93)	1.14		1.75 (0.70-4.35)	0.23		1.31 (0.46-3.81)	0.61	
<i>FOXL2</i>	0.90 (0.53-1.53)	0.70		0.92 (0.39-2.20)	0.85		0.97 (0.33-2.84)	0.95	
<i>BNIP3</i>	0.94 (0.58-1.55)	0.82		0.86 (0.39-1.88)	0.70		1.08 (0.38-3.12)	0.87	
<i>NEURL</i>	0.78 (0.41-1.50)	0.46		1.83 (0.77-4.36)	0.17		0.78 (0.18-3.43)	0.74	
<i>CACNA2</i>	0.99 (0.56-1.77)	0.99		0.70 (0.24-2.05)	0.52		2.18 (0.68-7.00)	0.19	
<i>THBS1</i>	1.12 (0.35-3.59)	0.85		0.48 (0.00-683)	0.62		26.5 (1.66-423)	0.02	
<i>TFPI2</i>	1.35 (0.58-3.16)	0.48		1.40 (0.32-5.95)	0.66		0.98 (0.22-4.42)	0.98	
<i>CACNA1G</i>	1.07 (0.54-2.10)	0.85		2.39 (0.55-10.3)	0.24		5.62 (1.49-21.3)	0.01	
<i>IGF2</i>	0.73 (0.31-1.70)	0.46		0.94 (0.22-4.03)	0.94		1.46 (0.19-11.1)	0.72	
<i>NEUROG1</i>	1.14 (0.69-1.89)	0.60		2.18 (0.97-4.87)	0.58		1.75 (0.65-4.72)	0.27	
<i>RUNX3</i>	0.75 (0.42-1.35)	0.34		0.94 (0.37-2.36)	0.89		0.94 (0.31-2.81)	0.91	
<i>SOSC1</i>	0.86 (0.52-1.43)	0.86		0.86 (0.40-1.86)	0.70		1.88 (0.59-6.01)	0.29	

Chapter 5

Emerging evidence for *CHFR*
as a cancer biomarker:
update and prospects

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Introduction

Novel insights in the biology of cancers have revealed a variety of promising biomarkers for early detection of cancer and for predicting prognosis and response to therapy. Despite these insights and a revolution in biotechnology, only few biomarkers are implemented in current daily practice. Examples of operational biomarkers are testing for *KRAS* mutation and expression of *EGFR* to predict response to monoclonal antibodies against EGFR in lung¹ and metastatic colon cancer². However, EGFR antibodies are most frequently used as second or third line therapy and the number of patients that might benefit from this treatment is small. The great majority of patients still receive standard first line therapy. For this reason the use of chemotherapeutic agents is still very inefficient and too many patients experience unneeded toxicity.

In this review, we raise attention to a biomarker for which multiple lines of evidence are emerging: checkpoint with FHA and ring finger (*CHFR*). *CHFR* is a mitotic checkpoint- and tumor suppressor gene and is inactivated in a diversity of solid malignancies. Here we summarize the current evidence of altered *CHFR* expression in cancer and discuss its promising role as a prognostic and predictive biomarker.

CHFR: an important regulator of cell cycle progression

One of the hallmarks of cancer is loss of chromosomal instability, mostly due to dysfunction of one or more cell cycle checkpoints. The spindle assembly checkpoint is a key player in controlling chromosomal integrity by sensing microtubule kinetochore attachment and allowing promotion of mitosis only when chromosomes are attached properly.

Checkpoint with FHA and RING finger domain, *CHFR*, is an early mitotic checkpoint gene, which is more frequently inactivated in cancer than all mitotic checkpoint control genes together³. In case of microtubule- and radiation damaging stress *CHFR* is able to reversibly delay passage into mitosis before chromosome condensation has taken place^{4,5}. *CHFR* localizes in the nucleus and becomes phosphorylated by protein kinase B (PBK/AKT), a member of the PI3K signaling pathway⁶. The nuclear distribution, mobility and function of *CHFR* is modulated by interaction with promyelocytic leukemia protein (PML) bodies^{7,8}. PML bodies are involved in various cellular processes such as cellular senescence, apoptosis and maintenance of chromosomal stability and also the function of *CHFR* is dependent upon the interaction with PML bodies⁷. *CHFR* is an E3 ubiquitin ligase which contains a FHA domain and a RING finger domain (Figure 5.1). The function of the FHA domain is largely unknown but is thought to mediate binding to phosphorylated proteins⁹ and is responsible for the anti-proliferative effect of *CHFR*. The RING finger domain is

able to ubiquitinate substrates such as polo-like kinase 1 (PLK1), Aurora A, MAD2 and CHFR itself⁹. By degradation of PLK1 CHFR inhibits the formation of the cyclin B1-Cdk kinase complex, which will result in a cell cycle arrest¹⁰. While evidence for degradation of PLK1 by CHFR in vivo is weak, ubiquitination of pro-mitotic protein Aurora A and thereby maintaining chromosomal stability is clearly shown in vitro and in vivo¹¹. The same accounts for ubiquitination of MAD2, which enables CHFR to control proper spindle attachment to kinetochores and thereby delays mitotic progression^{12,13,14}. The interaction between CHFR and Aurora A and MAD2 is accomplished by the cysteine rich domain of CHFR^{11,12,15}. Inside this cysteine rich region a RAR-binding zinc-finger (PBZ) is situated which is able to bind poly-(ADP-ribose)polymerases, important for checkpoint regulation and DNA damage response¹⁶.

More recently it was shown that CHFR also interacts with histone deacetylase HDAC1¹⁷. HDAC1 is able to repress p21 and by inhibiting the deacetylase activity of HDAC1 CHFR restores the p21-G1 checkpoint¹⁷⁻¹⁹.

Figure 5.1 *CHFR* gene and protein. Schematic representation of promoter CpG island methylation, mutation and polymorphisms with functional significance of:

A) *CHFR* gene encompassing 18 exons. CpG island is enlarged with CpG dinucleotides as vertical lines. TSS: transcription start site * mutation. # polymorphism.

B) CHFR protein consisting of 664 aminoacids. FHA: forhead-associated domain. RING: ringfinger domain. CR: cysteine rich domain. PBZ: RAR-binding zinc-finger domain. Mutations in black, polymorphism in gray.

CHFR is frequently inactivated in cancer

Although many cancer cells have an impaired or unregulated spindle checkpoint, mutations of mitotic checkpoint genes are rare. Lack of expression of *CHFR* in cancer cells was first described by Scolnick et al.³ in one neuroblastoma and two colorectal cancer cell lines. Nonfunctional *CHFR* in these cell lines resulted in a high mitotic index when exposed to microtubule stress compared to wild-type cancer cells, which was restored by reintroduction of functional *CHFR*. A role for *CHFR* in tumor suppression became clear when *CHFR* deficient mice spontaneously developed malignant tumors¹¹. *CHFR* knockout mice are viable and develop without defects but display invasive lymphomas and solid tumors (lung, liver, gastrointestinal) after 40 weeks. Furthermore, *CHFR* deficient mice show defects in chromosome segregation and aneuploidy. Also in a breast cancer cell line model decreased *CHFR* expression led to an accelerated growth rate, enhanced invasiveness, and amplified colony formation²⁰. Since then, disrupted *CHFR* expression has been described in multiple cancer tissues (Table 5.1).

The tumor suppressor role of *CHFR* is not entirely clear. Due to its checkpoint function *CHFR* was thought to participate in the acquisition of chromosomal defects and a chromosomal instability phenotype. Although this was observed in previously described cell lines models, in primary colorectal- and breast cancer tissue this could not be observed²⁵. To the contrary, *CHFR* inactivation was found to occur most frequently in cancers with microsatellite instability (MSI)²⁹ and *MLH1* promoter CpG island methylation. Comparable results have been observed in gastric cancer⁵⁵. The mechanism underlying the association between *CHFR* promoter methylation and MSI is unknown but might be explained the association with *MLH1* promoter methylation and a shared underlying DNA methylation defect. Future studies are needed to unravel the underlying mechanism, and a significant subset of MSS CRCs also has *CHFR* promoter methylation, which could be of clinical relevance.

Furthermore, a recent study shows an additional role for *CHFR* in regulating expression of pro-inflammatory chemokine interleukin-8⁵⁶. *CHFR* was able to inhibit the NFκB signaling pathway and IL-8, which subsequently resulted in decreased angiogenesis and cell migration⁵⁷⁻⁵⁹. Inactivation of *CHFR* may trigger NFκB signaling activity, which results in increased angiogenesis, a metastatic phenotype and a poor prognosis.

Table 5.1 CHFR inactivation in multiple cancers

Cancer	Aberration	Method, region analyzed	Percentage of primary cancers	ref
Breast cancer	Reduced expression	IHC	36% (51/142)	20
	Methylation	Bisulfite restriction analysis	8% (2/24) cell lines	21
Bladder cancer	Methylation	MLPA	18.7% (17/91)	22
Colorectal cancer	Methylation	COBRA	40% (25/63)	23
		MSP, -281 to +51 bp	37% (11/30)	24
		MSP, -220 to -14 bp	53% (27/51) (adenomas)	25
		MSP, -240 to -73 bp	36% (8/22)	26
		MSP, -226 to -82 bp	41% (29/71)	27
		qMSP, +221 to +325 bp	24% (217/888)	28
		MSP, -240 to -73 bp	31% (19/61)	29
Gastric cancer	Methylation	COBRA	39% (24/61)	30
		MSP, -9 to +98 bp	35% (25/71)	31
		MSP	44% (19/43)	32
		MSP, -163 to -8 bp	52% (24/46)	33
Nasopharyngeal cancer	Methylation	MSP, -220 -14 bp	61% (22/36)	34
		MSP, -220 -14 bp	59% (31/53)	35
Lung Cancer	Methylation	MSP, -220 -14 bp	19% (7/37)	36
	Mutation	MSP, -220 -14 bp	10% (2/20)	37
		MSP, -220 -14 bp	39% (62/157)	38
		MSP, -220 -14 bp	32.4% (100/308) serum, stage IV	39
			0.06% 3/53	
Esophageal Cancer	Methylation	MSP, -220 -14 bp	16.3% (7/43)	40
	Copy number loss	MSP, -220 -14 bp	24% (9/38)	41
		bisulfite pyrosequencing	31% (18/58)	42
		qPCR	59% (44/56)	43
Cervical cancer	Methylation	MSP, +168 to 318	12% (2/14)	44
Hepatocellular cancer	Methylation	MSP, -225 to -85 bp	35% (22/62)	45
Biliary tract carcinoma	Methylation	MSP, -281 to +51 bp	16% (6/37)	46
Oral squamous cell cancer	Methylation	MSP, -220 bp -14 bp	31% (4/13)	47
			34.7% (17/49)	
Cutaneous T-cell lymphoma	Methylation	CpG island microarray	19% (5/28)	48
Head and neck cancer	Methylation	COBRA	30% (16/54)	23
		MS-MLPA	25% (7/28)	49
Endometrial cancer	Methylation	MSP, +168 to 318 bp	12% (6/50)	50
Cutaneous T-cell lymphomas	Methylation	CpG island microarray	19% (5/28)	48

IHC: immunohistochemistry, (MS)-MLPA: (methylation specific) multiplex ligation-dependent probe amplification, MSP: methylation specific PCR, COBRA: combined bisulfite restriction analysis

CHFR is inactivated by genetic and epigenetic mechanisms

The original study of Scolnick and Halazonetis describes a sequence variation in the cysteine rich domain of *CHFR* in osteosarcoma cell line U2OS³ (Figure 5.1). The variation consisted of a G to A transition leading to substitution of Val 539 with Met and was initially interpreted as potential missense mutation with functional impairment but turned out to be a functional polymorphism.³⁶ The functional difference between wild-type and variant genotype was further studied in a series of 462 colorectal cancer patients and 245 healthy controls⁶⁰. This study showed that the A allele of the GA variant was associated with a reduced colorectal cancer risk and absence of distant metastasis. Additional studies to identify structural variations in the *CHFR* coding sequence led to the identification of three missense mutations in non-small cell lung cancer (NSCLC) that all three were associated with a defective mitotic checkpoint³⁹. Two mutations target the FHA en RING finger domain and the third is located in the cysteine rich region (Figure 5.1B). These mutations, however, were observed in only 3 out of 53 patients and are therefore rare events. Loss of the chromosomal region harboring *CHFR*, 12q24.33, occurs more frequently. In esophageal adenocarcinomas, *CHFR* DNA copy number loss occurs in 59% (44/56) of esophageal cancers⁴² and is associated with reduced *CHFR* expression.

In most cancers, however, *CHFR* expression is reduced due to promoter CpG island methylation (Figure 5.1A). The promoter region of *CHFR* contains a CpG island spanning -905 bp to +783 bp relative to the transcription start site (using the lower limit values 500bp for length, 55% for GC content and 0.65 for ObsCpG/ExpCpG⁶¹). *CHFR* promoter methylation and subsequent transcriptional silencing was first described in esophageal cancer⁴⁰, of which 16.3% (7/43) was hypermethylated while this was absent in adjacent normal tissues. Later it became clear that *CHFR* promoter methylation occurs in other cancers as well, among which colorectal-, gastric- and lung cancer (Table 5.1). Also in breast cancer *CHFR* becomes inactivated, however, only occasionally by promoter CpG island methylation²⁰.

In most studies *CHFR* promoter CpG island methylation is assessed by methylation- specific PCR (MSP), which is an accurate and sensitive test to detect aberrant methylation in the primary tumor. In recent years it has become clear that the core region of CpG methylation within the CpG island which is biologically and clinically relevant can vary per gene⁶². For *CHFR*, CpG methylation within region -240 bp to -79 relative to the transcription start site is associated with reduced expression and clinical parameters such as prognosis²⁹ (Cleven et al, submitted) and therefore identified as core region.

CHFR promoter methylation is associated with a poor prognosis and good response to microtubule inhibitors

CHFR promoter CpG island methylation as prognostic marker

In recent years it has become increasingly apparent that *CHFR* promoter CpG island methylation is associated with a poor prognosis in multiple cancer types. For NSCLC two independent groups published the association between *CHFR* promoter CpG island methylation and an increased risk for recurrence and poor survival^{38,51}. Also for colorectal cancer a small retrospective study (n=82) showed an association between *CHFR* promoter methylation and an increased risk of local recurrence as well as poor prognosis in stage III CRC⁵². We recently showed in a larger series of 173 patients that *CHFR* promoter methylation is an indicator of poor survival in stage II microsatellite stable CRC (n=82) and validated these results in an independent prospective cohort study (n=151) (Cleven et al, submitted).

Furthermore, different studies have shown the ability to detect *CHFR* promoter methylation in circulating tumor DNA in patients with non-small cell lung cancer (NSCLC)³⁸ and in peritoneal lavage in case of peritoneal metastasis in patients with gastric cancer^{31,32}. Peritoneal micrometastases in gastric cancer are associated with a poor prognosis but difficult to detect. Testing for *CHFR* promoter methylation in DNA from peritoneal fluid can help to detect^{63,64} malignant cells and select patients that might benefit from additional chemotherapy.

Together these studies underscore the potential of *CHFR* promoter CpG island methylation as a prognostic marker.

CHFR promoter CpG island methylation as predictor of response to therapy

In addition to being a biomarker for poor prognosis in CRC and NSCLC, *CHFR* promoter CpG island methylation has been shown to be a potential predictor of response to microtubules inhibitors (Table 5.2).

Microtubule inhibitors such as docetaxel and paclitaxel disrupt normal microtubule dynamics during cell division by binding to the beta-tubulin subunits. This will lead to a failure of microtubule separation and apoptosis. *CHFR* is able to block entry into prophase until chromosomal alignment is restored, which will lead to decreased effect of taxanes. Cells expressing *CHFR* have shown to be more viable upon treatment with microtubule inhibitors compared to cells not expressing *CHFR*⁶⁵.

The association between *CHFR* expression and a poor sensitivity to microtubule inhibitors was first shown by Satoh *et al.* in gastric cancer cell lines³⁰. Docetaxel is the second line therapy for gastric cancer; some patients

respond while others are resistant. Therefore, *CHFR* promoter CpG island methylation was hypothesized to be an important determinant of response to therapy. Different studies to test this hypothesis have been performed with conflicting results. A small study restricted to twelve patients, showed that cancers with *CHFR* promoter CpG island methylation had a better clinical response compared to cancers with unmethylated *CHFR*, of which the majority showed progressive disease³³. In a larger study (n=41), no clear relationship between *CHFR* promoter CpG island methylation and response to microtubule inhibitors was observed⁶⁶. Clinical response, however, was measured in metastatic lesions of which the methylation status of *CHFR* was not assessed. Since then, a potential role for *CHFR* promoter CpG island methylation in predicting response to microtubule inhibitors has been described in other cancer types as well. In NSCLC, *CHFR* promoter CpG island methylation is associated with high sensitivity to paclitaxel⁵³. Interestingly, sensitivity to paclitaxel is correlated with wild-type *EGFR* and smoking habit, and the combination of the three is a strong predictor of clinical response to paclitaxel in NSCLC^{51,53}.

Table 5.2 *CHFR* as marker of prognosis and response to microtubule inhibitor.

Cancer		Method ref	
Prognostic markers			
Lung cancer	Reduced CHFR expression associated with poor prognosis (n=157)	IHC	37
	Promoter methylation associated with poor prognosis (n=203)	MSP	51
Colorectal cancer	Promoter methylation associated with poor prognosis in stage II MSS BRAF wt CRC (n=66). Confirmed in second independent series (n=151)	MSP	52
	Promoter methylation associated with reduced recurrence free- and overall survival (n=82)	MSP	
Predictive markers			
Gastric cancer	Promoter methylation predicts good response to paclitaxel (cell lines n=4)	MSP	30
	No association between <i>CHFR</i> promoter methylation and sensitivity paclitaxel (n=12)		33
Cervical cancer	Promoter methylation predicts increased sensitivity to paclitaxel (cell lines, n=6))	MSP	43
Oral squamous cell carcinomas	Silencing CHFR (siRNA) increases taxane sensitivity (cell lines)	siRNA	46
Lung cancer	Promoter methylation associated with good response to paclitaxel (n=69)	MSP	53
	Unmethylated <i>CHFR</i> promoter associated with good response to EGFR TKIs (n=179)		38
Endometrial Cancer	<i>CHFR</i> promoter methylation associated with increased sensitivity to paclitaxel (cell lines, n=6)	MSP	50,54

IHC: immunohistochemistry, MSP: methylation specific PCR, siRNA: short interference RNA

Also in cervical adenocarcinomas, *CHFR* promoter CpG island methylation is proposed as biomarker to predict response to paclitaxel. In cervical adenocarcinomas, *CHFR* promoter CpG island methylation occurs in 14% of cases, in sharp contrast to cervical squamous cell carcinomas in which *CHFR* is unmethylated⁶⁷. Cervical adenocarcinomas respond to paclitaxel in 31% of cases, which is only 17% for other cervical cancers. Although this difference might involve *CHFR* promoter methylation, this relationship has only been shown in cervical cancer cell lines⁴³. The same accounts for *CHFR* promoter methylation in endometrial cancers treated with paclitaxel, where *CHFR* promoter methylation is associated with increased sensitivity to paclitaxel, but is only tested in cell lines^{50,54}. These results emphasize the need for bigger and better controlled patient series to investigate the role for *CHFR* promoter methylation as marker of response to taxanes.

Conclusion

In the last decade many studies have been performed to investigate *CHFR* promoter CpG island methylation as biomarker to predict prognosis and response to microtubule inhibitors in a diversity of cancers. There is compelling evidence that *CHFR* promoter methylation is a promising biomarker that can improve the management of multiple tumor types. Large, independent cohort studies and clinical trials to validate the prognostic and predictive value of *CHFR* promoter methylation are required and must be compared to established clinical markers such as for example the Tumor-Node-Metastasis (TNM) classification system. Simon *et al.* have proposed a less time consuming design in which archival material of prospective trials is used to investigate the performance of a single biomarker⁶⁸.

Still the majority of candidate-biomarkers reported in literature do not reach clinical use, mostly because they fail to pass the validation phase. This can be explained by heterogeneity of the tumors and a technical inability to consistently verify the presence of the biomarker in patient material and the lack of specificity for a particular disease⁹. Furthermore, comparison between studies is difficult because different methylation detection methods are used and different regions within the CpG island are analyzed. It is therefore that a standardized method to assess *CHFR* promoter CpG methylation within the core region of the CpG island is needed. MSP is a very accurate, easy to handle method that can be used in the majority of laboratories and therefore a good method of choice. Especially since the core region of methylation which is associated with expression and prognosis has been identified and can also be detected by MBD2-affinity sequencing (unpublished results).

Although work is still in progress, currently available results all point towards the same direction, making *CHFR* promoter CpG island methylation a

biomarker with great potential and the development of clinical trials to study its predictive and prognostic power a priority.

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Chapter 6

Stromal expression of hypoxia regulated
proteins is an adverse prognostic factor
in colorectal carcinomas

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Abstract

Hypoxia modifies the phenotype of tumors in a way that promotes tumor aggressiveness and resistance towards chemotherapy and radiotherapy. However, the expression and influence of hypoxia-regulated proteins on tumor biology are not well characterized in colorectal tumors. We studied the role of protein expression of hypoxia-inducible factor (HIF)-1 α , HIF-2 α , carbonic anhydrase 9 (CA9) and glucose transporter 1 (GLUT1) in patients with colorectal adenocarcinomas.

Expression of HIF-1 α , HIF-2 α , CA9 and GLUT1 was quantified by immunohistochemistry in 133 colorectal adenocarcinomas. The expression of hypoxia markers was correlated with clinicopathological variables and overall patient survival.

Expression of these hypoxia markers was detected in the epithelial compartment of the tumor cells as well as in tumor-associated stromal cells. Although tumor cells frequently showed expression of one or more of the investigated hypoxia markers, no correlation among these markers or with clinical response was found. However, within the tumor stroma, positive correlations between the hypoxia markers HIF-2 α , CA9 and GLUT1 were observed. Furthermore expression of HIF-2 α and CA9 in tumor-associated stroma were both associated with a significantly reduced overall survival. In the Cox proportional hazard model, stromal HIF-2 α expression was an independent prognostic factor for survival.

These observations show, that expression of hypoxia regulated proteins in tumor-associated stromal cells, as opposed to their expression in epithelial tumor cells, is associated with poor outcome in colorectal cancer. This study suggests that tumor hypoxia may influence tumor-associated stromal cells in a way that ultimately contributes to patient prognosis.

Introduction

Regulation of tissue oxygen homeostasis is critical for cell function, behavior and survival. Lack of oxygen (hypoxia), occurs early and remains a common feature of tumors throughout their development. Hypoxic areas in tumors contribute to a worse prognosis independent of treatment modality^{1,2}. One of the underlying mechanisms proposed to account for this poor prognosis is a contribution of hypoxia to the malignant status of tumors through promotion of metastasis, angiogenesis, and selection of cells with defects in apoptosis³. In colorectal cancer, the importance of hypoxia has been demonstrated by clinical studies in which hypoxia predicts for worse outcome and resistance towards chemotherapy and radiotherapy^{4,5}.

The majority of studies investigating hypoxia in human tumors has been carried out with oxygen sensing needle electrodes that are capable of direct oxygen measurements in tumors⁶. However, this procedure is invasive and restricted to accessible tumor sites⁷. This has led to the development and testing of alternative hypoxia markers including the 2-nitroimidazole compounds pimonidazole and EF-5 which undergo reduction upon binding within hypoxic cells⁸. Estimation of hypoxia by these so-called “exogenous” markers is made several hours following intravenous administration, by evaluating the degree of binding using specific antibodies in biopsies⁹. In addition, several “endogenous” markers have also been proposed. These markers consist of hypoxia regulated gene products and include among others hypoxia inducible factor (HIF)-1 α , HIF-2 α , carbonic anhydrase 9 (CA9), glucose transporter 1 (GLUT1), glucose transporter 3 (GLUT3), and vascular endothelial growth factor (VEGF)⁹⁻¹⁷.

HIF is a heterodimeric transcription factor composed of α and β subunits. HIF-1 β is expressed constitutively and is not sensitive to the hypoxic status of the cells, whereas the alpha subunit accumulates rapidly inside the hypoxic cells primarily because of the prevention of ubiquitination and subsequent protein degradation by the proteasome complex, which usually takes place in normoxic cells. There are three homologues of the alpha subunit (HIF-1 α , HIF-2 α , HIF-3 α)¹⁸. HIF-1 α and HIF-2 α are thought to play a significant role in tumor neovascularization, enhanced cellular proliferation, decreased apoptosis, and development of drug resistance to chemotherapeutic agents¹⁹. In colorectal cancer HIF-2 α also seems to play an important role in angiogenesis, the combined expression of HIF-2 α and HIF-1 α may play a role in tumor progression and prognosis^{10,20}.

CA9 is a transmembrane glycoprotein and a member of the carbonic anhydrase family that is regulated by HIF²¹. Carbonic anhydrases are zinc metalloenzymes that catalyze the reversible conversion of carbon dioxide to carbonic acid and are involved in respiration, calcification, acid-base balance, the formation of cerebrospinal fluid, saliva, and gastric acid. Increased CA9

expression is induced by hypoxia, as was shown in a wide spectrum of tumor types including tumors of the uterine cervix, head and neck, lung, bladder, breast, esophagus, colon and rectum²²⁻²⁹. CA9 expression has previously been demonstrated in colorectal tumors by Saarnio et al. (1998). CA9 staining intensity in colorectal tumors was higher in less advanced tumors (Dukes A + B)^{29,30}.

GLUT1 is a member of the glucose transporter family of proteins facilitating independent transport of glucose that is also regulated by HIF. Tumors have accelerated metabolism and increased requirements for ATP production, therefore cancer cells have high rates of glycolysis. Elevated GLUT1 expression under hypoxic conditions has been described for many cancers, including hepatic, pancreatic, breast, esophageal, brain, renal, lung, cutaneous, colorectal, endometrial, ovarian and cervical carcinoma³¹. In a study done by Haber et al in colorectal cancer, disease specific mortality was greater in patients with high expression of GLUT1 in tumors (>50% of cells were GLUT1 positive)³². Furthermore, GLUT1 expression in colorectal cancer was shown to be associated with a high incidence of lymph node metastasis³³.

The present study was performed to evaluate the expression of hypoxia regulated proteins and their association with clinicopathological response in colorectal adenocarcinomas. We applied a panel of hypoxia-related endogenous immunohistochemical markers on a series of 133 colorectal cancer tissues, and correlated their expression with morphologic tumor parameters, clinicopathological features and overall patient survival.

Materials and methods

Patients

Patients were 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 colonic cancer by conventional surgery or the no-touch isolation technique³⁴. The other trial was conducted to compare survival in rectal cancer patients with or without 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. Although the results from this study can therefore not be extrapolated to current practice, it does enable unbiased study of the influence of hypoxic conditions on tumor biology. In the current study, we included only the patients who did not undergo preoperative radiotherapy. For immunohistochemical analysis tumor tissues from 133 patients with primary colorectal cancer were available. The distribution of age, gender, tumor stage, location and type of tumor, frequency

of events and mean follow-up time of the patients in this study are representative for the patients in the trial (see Table 6.1).

Table 6.1 Clinicopathological variables of study population.

Age	
<69 years	n=64 (48%)
>69 years	n=69 (52%)
Sex	
Male	n=55 (41%)
Female	n=78 (59%)
Tumor-size	
<46mm	n=57 (43%)
>46mm	n=76 (57%)
Tumor location	
Proximal	n=52 (39%)
Distal	n=81 (61%)
Tumor	
Colon	n=91 (68%)
Rectum	n=42 (32%)
Differentiation	
Well	n=14 (11%)
Moderate/poor	n=119 (89%)
TNM	
1	n=0 (0%)
2	n=83 (63%)
3	n=35 (26%)
4	n=15 (11%)
Tumortype	
Exophytic	n=15 (11%)
Sessile	n=19 (14%)
Ulcerative	n=99 (74%)

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, during the next years 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.

After surgery, tumor tissues and lymph nodes were fixed in buffered formalin, sectioned, and embedded in paraffin. Experienced pathologists documented the histopathological characteristics of the tumors, including tumor stage, differentiation grade, size, (lymph-)angioinvasion, perineural invasion and

lymphnode involvement. Tumor stage was defined according to the TNM staging system.

Immunohistochemistry

Formalin fixed, paraffin-embedded tissues consisting of both normal mucosa and tumor were used for immunohistochemical staining. Serial sections (4 μ m) from each patient were stained for HIF-1 α , HIF-2 α , CA9 and GLUT1 after deparaffination. Endogenous peroxidase activity was blocked by pre-incubating in 0.6% hydrogen peroxide for 20 min.

The following staining protocols for the different antibodies were used:

HIF-1 α staining: Antigen retrieval was performed by microwave 750 W for 2 min. followed by 20 min. at 90 W in 1mM TE buffer pH 9.0, followed by 30 min. cooling in buffer. Slides were blocked in 25% normal serum for 10 min. Sections were incubated overnight (4°C) with primary antibody HIF-1 α (1:120) (anti-HIF-1 α monoclonal: 610958 BD, USA).

HIF-2 α staining: Antigen retrieval was performed by microwave treatment (750 W for 20 min in 1mM TE buffer pH 8.0), followed by 30 min. cooling in buffer. Slides were blocked in 25% normal serum for 10 min. Sections were incubated with primary antibody HIF-2 α (1:500) for 100 min. (anti-HIF2 alpha monoclonal: ab8365 AbCam, UK).

CA9 staining: Slides were blocked in 25% normal serum for 10 min., then incubated for 45 min. with primary CA9 antibody MoAb M75 (1:50) (anti-human CA9, kindly supplied by Dr. S. Pastorekova) at room temperature²⁷.

GLUT1 staining: Microwave treatment (750 W for 15 min. in Citrate buffer pH 6.0), followed by 30 min. cooling in buffer. Slides were blocked in 25% normal serum for 10 min. Sections were incubated (1:100) with primary antibody GLUT1 (rabbit polyclonal anti-human GLUT1, A3536, DakoCytomation, Denmark) for 2 hr. followed by incubation with Swine Anti-Rabbit Immunoglobuline/Biotinylated (E0431, DakoCytomation, Denmark) (1:250) for 30 min. at room temperature, and StreptABComplex/HRP (K0377, DakoCytomation, Denmark) (1:200) for 30 min. at room temperature.

As a negative control for all antibodies, TBS buffer instead of primary antibody was used. Visualization was performed using Dako Envision, Peroxidase, mouse System (K4001, DAKO, Denmark). For GLUT1, Diaminobenzidine was applied for 10 min. The slides were counterstained with hematoxylin and mounted.

Evaluation of staining

Immunohistochemical staining was evaluated on the basis of 1) localisation in tumor epithelial or stromal cells and 2) subcellular localisation. For the tumor stroma, only the tumor-associated stromal cells were taken into account, not

the tumor infiltrating inflammatory cells or the lamina propria of the normal mucosa.

If nuclear staining was present in >5% of the tumor epithelial cells or tumor-associated stromal cells, the sample was considered positive for HIF-1 α and HIF-2 α . If membranous staining occurred in >5% of the tumor epithelial cells or stromal cells, samples were considered positive for CA9 and GLUT1. Staining results were also checked by determining the extent and intensity of staining. Because this did not change the categorization, we used the 5% cut-off value for further analysis²⁰.

Data analysis

For the data analyses we used three groups of expression patterns for HIF-1 α , HIF-2 α , CA9 and GLUT1 expression, namely: 1 Stromal expression (= defined as purely stromal or a combination of stromal and epithelial expression), 2 Epithelial expression (=defined as epithelial only or a combination of stromal and epithelial expression), and 3 Negative.

The correlations between HIF-1 α , HIF-2 α , CA9, GLUT1 and various clinicopathological parameters were determined by the Pearson Chi-Square and Fisher's exact test as appropriate. To evaluate the relationship between HIF-1 α , HIF-2 α , CA9, GLUT1 and patient survival, Kaplan-Meier survival curves were calculated. 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: sex, age, tumor size, tumor location, TNM stage, differentiation grade, HIF-1 α , HIF-2 α , CA9 and GLUT1 (epithelial and stromal expression separately). All p-values are two sided and p-values <0.05 were considered statistically significant. SPSS 10.0 software was used for data analyses.

To check for differences between colon and rectum tumors, all survival analyses were performed separately for these two categories.

Results

We evaluated the staining characteristics of the potential hypoxia markers HIF-1 α , HIF-2 α , CA9 and GLUT1 in 133 human colorectal adenocarcinomas. On analyzing these four hypoxia associated proteins, it became clear that many tumors displayed positive staining in the tumor epithelial cells as well as positive staining in tumor-associated stromal cells. We therefore separately evaluated the staining patterns of HIF-1 α , HIF-2 α , CA9 and GLUT1 in tumor epithelial cells and tumor-associated stromal cells. All tumors showed

immunohistochemical expression of at least one of the analysed hypoxia markers. Overall HIF-1 α was positive in 43% of cases, whereas 83% of the tumors were positive for HIF-2 α , 89% of the tumors showed CA9 protein expression and GLUT1 expression was positive in 85% of the cases.

Expression of hypoxia proteins in tumor epithelial cells

In order to evaluate the association of hypoxia and the clinical outcome of colorectal cancer, quantification of the expression of hypoxia markers within the tumor epithelial cell compartment was performed. Overall, 29% of the patients showed HIF-1 α protein expression within the tumor epithelial cells (Figure 6.2A). In these cells, HIF-1 α expression was observed exclusively in the nuclei, often in areas surrounding tumor necrosis (Figure 6.1A), a pattern that is typical of hypoxia induced expression. Surprisingly, none of the 133 tumors showed any staining for HIF-2 α in tumor epithelial cells. This lack of expression may be associated with the intestinal cell lineage as expression was also not detected in any adjacent normal intestinal epithelial cells. Epithelial CA9 expression was observed in 78% of all tumors (Figure 6.2C). Expression in the tumor cells was restricted to a membranous type of expression (Figure 6.1E) but was typically not perinecrotic. GLUT1 was also present in tumor epithelial cells in 83% of the cases (Figure 6.2D), where it showed a clear and strong membranous staining, typically surrounding areas of tumor necrosis (Figure 6.1G).

Expression of hypoxia proteins in the tumor-associated stromal cells

Stromal HIF-1 α expression was observed in 32% of tumors, of which 18% showed combined staining of tumor epithelium and stroma and 14% showed exclusive staining in the stromal cells (Figure 6.2A). Staining was observed surrounding areas of tumor necrosis (Figure 6.1B) and was confined to the nuclei.

In contrast with the lack of HIF-2 α expression in tumor epithelial cells, HIF-2 α staining was frequently seen within the tumor stroma, both in tumor-associated stromal cells and inflammatory cells (Figure 6.1C and 6.1D). Expression was predominantly nuclear, only occasionally accompanied by additional cytoplasmic staining. In further analysis, HIF-2 α staining of inflammatory cells in the tumor stroma was disregarded, because HIF-2 α expression in inflammatory cells did not influence the effect of HIF-2 α expression in tumor-associated stromal cells on patient outcome (Figure 6.3A). Overall, 83% of the tumors showed positive staining for HIF-2 α in stromal cells, typically surrounding areas of necrosis (Figure 6.2B). In the remaining 17% of the tumors where no expression of HIF-2 α was observed, some staining was found in inflammatory cells in the lamina propria of the normal mucosa. Stromal

membranous CA9 expression (Figure 6.1F) was observed in 37% of all tumors. Interestingly, of the tumors that lacked CA9 expression in tumor epithelial cells, half were positive for CA9 in the stroma (Figure 6.2C). GLUT1 expression was only occasionally detected in stromal cells and restricted to a membranous type of expression (Figure 6.1H). Stromal cell expression of GLUT1 was present in 14 % of the cases (Figure 6.2D).

Figure 6.1 Expression patterns of hypoxia regulated proteins in colorectal tumors. black arrow: (A, E, G) epithelial tumor cell expression, (B, D, F, H) tumor-associated stromal cell expression, (C) inflammatory cell expression. (A) HIF-1α expression in the nuclei of epithelial tumor cells. (B) HIF-1α expression in the nuclei of tumor-associated stromal cells. (C) HIF-2α expression in inflammatory cells located within the tumor-associated stroma. (D) HIF-2α expression in tumor-associated stromal cells. (E) CA9 expression in epithelial tumor cells. (F) CA9 expression in tumor-associated stromal cells. (G) Expression of GLUT1 in tumor epithelial cells. (H) GLUT1 expression in tumor-associated stromal cells. (A, B, D, F and H original magnifications $\times 40$; C, E and G original magnifications $\times 20$.)

No staining of stromal cells within the lamina propria of surrounding normal mucosa was found for any of these hypoxia inducible proteins.

Figure 6.2 Quantification of expression patterns of hypoxia associated proteins in 133 colorectal cancers. (A) Quantification of HIF-1 α expression patterns. (B) Quantification of HIF-2 α expression patterns. (C) Quantification of CA9 expression patterns. (D) Quantification of GLUT1 expression patterns.

Correlations between HIF-1 α , HIF-2 α , CA9, GLUT1 and clinico-pathological variables

To investigate the functional relevance of HIF-1 α and HIF-2 α expression, we tested the correlations of the expression of these transcription factors with the expression of their downstream gene products CA9 and GLUT-1. HIF-1 α expression showed no correlation with HIF-2 α , GLUT1 or CA9 when analyzed by either epithelial or stromal expression patterns. This suggests that HIF-1 α expression is not primarily responsible for induction of CA9 and GLUT1 expression and that these genes are regulated by factors other than HIF-1 α . In contrast, a significant correlation was found between HIF-2 α and CA9 ($p=0.02$, Table 6.2) within the tumor stroma. Furthermore, GLUT1 and CA9 expression within the tumor stroma were significantly correlated ($p=0.002$, Table 6.2). These data suggest that HIF-2 α upregulation in tumor stroma may be responsible for upregulation of CA9 .

Table 6.2 Correlations between stromal expression of HIF-1 α , HIF-2 α , CA9, GLUT1.

	HIF-2 α		p-value		HIF-1 α		p-value
	Stroma	Negative			Stroma	Negative	
HIF-1α				GLUT1			
Stroma	37/43 (86%)	6/43 (14%)	0.6	Stroma	5/19 (26%)	14/19 (74%)	0.5
Negative	74/90 (82%)	16/90 (18%)		Negative	38/114 (33%)	76/114 (67%)	
	HIF-2 α				HIF-1 α		
	Stroma	Negative			Stroma	Negative	
GLUT1				CA9			
Stroma	17/19 (89%)	2/19 (11%)	0.7	Stroma	18/48 (38%)	30/48 (62%)	0.3
Negative	94/114 (82%)	20/114 (18%)		Negative	25/85 (29%)	60/85 (71%)	
	HIF-2 α				GLUT1		
	Stroma	Negative			Stroma	Negative	
CA9				CA9			
Stroma	45/48 (94%)	3/48 (6%)	0.02	Stroma	13/48 (27%)	35/48 (73%)	0.002
Negative	66/85 (78%)	19/85 (22%)		Negative	6/85 (7%)	79/85 (93%)	

No significant correlation was found between stromal or epithelial expression of HIF-1 α , HIF-2 α or GLUT1 and any of the following clinicopathological characteristics: age, sex, tumor size, location in the colon (proximal, distal), differentiation (well, moderately and poorly), TNM stage (2,3 and 4). Only CA9 epithelial expression showed a significant correlation with TNM stage, with reduced CA9 expression in the more advanced tumors. These results suggest that the biological responses to hypoxia in colorectal cancer are largely independent of these clinicopathological variables.

Relationship between hypoxia markers and patient survival

An important objective of our study was to analyze the relationship between hypoxia regulated proteins and patient survival. Previous studies in other tumor types have indicated that expression of the hypoxia induced proteins investigated here correlate with poor overall survival. This is presumed to be due to an adverse effect of hypoxia on the tumor cells themselves. Since we also observed frequent staining within the tumor stroma, we further investigated the potential relationship between stromal hypoxia and overall survival.

HIF1- α expression in tumor-associated stromal cells and epithelial tumor cells showed no significant correlation with patient survival. Similarly, CA9 epithelial expression had no impact on patient survival. GLUT1 epithelial negative cases showed a significantly poorer survival compared to GLUT1 positive epithelial cases ($p=0.02$, Figure 6.3B).

Figure 6.3 (A) Survival curve HIF-2α expression including inflammatory cells (IC) $p=0.06$ ($n=133$). (B) Survival curve GLUT1 epithelial expression $p=0.02$ ($n=133$). (C) Survival curve HIF-2α (without inflammatory cells) $p=0.008$ ($n=133$). (D) Survival curve HIF-2α stratified for TNM stage 2 $p<0.05$ ($n=83$). (E) Survival curve HIF-2α stratified for TNM stage 3 $p=0.4$ ($n=35$). (F) Survival curve CA9 $p=0.01$ ($n=133$).

In contrast to the lack of association between markers of hypoxia in the tumor cells and clinical response, we found strong evidence that stromal hypoxia may be associated with poor outcome. Patients with stromal HIF-2α expression showed a statistically significant poorer survival compared to HIF-2α negative patients ($p=0.008$, Figure 6.3C). This trend was maintained in TNM stage 2 and stage 3 (Figure 6.3D and 6.3E). The same trend was observed for stromal CA9 expression ($p=0.01$, Figure 6.3F). Patients with stromal GLUT1 expression also showed a tendency for poorer overall survival compared to patients negative for stromal GLUT1 expression, although this did not reach statistical significance. There was no difference in survival between cases with exclusive

stromal expression and cases with combined stromal and epithelial expression for any of the four applied markers (data not shown).

Multivariate analysis

An overall model to test the most relevant prognostic factors for patient outcome was performed using a multistep Cox-regression model. After stepwise multivariate analysis, stromal HIF-2 α positivity (Hazard Ratio (HR) 4.61), tumor epithelial GLUT1 positivity (HR 0.44), TNM stage 3 (HR 2.74) and stage 4 (HR 16.09) and age >69 years (HR 2.09) were statistically significant independent prognostic markers. Poorly and moderately differentiated tumors had a HR of 3.12 compared to well differentiated tumors, although this did not reach statistical significance ($p=0.07$, Table 6.3). Multivariate analysis restricted to stage 2 and 3 cases, revealed stromal HIF-2 α positivity as the strongest independent prognostic marker, with a HR 4.87 ($p=0.01$), followed by TNM stage 3 (HR 2.29, $p=0.01$), stromal CA9 expression (HR 1.95, $p<0.05$), age >69 years (HR 1.94, $p<0.05$) and epithelial GLUT1 expression (HR 0.26, $p=0.001$).

There were no differences in univariate or multivariate survival analysis between colon and rectum tumors.

Table 6.3 Cox proportional hazard model.

		Event: CRC mortality HR (95%CI)	p-value
HIF-2α	Positive*	4.61 (1.40-15.50)	0.01
GLUT1	Epithelial [†]	0.44 (0.22-0.86)	0.02
TNM	3 [‡]	2.74 (1.42-5.29)	0.003
	4 [‡]	16.09 (7.00-39.95)	0.000
Differentiation	Moderately/poorly**	3.12 (0.93-10.48)	0.07
Age	>69 years ^{††}	2.09 (1.16-3.74)	0.01

CRC, colorectal cancer; HR, Hazard Ratio=Relative Risk. *Reference group = negative HIF-2 α cases; [†]Reference group = negative GLUT1 cases; [‡]Reference group = TNM stage 2; **Reference group = well differentiated; ^{††}Reference group = Age<69 years.

Discussion

This is the first comprehensive study analyzing the expression of endogenous hypoxia markers HIF-1 α , HIF-2 α , CA9 and GLUT1 in colorectal cancer. All 133 tumors in this study showed expression of at least one of the hypoxia-regulated proteins, suggesting that all tumors may exhibit at least some degree of hypoxia. In contrast to what was initially expected, our data suggest that HIF-1 α immunostaining does not appear to be a reliable indicator of hypoxia in colorectal cancer and furthermore does not correlate with patient outcome. Although HIF-1 α did display staining characteristics consistent with hypoxia

(peri-necrotic staining of cell nuclei), its expression in either stromal or tumor cells did not correlate with the expression of its downstream genes CA9 and GLUT1. Consistent with our results, HIF-1 α staining was previously reported to be positive in 44.8% of patients with colorectal cancer, but had no correlation with HIF-2 α expression and no prognostic value⁵. Several explanations for the lack of correlation between HIF-1 α and CA9 or GLUT1 in the tumor cells are possible. HIF-1 α is susceptible to degradation after prolonged hypoxia due to upregulation of the Prolyl Hydroxylase Domain proteins³⁵. *In vitro* studies suggest that nutrient deprivation may also decrease HIF-1 α expression. Furthermore, the half-life of CA9 is sufficiently long (>>24hrs) that once formed it remains present for many days in the absence of continued HIF-1 α expression³⁶.

As for HIF-1 α , our data indicate that tumor epithelial cell expression of CA9, although frequently occurring, is not related to poorer survival either. In fact, we found that epithelial CA9 expression decreased with advancing TNM stage, with 78% of CA9 epithelial positive cases in TNM stage 2 compared to 53% of CA9 epithelial positive cases in TNM stage 4 ($p=0.02$). It is well known that in colorectal cancer TNM Stage 2 tumors have a better overall survival than Stage 4 tumors³⁷. The lack of correlation between HIF-1 α and CA9 expression in tumor epithelial cells suggests that the CA9 positivity may also be unrelated to hypoxia. This is supported by the finding that in cases with only tumor epithelial CA9 staining (53%), the expression was usually not perinecrotic. On the other hand, tumor epithelial cell expression of GLUT1 in our study was typically found in perinecrotic zones, and therefore may indeed be indicative of tumor hypoxia. However, GLUT1 epithelial positivity turned out to be a marker for good prognosis compared to epithelial negative GLUT1 cases. This is somewhat surprising, since GLUT1 expression has been previously reported to be associated with a high incidence of lymphnode metastases³⁸. Although further analyses is clearly necessary to determine the role for epithelial GLUT1 expression within different TNM stages of colorectal cancer, our data do not support the premise that epithelial GLUT1 expression is a poor prognostic factor. Taken together, the immunostaining patterns of these four markers within the tumor epithelial cell compartment do not consistently reflect tumor hypoxia, and tumor epithelial cell hypoxia does not seem to contribute to a poorer patient prognosis in colorectal cancer.

In direct contrast to these results, our study did indicate that the tumor stroma may be frequently hypoxic. We found that 84% of all tumors demonstrated staining of HIF-2 α within the tumor-associated stromal cells. HIF-2 α appears to be biologically active in stromal cells because HIF-2 α correlated significantly with stromal CA9 expression ($p=0.02$) in these tumors. Most importantly, tumors showing stromal HIF-2 α expression also had a worse overall survival when compared with HIF-2 α negative cases. After stratification for other factors

in a multivariate analysis (sex, age, TNM stage, differentiation grade, HIF-1 α , GLUT1 and CA9), stromal HIF-2 α expression was a strong (HR 4.61, $p=0.01$) independent unfavorable prognostic factor. The negative impact of HIF-2 α expression on patient survival in colorectal cancer was recently also described by others. However, in this study of curatively resected colorectal carcinoma patients, the authors did not study the contribution of the epithelial and stromal tumor compartments separately²⁰.

As might be expected given the correlation between HIF-2 α and CA9 in tumor-associated stromal cells, our data also indicated that stromal CA9 expression was associated with a worse overall survival. Furthermore, stromal CA9 expression proved to be an independent unfavorable prognostic factor in cases having stage 2 or 3 colorectal cancer. This finding may not be unique to colorectal cancer. In invasive breast cancer, stromal CA9 expression levels have been associated with a higher relapse rate and a worse overall survival³⁹. Together our data suggest the intriguing possibility that hypoxia within the tumor associated stroma may contribute to a poor outcome in patients with colorectal cancer who are treated by surgery alone. Although it is commonly accepted that the genetic and epigenetic changes in tumor cells are responsible for the differences in patient response, there are also data to suggest that non-tumor cells may play an important role as well. The induction of CA9 by hypoxia could help in maintaining normal intracellular pH, preventing apoptosis, and thus provide a survival advantage to the stromal cells, and indirectly be of benefit to the adjacent tumor cells. A reduction in extracellular pH might also provide an advantage to tumor cells as it helps in breakdown of extracellular matrix, migration and invasion of tumor cells, as well as induction and expression of growth factors. This explanation is consistent with a report from Sivridis et al., who showed that cancer-stromal cell interactions may be favoured by the altered microenvironmental conditions of hypoxia and acidity⁴⁰. The strong association of markers of intratumoral hypoxia and pH within the tumor stroma indicates an interesting link between cancer cell metabolism and the induction of a supportive stroma that favors cancer cell invasion and migration^{30,31,41-44}.

Recent data have provided further compelling evidence that tumor-associated stromal cells are important in cancer growth and metastasis⁴⁵. In colorectal tumors not only epithelial but also stromal elements demonstrate genetic instability and that such stromal alterations might influence the genesis of sporadic colorectal carcinomas⁴⁶. Weinberg et al. showed that co-injection of carcinoma associated stromal cells (myofibroblasts) promoted the growth of invasive breast carcinomas. This effect was mediated in part through increased secretion of stromal cell-derived factor-1 (SDF-1). This is particularly interesting since SDF-1 and its receptor have been shown to be upregulated by hypoxia⁴⁷. The Weinberg study also indicated that the tumor associated stromal cells

could promote angiogenesis by recruiting endothelial progenitor cells (EPC's) into carcinomas⁴⁸. Stromal cells within the tumor microenvironment have been shown to acquire new properties, including the capacity to promote phenotypic and genetic progression in adjacent epithelial cells^{30,49}. For example, epigenetic changes have been shown to occur in stromal cells of breast cancer during tumorigenesis, in a tumor stage- and cell type-specific manner⁵⁰. Alterations of stromal cells may therefore be either a consequence or contributor to an abnormal tumor micro-environment. Our study indicates that hypoxia may be a potential mechanism influencing the phenotype of tumor stroma, ultimately leading to a worse patient prognosis.

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Chapter 7

Promoter CpG island hypermethylation of
cancer associated genes is associated with
stromal expression of hypoxia markers in
colorectal cancer

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Abstract

It is becoming increasingly clear that epigenetic alterations, such as promoter CpG island methylation of important cancer related genes, play a crucial role in the cellular responses to hypoxia, another hallmark of cancer. This study investigates whether hypoxia in CRC is associated with promoter CpG island methylation of genes involved in important tumor biological processes such as genomic instability, limitless replication potential, tissue invasion and metastasis, insensitivity to anti-growth signals, self-sufficiency in growth signals, sustained angiogenesis and evading apoptosis.

Expression of HIF-1 α , HIF-2 α , CA9 and GLUT1 was quantified by immunohistochemistry in 173 colorectal adenocarcinomas. Promoter CpG island methylation was determined using methylation-specific PCR (MSP) for the following genes: *MGMT*, *CHFR*, *HLTF*, *MLH1*, *RASSF1A* (genomic instability), *p14ARF*, *p16INK4A* (limitless replication potential), *ADAM23*, *TFPI2* (tissue invasion and metastasis), *APC* (insensitivity to anti-growth signals), *SOCS1* (self-sufficiency in growth signals), *THBS1* (sustained angiogenesis), *BNIP3*, *NEURL*, *RUNX3*, *RAB32* (evading apoptosis). Associations between these variables were determined by T-test of means, Pearson Chi-Square and Fisher's exact test as appropriate.

Overall we did not find a correlation between mean number of promoter methylated genes and expression of hypoxia regulated proteins. However, methylation of individual genes was associated with stromal rather than epithelial expression of hypoxia regulated proteins: stromal expression of CA9 was more often observed in *CHFR* and *ADAM23* methylated tumors (52% (35/67) and 58% (35/60)) compared to tumors unmethylated for these two genes (34% (29/86) and 28% (22/79), respectively $p=0.03$ and $p<0.001$). Stromal expression of HIF-2 α was more often observed in *APC* and *SOCS1* methylated tumors (82% (46/56) and 79% (77/98), respectively) compared to tumors unmethylated for these two genes (67% (73/109) and 61% (39/64) respectively, $p=0.04$ and $p=0.02$). Only *BNIP3* methylated CRCs showed more often CA9 epithelial expression (87% (78/90)) compared to its unmethylated counterpart (73% (50/69), $p=0.03$).

Stromal expression of hypoxia regulated proteins HIF-2 α and CA9 was associated with promoter CpG island methylation of genes involved in important tumor biological processes. Tumors with hypoxic features do not appear to show increased overall CpG island promoter methylation, but rather harbour specific methylated cancer biology related genes, which could contribute to the more aggressive behaviour of these tumors.

Introduction

Hypoxia has been recognized as one of the fundamental features of solid tumors like colorectal cancer (CRC). It plays a critical role in various cellular and physiologic events, including apoptosis, cell proliferation and angiogenesis¹⁻⁴. Also it is important from a clinical point of view, as hypoxic tumors show resistance to radiation therapy and chemotherapy, and a poorer prognosis by increasing risk of invasion and metastasis¹⁻⁴. The cellular responses to hypoxia are at least partially orchestrated by activation of the hypoxia-inducible factors (HIFs) and its many downstream genes including carbonic anhydrase 9 (CA9) and glucose transporter 1 (GLUT1)². In the previous chapter we studied the role of protein expression of HIF-1 α , HIF-2 α , CA9 and GLUT1 in colorectal adenocarcinomas. We concluded that expression of hypoxia regulated proteins in tumor associated stroma, as opposed to their expression in cancer cells, is associated with poorer outcome, suggesting that tumor hypoxia may influence tumor associated stromal cells in a way that ultimately effects patient prognosis.

It is becoming increasingly apparent that epigenetic alterations, such as promoter CpG island methylation of important cancer related genes, also play a crucial role in the cellular responses to hypoxia⁵. Hypoxia could be a consequence of tumor progression through increased CpG island methylation and silencing of important tumor suppressor genes, or conversely, hypoxia could induce cancer related epigenetic changes. Such epigenetic changes may co-operate with hypoxia induced proteins to contribute to the maintenance of a hypoxia-adapted cellular phenotype, which is associated with an aggressive tumor behavior⁵. There are four current opinions on the interaction of epigenetics and hypoxia: (1) HIF stabilisation is influenced by the epigenetically controlled expression of several HIF-degrading proteins, for example silencing of VHL by promoter CpG island hypermethylation in renal cell carcinomas⁶. (2) Epigenetic mechanisms such as histone acetyltransferase enzymes regulate HIF binding by maintaining a transcriptionally active chromatin confirmation within and around HIF binding regions⁷. (3) Histone demethylase enzymes are direct HIF target genes and therefore play a role in the regulation of transcription during the hypoxic response⁸. (4) Significant global changes in histone modifications and promoter CpG island methylation occur in response to hypoxic exposure.

Studying the relation between hypoxia and epigenetic mechanisms should address the notion that the extent of promoter CpG island methylation of tumor suppressor genes and other important cancer related genes influences the ability of a tumor to adapt to hypoxia^{5,9}. An example of this phenomenon is found in both CRC and pancreatic cancer, where hypoxic induction of BCL-2/adenovirus E1B-19 kDa-interacting protein (*BNIP3*), which is known to

promote apoptosis, is blocked by promoter CpG island methylation, thereby facilitating the evasion of apoptosis during hypoxia^{10,11}. Previously we observed that promoter CpG island methylation of *CHFR* is related to a poorer survival. Loss of function of *CHFR* causes genomic instability¹²⁻¹⁴, which is a feature of tumor progression leading to increasing hypoxia, but may also contribute to intrinsic resistance of tumor cells to hypoxia⁴. Comparable to promoter CpG island methylation of *BNIP3*, silencing of *CHFR* could contribute to a more efficient adaptation of a tumor to hypoxia, eventually resulting in a more aggressive tumor behavior.

The aim of the current study was to find out whether expression of hypoxia regulated proteins in CRC is related to promoter CpG island methylation of 16 genes which are frequently methylated in CRC, and also involved in main pathways of tumor progression, the so-called hallmarks of cancer¹⁵ (Table 7.1): *MGMT*, *CHFR*, *HLTF*, *MLH1*, *RASSF1A* (genomic instability), *p14ARF*, *p16INK4A* (limitless replication potential), *ADAM23*, *TFPI2* (tissue invasion and metastasis), *APC* (insensitivity to anti-growth signals), *SOCS1* (self-sufficiency in growth signals), *THBS1* (sustained angiogenesis), *BNIP3*, *NEURL*, *RUNX3*, *RAB32* (evading apoptosis). Methylation status of these genes was determined using methylation-specific PCR (MSP) in a series of 173 CRCs. Expression of HIF-1 α , HIF-2 α , CA9 and GLUT1 was quantified by immunohistochemistry in this series of CRC's. Since we previously described the importance of stromal expression of hypoxia regulated proteins for patient survival in CRC, in the current study we also investigated whether promoter CpG island hypermethylation was related to differential expression of hypoxia regulated proteins, in either the epithelial or stromal tumor compartments.

Table 7.1 Promoter CpG island hypermethylated genes related to the hallmarks of cancer

Cancer Hallmarks	Promoter CpG island hypermethylated genes	Reference
Genomic instability	<i>MGMT</i> , <i>CHFR</i> , <i>HLTF</i> , <i>MLH1</i> , <i>RASSF1A</i>	12-14, 40-56
Limitless replication potential	<i>p14ARF</i> , <i>p16INK4A</i>	57-60
Tissue invasion and metastasis	<i>ADAM23</i> , <i>TFPI2</i>	15, 61-68
Insensitivity to anti-growth signals	<i>APC</i>	69-71
Self-sufficiency in growth signals	<i>SOCS1</i>	72-74
Sustained angiogenesis	<i>THBS1</i>	75-77
Evading apoptosis	<i>BNIP3</i> , <i>NEURL</i> , <i>RUNX3</i> , <i>RAB32</i>	78-86 10, 37-39, 87-91

Materials and methods

Patient population

Patients were entered in two multi-center prospective clinical trials in the Netherlands, between 1979 and 1981. One trial was designed to compare patient survival after treatment of colon cancer by conventional surgery or the

no-touch isolation technique¹⁶. 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 undergo 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. This study population therefore enables unbiased study of the influence of hypoxic conditions on tumor biology.

Tumor tissues were fixed in buffered formalin, sectioned, and embedded in paraffin. Experienced pathologists documented the histopathological characteristics of the tumors (see previous chapters). 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 recurrence of disease and disease-related death. After five year follow up period, only time and cause of death were registered. Follow-up was complete for all patients. Failure was defined as death due to recurrent disease, excluding postoperative mortality within 30 days and non-disease related death.

For immunohistochemical and molecular analysis, tumor tissues from 173 CRC patients were available. The distribution of age, gender, tumor stage, location and type of tumor, frequency of events and mean follow-up time of the patients in this study are representative for the patients in the trial.

Immunohistochemistry

Immunohistochemistry and evaluation of the data was performed as described previously¹⁷. In brief serial formalin fixed, paraffin-embedded tissues sections (4 µm) were stained for HIF-1α, HIF-2α, CA9 and GLUT1. Localisation (epithelial or stromal) was scored separately. For the category stromal staining only the stromal myofibroblasts were taken into account, not the tumor infiltrating inflammatory cells or the lamina propria of the normal mucosa. If staining was present in >5% of the tumor stromal cells, the sample was considered positive for stromal expression. If membranous staining occurred in >5% of the tumor epithelial cells, samples were considered positive for epithelial expression¹⁸.

Promoter CpG island methylation 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 *MLH1* (mutL homolog1, colon cancer, nonpolyposis type 2 (E.Coli)), *p16INK4A* (cyclin dependent kinase inhibitor 2A),

p14ARF (cyclin dependent kinase inhibitor 2A), *MGMT* (O-6-methylguanine-DNA methyltransferase), *RASSF1A* (Ras association (RalGDS/AF-6) domain family member 1), *APC* (adenomatous polyposis coli), *HLTF* (helicase-like transcription factor), *CHFR* (checkpoint with forkhead and ring finger domain), *ADAM23* (ADAM metalloproteinase domain 23), *RAB32* (Rab32, a member RAS oncogene family), *BNIP3* (BCL2-adenovirus E1B 19kDa interacting protein 3), *NEURL* (neuralized homolog (Drosophila)), *THBS1* (thrombospondin 1), *TFPI2* (tissue factor inhibitor 2), *RUNX3* (runt-related transcription factor 3) and *SOCS1* (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). Methylation Specific PCR (MSP) was performed as described in detail elsewhere^{19,20}. 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. For primers and PCR conditions see chapter 4.

Data-analysis

The 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 16 genes (Table 7.1). Relationships between mean methylation index and expression of HIF-1 α , HIF-2 α , CA9, GLUT1 was determined by T-test of means. Correlations between HIF-1 α , HIF-2 α , CA9, GLUT1 and the cancer hallmark genes were determined by the Pearson Chi-Square and Fisher's exact test as appropriate. All p-values are two sided and p-values <0.05 were considered statistically significant. Patients with unknown and unspecified scores have been omitted from analyses for that specific variable. SPSS 15.0 software was used for data analyses.

Results

Overall, no correlation was observed between epithelial and stromal expression of HIF-1 α , HIF-2 α , CA9 and GLUT1 and the methylation index in these subgroups of CRC, which varies between 0.36 and 0.45 (Table 7.2). However

when categorizing each gene in important tumor biology pathways (Table 7.1), we found that promoter CpG island methylation of a least one gene within each group was associated with stromal expression of hypoxia regulated proteins but not with epithelial expression (Table 7.3). Within the group of genomic instability associated genes (*MGMT*, *CHFR*, *HLTF*, *MLH1*, *RASSF1A*), stromal expression of hypoxia regulated protein CA9 was more often observed in *CHFR* methylated tumors 52% (35/67) compared to unmethylated *CHFR* tumors 34% (29/86), $p=0.03$. Epithelial expression of hypoxia regulated proteins was not related to methylation of genes involved in genomic instability. Within the group of methylated genes involved in limitless replication potential (*p14ARF*, *p16INK4A*), we found a borderline significant correlation that stromal expression of HIF-2 α was more often observed in *p16INK4A* methylated tumors 80% (56/70) compared to unmethylated *p16INK4A* tumors 65% (58/89), $p=0.05$. In line with the group of genes involved in genomic instability, epithelial expression of hypoxia regulated proteins was not associated with the methylation status of genes related to limitless replication potential. Between hypoxia regulated proteins and genes involved in tissue invasion and metastasis (*ADAM23*, *TFPI2*), again stromal expression of hypoxia regulated protein CA9 was more often observed in *ADAM23* methylated tumors 58% (35/60) compared to *ADAM23* unmethylated tumors 28% (22/79), $p<0.001$.

Table 7.2 Mean methylation index (MI) of *MGMT*, *CHFR*, *HLTF*, *MLH1*, *RASSF1A*, *p14ARF*, *p16INK4A*, *ADAM23*, *TFPI2*, *APC*, *SOCS1*, *THBS1*, *BNIP3*, *NEURL*, *RUNX3*, *RAB32* according to positive expression of hypoxia regulated proteins in the overall study population.

	MI	p-value		MI	p-value
HIF-1α			HIF-1α		
Stroma	0.38		Epithelial	0.43	
Negative	0.45	0.09	Negative	0.43	0.98
HIF-2α					
Stroma	0.38				
Negative	0.35	0.39			
CA9			CA9		
Stroma	0.41		Epithelial	0.39	
Negative	0.36	0.19	Negative	0.35	0.45
GLUT1			GLUT1		
Stroma	0.37		Epithelial	0.40	
Negative	0.40	0.55	Negative	0.40	0.95

Likewise *APC*, which is involved in insensitivity to anti-growth signals, showed more often 82% (46/56) HIF-2 α stromal expression in methylated tumors compared to *APC* unmethylated tumors 67% (73/109), $p=0.04$. Furthermore within the group of genes involved in self-sufficiency in growth signals, HIF-2 α stromal expression was more often observed within *SOCS1* methylated tumors

79% (77/98) compared to their unmethylated counterparts 61% (39/64), $p=0.04$. These results underscore that in particular stromal and not epithelial expression of hypoxia regulated proteins is associated with the loss of function by promoter CpG island methylation of genes involved in important tumor biological processes like genomic instability, tissue invasion and metastasis.

THBS1 promoter CpG island methylation was an infrequent event within our study population ($n=8$), and furthermore we did not observe a correlation between hypoxia regulated proteins and *THBS1* methylation involved in sustained angiogenesis. Four genes (*BNIP3*, *NEURL*, *RUNX3*, *RAB32*) were related to evading apoptosis, of which only *BNIP3* methylated tumors showed more often 87% (78/90) CA9 epithelial expression compared to *BNIP3* unmethylated tumors 73% (50/69, $p=0.03$). Within this group of genes, stromal expression of hypoxia regulated proteins was not related to promoter CpG island methylation.

Regarding HIF-1 α , neither stromal nor epithelial expression showed correlation with promoter CpG island methylation of genes listed in Table 7.3. The results for GLUT1 were less clear-cut. Although GLUT1 stromal expression appeared to be a feature of *THBS1* methylated tumors 43% (3/7) compared to *THBS1* unmethylated tumors 12% (13/105), $p=0.06$, it was more often observed in *p16INK4A* unmethylated tumors 23% (15/66) compared to *p16INK4A* methylated tumors 8% (5/61), $p=0.03$. As already stated in chapter 5, these findings for GLUT1 should be interpreted with caution, since the frequency of stromal GLUT1 expression is very low ($n=20$) compared to stromal expression of HIF-2 α ($n=119$) and CA9 ($n=66$).

Table 7.3 Percentage of positive stromal or epithelial expression of HIF-1 α , HIF-2 α , CA9 and GLUT1 according to methylation status of *MGMT*, *CHFR*, *HLTF*, *MLH1*, *RASSF1A*, *p14ARF*, *p16INK4A*, *ADAM23*, *TFPI2*, *APC*, *SOC1*, *THBS1*, *BNIP3*, *NEURL*, *RUNX3*, *RAB32*.

		HIF-1 α			HIF-2 α			CA9			GLUT1		
		Stroma	Epithelial	p-value	Stroma	Epithelial	p-value	Stroma	Epithelial	p-value	Stroma	Epithelial	p-value
<i>MGMT</i>	U	26/67 (39%)	22/67 (33%)	0.20	81/112 (72%)	38/53 (72%)	0.83	42/107 (39%)	85/107 (79%)	0.67	13/85 (15%)	72/85 (85%)	0.16
	M	10/40 (25%)	12/40 (30%)	0.83	38/53 (72%)	38/53 (72%)	0.83	24/53 (45%)	44/53 (83%)	0.50	7/45 (16%)	33/45 (73%)	0.16
<i>CHFR</i>	U	17/53 (32%)	16/53 (30%)	1.00	64/88 (73%)	64/88 (73%)	1.00	29/86 (34%)	70/86 (81%)	0.03	11/69 (16%)	54/69 (78%)	0.66
	M	17/51 (33%)	16/51 (31%)	1.00	52/70 (74%)	52/70 (74%)	0.86	35/67 (52%)	53/67 (79%)	0.84	9/57 (16%)	47/57 (83%)	0.66
<i>HLTF</i>	U	17/50 (34%)	14/50 (28%)	1.00	66/93 (71%)	66/93 (71%)	0.67	35/69 (51%)	74/69 (83%)	0.51	10/69 (15%)	54/69 (78%)	0.66
	M	17/53 (32%)	17/53 (32%)	1.00	66/93 (71%)	66/93 (71%)	0.67	30/66 (45%)	50/66 (76%)	0.72	10/66 (15%)	46/66 (69%)	0.66
<i>MLH1</i>	U	25/66 (38%)	21/66 (32%)	0.30	81/114 (71%)	81/114 (71%)	0.71	45/110 (41%)	90/110 (82%)	0.51	14/86 (16%)	69/86 (80%)	0.66
	M	11/41 (27%)	13/41 (32%)	1.00	38/51 (75%)	38/51 (75%)	0.71	21/50 (42%)	39/50 (78%)	0.84	6/44 (14%)	36/44 (82%)	1.00
<i>RASSF1A</i>	U	31/85 (37%)	29/85 (34%)	0.20	96/130 (74%)	96/130 (74%)	0.29	53/126 (42%)	104/126 (83%)	0.84	16/104 (15%)	82/104 (79%)	0.40
	M	4/21 (19%)	4/21 (19%)	0.29	23/34 (68%)	23/34 (68%)	0.29	13/33 (39%)	24/33 (73%)	0.84	4/25 (16%)	22/25 (88%)	0.40
<i>P14ARF</i>	U	16/50 (32%)	15/50 (30%)	1.00	66/92 (72%)	66/92 (72%)	0.68	34/86 (40%)	66/86 (77%)	0.52	9/65 (14%)	53/65 (82%)	0.83
	M	19/56 (34%)	19/56 (34%)	1.00	52/72 (72%)	52/72 (72%)	0.68	32/73 (44%)	62/73 (85%)	0.63	11/64 (17%)	51/64 (80%)	0.83
<i>P16INK4A</i>	U	18/46 (39%)	14/46 (30%)	0.29	58/89 (65%)	58/89 (65%)	1.00	32/86 (37%)	69/86 (80%)	0.05	15/66 (23%)	53/66 (80%)	1.00
	M	16/58 (28%)	17/58 (29%)	0.29	56/70 (80%)	56/70 (80%)	1.00	31/69 (45%)	57/69 (83%)	0.41	5/61 (8%)	49/61 (80%)	1.00
<i>ADAM23</i>	U	17/50 (34%)	13/50 (26%)	1.00	65/83 (78%)	65/83 (78%)	0.05	22/79 (28%)	61/79 (77%)	0.05	10/61 (16%)	47/61 (77%)	0.64
	M	13/41 (32%)	14/41 (34%)	0.49	40/61 (66%)	40/61 (66%)	0.13	35/60 (58%)	53/60 (88%)	0.00	6/51 (12%)	42/51 (82%)	0.64
<i>TFPI2</i>	U	3/12 (25%)	2/12 (17%)	0.75	13/17 (77%)	13/17 (77%)	0.33	7/17 (41%)	14/17 (82%)	1.00	2/17 (12%)	14/17 (82%)	1.00
	M	27/83 (33%)	28/83 (34%)	0.33	89/121 (74%)	89/121 (74%)	0.33	51/118 (43%)	93/118 (79%)	1.00	16/97 (17%)	79/97 (81%)	1.00
<i>APC</i>	U	25/68 (37%)	23/68 (34%)	0.40	73/109 (67%)	73/109 (67%)	0.67	39/104 (38%)	80/104 (77%)	0.04	14/82 (17%)	66/82 (81%)	1.00
	M	11/39 (28%)	11/39 (28%)	0.40	46/56 (82%)	46/56 (82%)	0.67	27/56 (48%)	49/56 (88%)	0.24	6/48 (13%)	39/48 (81%)	1.00
<i>SOC1</i>	U	11/29 (38%)	11/29 (38%)	0.65	39/64 (61%)	39/64 (61%)	0.49	26/60 (43%)	50/60 (83%)	0.02	9/43 (21%)	34/43 (79%)	0.82
	M	24/75 (32%)	23/75 (31%)	0.65	77/98 (79%)	77/98 (79%)	0.49	38/97 (39%)	76/97 (78%)	0.62	11/84 (13%)	68/84 (81%)	0.82
<i>THBS1</i>	U	33/88 (38%)	27/88 (31%)	0.09	97/132 (74%)	97/132 (74%)	0.67	52/129 (40%)	104/129 (81%)	0.02	13/105 (12%)	85/105 (81%)	1.00
	M	0/7 (0%)	1/7 (14%)	0.67	6/8 (75%)	6/8 (75%)	0.67	2/8 (25%)	10/29 (34%)	0.48	3/7 (43%)	6/7 (86%)	1.00
<i>BNIP3</i>	U	16/44 (36%)	12/44 (27%)	0.68	55/72 (76%)	55/72 (76%)	0.41	33/69 (48%)	50/69 (73%)	0.30	12/56 (21%)	47/56 (84%)	0.50
	M	19/62 (31%)	22/62 (36%)	0.41	63/92 (69%)	63/92 (69%)	0.41	33/90 (37%)	78/90 (87%)	0.19	8/73 (11%)	57/73 (78%)	0.50
<i>NEURL</i>	U	30/82 (37%)	28/82 (34%)	0.34	94/129 (73%)	94/129 (73%)	0.83	54/126 (43%)	99/126 (79%)	0.03	17/101 (17%)	83/101 (82%)	0.41
	M	6/24 (25%)	5/24 (21%)	0.34	25/33 (76%)	25/33 (76%)	0.83	11/31 (36%)	27/31 (87%)	0.54	3/27 (11%)	20/27 (74%)	0.41
<i>RUNX3</i>	U	22/61 (36%)	19/61 (31%)	0.84	77/104 (74%)	77/104 (74%)	0.85	40/99 (40%)	78/99 (79%)	0.03	12/78 (15%)	65/78 (83%)	0.81
	M	14/42 (33%)	14/42 (33%)	0.84	37/51 (73%)	37/51 (73%)	0.85	21/51 (41%)	42/51 (82%)	1.00	8/46 (17%)	37/46 (80%)	0.81
<i>RAB32</i>	U	32/88 (36%)	27/88 (31%)	0.17	102/142 (72%)	102/142 (72%)	0.78	57/137 (42%)	109/137 (80%)	0.59	17/108 (16%)	86/108 (80%)	0.36
	M	3/17 (18%)	6/17 (35%)	0.78	16/20 (80%)	16/20 (80%)	0.78	9/20 (45%)	18/20 (90%)	0.81	3/20 (15%)	18/20 (90%)	0.36

Discussion

The interactions between epigenetic changes in tumors and the phenomenon of tumor hypoxia are complex, including HIF-dependent and independent mechanisms, and vary from HIF stabilization through epigenetic control of HIF degrading proteins, through activity of histone acetyltransferases and histone demethylases involved in binding and transcriptional activity of HIF, to global epigenetic alterations such as histone modification and promoter CpG island methylation.

In this study, we emphasized on the latter aspect and formulated the hypothesis that hypoxia in CRC is related to promoter CpG island methylation of genes which play a role in executing important mechanisms of cancer progression, the so-called hallmarks of cancer. We studied a panel of hypoxia-related markers including HIF-1 α , HIF-2 α , CA9 and GLUT1, and correlated their expression with promoter CpG island methylation of 16 genes frequently methylated in CRC including: *MGMT*, *CHFR*, *HLTF*, *MLH1*, *RASSF1A*, *p14ARF*, *p16INK4A*, *ADAM23*, *TFPI2*, *APC*, *SOCS1*, *THBS1*, *BNIP3*, *NEURL*, *RUNX3*, *RAB32*. The results show interesting correlations between hypoxia and DNA promoter hypermethylation. Although overall hypoxia was not associated with a higher methylation index, stromal expression of hypoxia regulated proteins HIF-2 α or CA9 was related to methylation of at least one gene within almost every group of cancer related biological pathways (Table 7.1) as follows: *CHFR*, $p=0.03$ (genomic instability), *p16INK4A*, $p=0.05$ (limitless replication potential), *ADAM23*, $p<0.001$ (tissue invasion and metastasis), *APC* $p=0.04$ (insensitivity to anti-growth signals), *SOCS1*, $p=0.04$ (self-sufficiency in growth signals). These results have to be interpreted with caution, since we studied relatively many genes ($n=16$) within small subgroups of CRCs, possibly resulting in false positive results due to multiple testing. Therefore our results should be interpreted accordingly and need further validation in additional studies²¹. Although our data need to be validated, the fact that expression of HIF-2 α and its downstream gene product CA9 were associated with methylation of genes involved in common cancer related biological pathways, could reflect a mechanism by which hypoxic tumors, behave more aggressively and eventually influence patient prognosis. The current data and *in vitro* studies by others^{22,23} make it tempting to speculate about the sequence of events during hypoxia in which epigenetic mechanisms such as promoter CpG island methylation may play a role.

Conflicting experimental results can be derived from the literature. An inverse relationship between the presence of extensive tumor hypoxia and the incidence of promoter CpG island methylation was shown in human colorectal cell lines, in which exposure to hypoxia for 24 hours induced a reduction of 15-20% in global promoter CpG island methylation²⁴. In contrast, an altered

cellular phenotype in response to chronic hypoxia was demonstrated, with significant increases in global levels of promoter CpG island methylation, and concomitant increased expression of DNA methyltransferase⁹. Although the effects of hypoxia on epigenetic changes apparently can occur in a bi-directional mode, with either decrease or increase in promoter CpG island methylation, these results nonetheless indicate that epigenetic changes may represent an additional level of cellular adaptation to hypoxia, in addition to the HIF induced cellular response.

In theory, three possible scenarios could be put forward to account for the relation between tumor hypoxia and epigenetic alterations: firstly, DNA promoter hypermethylation and silencing of relevant tumor suppressor or DNA repair genes, by interfering with their control functions, can lead to conditions of genomic instability, evasion of apoptosis and limitless replication, resulting in tumor growth and subsequent hypoxia. Conversely, hypoxia in a growing tumor mass could induce epigenetic changes, such as gene silencing through methylation. Lastly, promoter hypermethylation of specific genes could offer protection against the deleterious effects of hypoxia on tumor cells, thus rendering a selective growth advantage in tumors with the appropriate methylation status^{5,9}.

Promoter CpG island methylation of the 16 genes we analyzed in the present study has also been detected in early lesions such as hyperplastic polyps and adenomas²⁵⁻²⁷, in which hypoxia does not play any significant role², suggesting that these genes are already methylated before hypoxia has occurred, rather than methylation being a consequence of hypoxia. This is in line with the theory that by silencing of crucial cancer biology genes, tumors are able to adapt and behave more aggressively during hypoxia^{4,28}. Strengthening this theory is the fact that within our series of CRCs, promoter CpG island methylation of *CHFR* and stromal expression of HIF-2 α and CA9 were both shown to be indicators of a more aggressive tumor behavior, since they were both related to a poorer patient survival (Chapters 4&6). We concluded that expression of hypoxia regulated proteins HIF-2 α and CA9 in tumor associated stroma, as opposed to their expression in epithelial cancer cells, is associated with poorer outcome, suggesting that tumor hypoxia may influence tumor associated stromal cells in a way that ultimately effects patient prognosis. The results of the current study add to this observation, in that tumors with stromal expression of HIF-2 α and CA9, show promoter CpG island methylation of crucial cancer pathway genes occurs. As our study is correlative and descriptive in nature, and based on patient material, we are unable to pinpoint the precise mechanisms involved. There are however some indications from *in vitro* data, which have also shown that tumor stroma is involved in effecting tumor biology, e.g. by downregulation of caspases, crucial apoptosis mediators²⁹⁻³². Interestingly, this downregulation was established by promoter CpG island methylation, as it was shown that the

DNA-methylation inhibitor 5-azadeoxycytidine could reverse methylation and restore caspase expression³³.

Apart from the discussion whether hypoxia should be considered a consequence or cause of epigenetic changes such as promoter CpG island methylation, the third aspect, protection of tumor cells against hypoxic cell damage, is also relevant. It has been shown that hypoxia, through induction of cell death, selects for cells with defective cell death regulators such as *BNIP3* and *TP53*, thus evading apoptosis³⁴. One way of achieving this situation is by promoter CpG island methylation and silencing of *BNIP3*. In the current study we found that *BNIP3* methylated tumors more often showed CA9 epithelial expression (88%), compared to *BNIP3* unmethylated tumors (73%), $p=0.03$. Conceivably, the combination of *BNIP3* methylation and CA9 epithelial expression reflects a synergistic mechanism to evade apoptosis, since CA9 expression maintains intracellular pH, thereby preventing tumor cell apoptosis³⁵. This is in line with the concept that in hypoxic tumors there is a selective growth and survival advantage for cells with defects in the apoptotic machinery, by silencing of *BNIP3* due to promoter CpG island methylation and *TP53* mutations^{11,36}. Epigenetic silencing of *BNIP3* by promoter CpG island methylation has been reported in several cancer types and contributes to resistance to hypoxia-induced cell death³⁷⁻³⁹. The exact role of *BNIP3* in CRC is unknown. In the next chapter we attempt to elucidate whether *BNIP3* methylation and *TP53* mutations in tumor cells, are related to patient outcome and apoptotic activity in hypoxic CRCs.

In summary, stromal expression of hypoxia regulated proteins HIF-2 α and CA9 was associated with promoter CpG island methylation of genes that participate in all of the major tumor biological pathways that were studied. Tumors with hypoxic features do not appear to show increased overall methylation, but rather harbour a selection of methylated genes, which could individually contribute to the more aggressive behaviour of these tumors. Further clinical and biological studies are necessary to validate our data and to unravel the precise mechanisms involved.

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Chapter 8

Poorer outcome in stromal HIF-2 α and
CA9 positive colorectal
adenocarcinomas is associated with
Wild-type *TP53* but not with *BNIP3*
promoter hypermethylation or
apoptosis

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Abstract

Stromal expression of hypoxia inducible factor 2 α (HIF-2 α) and Carbonic Anhydrase 9 (CA9) are associated with a poorer prognosis in colorectal cancer (CRC). Tumor cell death, regulated by a hypoxic stromal microenvironment could be of importance in this respect. Therefore, we correlated apoptosis, *TP53* mutational status and *BNIP3* promoter hypermethylation of CRC cells with HIF-2 α and CA9 related poor outcome.

In a series of 195 CRCs, *TP53* mutations in exons 5-8 were analyzed by direct sequencing and promoter hypermethylation of *BNIP3* was determined by methylation specific PCR (MSP). Expression of HIF-2 α , CA9, p53, BNIP3 and M30 were analyzed immunohistochemically.

Poorer survival of HIF-2 α and CA9 stromal positive CRCs was associated with wild-type *TP53* ($p=0.001$ and $p=0.0391$), but not with *BNIP3* methylation. Furthermore, apoptotic levels were independent of *TP53* status, but lower in unmethylated *BNIP3* CRCs ($p=0.004$).

It appears that wild-type *TP53* in CRC cells favours progression of tumors expressing markers for hypoxia in their stroma, rather than in the epithelial compartment. Preserved BNIP3 function in CRC cells lowers apoptosis, and may thus be involved in alternative cell death pathways, such as autophagic cell death. However, *BNIP3* silencing in tumor cells does not impact on hypoxia driven poorer prognosis.

These results suggest that the biology of CRC cells can be modified by alterations in the tumor microenvironment under conditions of tumor hypoxia.

Introduction

Hypoxia has been reported to influence tumor biology in opposing ways. It can directly induce cell death by activating apoptosis or autophagy, yet hypoxic zones in solid tumors also harbour viable cells resistant to treatment which contribute to poor patient outcome¹. Hypoxia influences the expression of genes involved in cell death and energy homeostasis mainly by stabilization and activation of the Hypoxia Inducible Factor (HIF) family of transcription factors, influencing angiogenesis (*VEGF*), glycolysis (*GLUT1*), pH regulation (*CA9*), apoptosis (*TP53*, *BNIP3*) and autophagy (*BNIP3*)^{2,3}.

A common hallmark of solid tumors under hypoxic stress is an increased ATP requirement, which is supplied by induction of anaerobic glycolysis. This subsequently leads to a high production of intracellular lactate, requiring regulation of intracellular pH, a process mediated partly by HIF dependent upregulation of Carbonic Anhydrase 9 (*CA9*). *CA9* catalyzes the extracellular trapping of acid by hydrating cell-generated CO₂ into HCO₃⁻ and H⁺⁴. Consequently, *CA9* expression may serve as a marker for metabolic adaptation during hypoxia.

Upregulation of p53 and *BNIP3* proteins, which stimulate cell death via apoptosis and/or autophagy, appears contradictory to the adverse association between tumor hypoxia and prognosis. However, it was suggested that through induction of cell death, hypoxia selects for cells with defective cell death regulators such as *TP53*⁵. In non-selected cells hypoxia can induce expression of p53 and activate p53 mediated G₀/G₁ arrest or apoptosis, although secondary effects such as extracellular acidosis and glucose deprivation are necessary for p53 mediated apoptosis⁵⁻⁸. Over 50% of human tumors contain somatic mutations in the *TP53* gene, resulting in a defective apoptotic response^{9,10}. Therefore, *TP53* mutations are expected to decrease the susceptibility of tumor cells to hypoxia induced cell death, as shown in vitro^{5,11}. *BNIP3* is a Bcl-2 family member, containing a single Bcl-2 homology 3 (BH3) domain and a transmembrane domain localizing it to the outer mitochondrial membrane^{12,13}. It is activated by HIF during hypoxia and initiates programmed cell death through apoptosis or autophagy^{14,15}. Epigenetic silencing of *BNIP3* by promoter hypermethylation has been reported in several cancer types and contributes to resistance to hypoxia-induced cell death¹⁶⁻¹⁸. The role of *BNIP3* in CRC is unknown, although 66% of tumors show *BNIP3* silencing by promoter hypermethylation^{19,20}.

Usually, the effects of hypoxia in solid tumors are studied within the tumor cells themselves, neglecting the hypoxic response in tumor associated stroma. In a previous study we found that hypoxia within the tumor associated stroma is indeed correlated with a poorer outcome in patients with colorectal cancer who are treated by surgery alone. In a multivariate model, stromal expression of

HIF-2 α and CA9 both were independent adverse prognostic factors, whereas HIF-1 α was not. Furthermore, expression of hypoxia related proteins HIF-1 α , GLUT1 and CA9 in the tumor cells self was not associated with poorer patient survival²¹.

Our previous findings indicate that hypoxic conditions may modulate the tumor stroma in such a way, that a more aggressive tumor behavior is facilitated, ultimately leading to decreased patient survival.

The present study attempts to elucidate whether changes in the epithelial cell compartment of CRC, such as apoptosis and concomitant (epi-)genetic changes that are confined to the tumor cells, are related to hypoxia related changes in the stromal compartment. For this purpose, we correlated alterations of *TP53* and *BNIP3* in tumor cells with expression of hypoxia related proteins HIF-2 α and CA9 in relation with patient outcome and apoptotic activity in CRCs.

Patients and methods

Patient population

Patients were entered in two multi-center prospective clinical trials in the Netherlands, between 1979 and 1981. One trial was designed to compare patient survival after treatment of colon cancer by conventional surgery or the no-touch isolation technique²². 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 undergo 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. This study population therefore enables unbiased study of the influence of hypoxic conditions on tumor biology.

Tumor tissues were fixed in buffered formalin, sectioned, and embedded in paraffin. Experienced pathologists documented the histopathological characteristics of the tumors (Table 8.1). 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 recurrence of disease and disease-related death. After five year follow up period, only time and cause of death were registered. Follow-up was complete for all patients. Failure was defined as death due to recurrent disease, excluding postoperative mortality within 30 days and non-disease related death.

For immunohistochemical and molecular analysis, tumor tissues from 195 CRC patients were available. The distribution of age, gender, tumor stage, location and type of tumor, frequency of events and mean follow-up time of the patients in this study are representative for the patients in the trial (see Table 8.1).

Table 8.1 TP53 mutation analyses, BNIP3 methylation and clinicopathological characteristics.

	TP53					BNIP3				
	Wt		Mutant		P	U		M		P
	No	%	No	%		No	%	No	%	
Total patients	78	(50)	77	(50)		92	(47)	103	(53)	
Age					.297					.759
<69 years	35	(45)	41	(53)		44	(48)	47	(46)	
>69 years	43	(55)	36	(47)		48	(52)	56	(54)	
Sex					.942					.788
Male	35	(45)	35	(45)		42	(46)	49	(48)	
Female	43	(55)	42	(55)		50	(54)	54	(52)	
Tumor location					.202					.016
Proximal	32	(41)	24	(31)		25	(27)	45	(44)	
Distal	46	(59)	53	(69)		67	(73)	58	(56)	
Tumor					.819					.967
Colon	49	(63)	47	(61)		61	(66)	68	(66)	
Rectum	29	(37)	30	(39)		31	(34)	35	(34)	
Differentiation					.913					.959
Well	8	(10)	8	(10)		10	(11)	11	(11)	
Moderate/poor	70	(90)	69	(90)		82	(89)	92	(89)	
TNM					.471					.001
1	1	(1)	2	(2)		3	(4)	1	(1)	
2	40	(51)	46	(60)		61	(66)	55	(53)	
3	28	(36)	19	(25)		14	(15)	40	(39)	
4	9	(12)	10	(13)		14	(15)	7	(7)	

Wt, Wild-type; U, Unmethylated; M, Methylated. Location: proximal or distal to splenic flexure.

Genomic DNA Isolation

Genomic DNA was extracted from CRC tissues using PureGene™ Genomic DNA Isolation Kit (Gentra Systems) based on the manufacturer's protocol.

TP53 sequencing

Mutation analyses of TP53 exons 5-8 was performed using a semi-nested PCR approach, (see supplemental Table 8.1S for primer sequences). CaCo2 (exon 6, codon 204 non-sense mutation) was included as a control. Direct sequencing of PCR products was performed using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) and analysed on the ABI 3730 DNA Analyzer (Applied Biosystems). Mutation detection was performed using Mutation Surveyor DNA Variant Analysis Software v3.0 (SoftGenetics LLC, USA). The results of the mutation analyses are listed in Table 8.2.

Furthermore, we assessed whether *TP53* missense-mutants were transcriptionally active or not, based on the IARC prediction models (<http://www.p53.iarc.fr/MutationValidationCriteria.asp>). Missense mutations were classified as either transactivation incompetent or transactivation competent missense mutations.

Table 8.2 Frequency and type of *TP53* alterations.

<i>TP53</i> Mutation	No	%
Wild type	72	(46)
Tr-Incom. Missense	57	(37)
Tr-Com. Missense	8	(5)
Silent	4	(3)
Non-sense	5	(3)
Deletion	4	(3)
Insertion	3	(2)
Polymorphism	2	(1)
Total	155	(100)

Tr-Incom, Transactivation incompetent; miss-mutations, missense mutation;
Tr-Com, Transactivation competent.

BNIP3 Promoter methylation analysis

BNIP3 promoter methylation was determined by sodium bisulfite modification of genomic DNA using the EZ DNA methylation kit (ZYMO research Co., Orange, CA). Methylation Specific PCR (MSP) was performed as described in detail elsewhere^{23,24}. DNA was first amplified with *BNIP3* flanking PCR primers that amplify bisulfite modified DNA but do not preferentially amplify methylated or unmethylated DNA. The resulting template was used as the template for *BNIP3* MSP. For primer sequences see supplemental Table 8.1S. All PCRs were performed with a control for unmethylated *BNIP3* alleles (normal lymphocyte DNA) and a positive control for methylated *BNIP3* 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.

Immunohistochemistry

Serial formalin fixed, paraffin-embedded tissues sections (4 µm) were stained for HIF-2α and CA9 as described previously²¹.

Briefly, *HIF-2α staining*: Antigen retrieval was performed by microwave treatment (750 W for 20 min in 1mM TE buffer pH 8.0), followed by 30 min. cooling in buffer. Slides were blocked in 25% normal serum for 10 min.

Sections were incubated with primary antibody HIF-2 α (1:500) for 100 min. (anti-HIF2 alpha monoclonal: ab8365 AbCam, UK).

CA9 staining: Slides were blocked in 25% normal serum for 10 min., then incubated for 45 min. with primary CA9 antibody MoAb M75 (1:50) (anti-human CA9, kindly supplied by Dr. S. Pastorekova) at room temperature.

In addition, serial sections were stained for p53, BNIP3 and M30, as follows:

p53 staining: Antigen retrieval was performed by microwave treatment (750 W for 15 min. in Antigen Retrieval (Dako)), followed by 30 min. cooling in buffer. Slides were blocked in 25% normal serum for 10 min. Sections were incubated for 45 min at room temperature with primary antibody p53 (1:500) (anti-p53 monoclonal (DO-7), M7001 DAKO, Denmark).

BNIP3 staining: Antigen retrieval was performed by microwave treatment (750 W for 15 min. in Antigen Retrieval (Dako)), followed by 30 min. cooling in buffer. Slides were blocked in 25% normal serum for 10 min. Sections were incubated for 180 min at room temperature with primary antibody BNIP3 (1:400) (anti-BNIP3 monoclonal (Ana40): ab10433, UK).

M30 staining: Antigen retrieval was performed by microwave treatment (750 W for 10 min. in Antigen Retrieval (Dako)), followed by 30 min. cooling in buffer. Slides were blocked in 25% normal serum for 10 min. Sections were incubated for 45 min at room temperature with primary Cytodeath antibody M30 (1:50) (mouse monoclonal (CloneM30), Roche Applied Science, Germany).

Each staining protocol was started with pre-incubating in 0.6% hydrogen peroxide for 20 min to block endogenous peroxidase activity. Furthermore, as a negative control, TBS buffer instead of primary antibody was used. Visualization was performed using Dako Envision, Peroxidase, mouse System (K4001, DAKO, Denmark). Powerenvision Poly-HRP (50510-60307, Immunologic, the Netherlands) was used for M30 visualization. The slides were counterstained with hematoxylin.

Evaluation of IHC

Evaluation for HIF-2 α and CA9 staining as described previously in detail²¹. Briefly, localisation (epithelial or stromal) was scored separately. For the category stromal staining only the stromal myofibroblasts were taken into account, not the tumor infiltrating inflammatory cells or the lamina propia of the normal mucosa. If nuclear staining was present in >5% of the tumor epithelial cells or stromal cells, the sample was considered positive for HIF-2 α .

If membranous staining occurred in >5% of the tumor epithelial cells or stromal cells, samples were considered positive for CA9²⁵.

TP53 and BNIP3 staining were considered positive by the presence of nuclear staining for TP53 and cytoplasmic staining for BNIP3 respectively, in >5% of tumor cells.

M30 expression was documented as the number of positive M30 cells per square millimeter of tumor cells (counted in 10 high-power fields ($\times 100$) per tumor) (Figure 8.1A, B). Apoptosis was categorized as Low apoptosis when the number of M30 positive cells ≤ 10 (mean), and High apoptosis when the number of M30 positive cells $> 10^{26,27}$.

Figure 8.1 (A) Nuclear expression of TP53 in colorectal tumor epithelial cells. (B) Cytoplasmic expression of BNIP3 in colorectal tumor epithelial cells. (C,D) Expression of M30 in colorectal tissue. (C) Expression of M30 in normal colon tissue. (D) Expression of M30 in tumor epithelial cell. (A,B) Original magnifications $\times 20$. (C-D) Original magnifications $\times 40$. Black bar pictures A, B = 200 μm , C, D = 100 μm .

Data-analysis

Correlations between HIF-2 α , CA9, *BNIP3*, *TP53*, M30 and clinicopathological parameters were determined by the Pearson Chi-Square and Fisher's exact test where appropriate. To evaluate the relationship between HIF-2 α , *BNIP3*, *TP53* and survival, Kaplan-Meier survival curves were calculated. Differences between groups were determined by using the Log-rank test. The endpoint for analyses was overall survival starting from the day of surgery. All p-values are two sided and p-values < 0.05 were considered statistically significant. Correction for multiple comparisons was performed using the Bonferroni

procedure. Patients with unknown and unspecified scores have been omitted from analyses for that specific variable. SPSS 12.0 software was used for data analyses.

Results

TP53 mutations

TP53 mutation analysis was successful in 155/195 (79%) of CRCs. CRCs were classified as having no *TP53* mutations 72/155 (46%), silent mutations 4/155 (3%) or known common polymorphism (exon 6, codon 213, CGA>CGG, R/R, refSNP rs1800372) 2/155 (1%) (Table 8.2). 37% (57/155) of CRCs were classified as transactivation incompetent missense mutations and 5% (8/155) of CRCs were classified as transactivation competent missense mutations. Correlations between *TP53* and other variables did not change with respect to the predicted presence or absence of transcriptional activity of *TP53* mutants. Therefore, in further analyses CRCs were classified as *TP53* wild-type when no mutations, silent mutations or a known common polymorphism were found, and as mutant *TP53* when CRCs had either missense, nonsense or frame shift mutations. Using this classification, 77/155 (50%) of CRC's showed *TP53* mutations (Table 8.1), which is in agreement with data published by others²⁸⁻³⁷. p53 protein expression was only observed in the nucleus of epithelial cells (Figure 8.1A). There was a significant correlation between the absence of p53 protein expression and wild-type *TP53* versus the presence of p53 protein in mutant *TP53*, $p=0.029$ (data not shown). No correlation between *TP53* mutation status and clinicopathological data was observed (Table 8.1).

TP53 mutations, patient survival and apoptosis

Overall no significant survival difference between *TP53* wild-type and mutant CRCs was observed (data not shown). However, the previously reported association between HIF-2 α or CA9 positive CRCs and poor prognosis was found to exist exclusively in *TP53* wild-type CRCs ($p=0.001$ and $p=0.0829$ respectively, Figure 8.2 A+B). Furthermore, there was a significant difference in survival between stromal CA9 expression (38% 5-years survival) versus epithelial CA9 expression (71% 5-years survival) within *TP53* wild-type tumors, $p=0.0391$ (data not shown). Overall levels of HIF-2 α or CA9 expression were not different between wild-type and mutant *TP53* CRCs (Data not shown). Survival of *TP53* mutant CRCs was not related to HIF-2 α nor CA9 expression ($p=0.9312$ and $p=0.8456$, respectively, Figure 8.2 E+F). These data suggest that wild-type *TP53* CRCs are less susceptible to the adverse effects of hypoxia. Since *TP53* can induce apoptosis during hypoxia, we assessed the extent of apoptosis (M30 staining, Figure 8.1C+D). Overall we found no

differences in apoptotic levels between *TP53* wild-type and *TP53* mutant CRCs (Table 8.3), or between HIF-2 α and CA9 positive or negative CRCs (data not shown). This was found regardless of *TP53* mutation status. These results indicate that presence or absence of functional p53 protein is not decisive for determining the extent of apoptosis in CRCs.

BNIP3 methylation

BNIP3 promoter methylation analysis was successful in 195/195 CRC's (100%). Overall 53% (n=103/195) of CRCs showed *BNIP3* promoter hypermethylation (Table 8.1), which is in agreement with data published by others^{12,20}.

The relationship between protein expression of BNIP3 and *BNIP3* promoter methylation status was analyzed in a randomly selected subset of patients (n=31). BNIP3 protein expression was only observed in the cytoplasm of epithelial cells (Figure 8.1B). *BNIP3* promoter methylated CRCs less frequently demonstrated BNIP3 protein expression than unmethylated CRCs (25% vs. 75%, respectively).

BNIP3 methylation, patient survival and apoptosis

Overall there was no significant survival difference between *BNIP3* methylated and unmethylated CRCs (data not shown). Although in HIF-2 α stromal negative CRCs, *BNIP3* methylation occurred in 61% (33/54), and did not influence prognosis whereas in the HIF-2 α positive tumors methylation was observed in almost equal frequency, 52% (68/132) but was associated with poorer patient survival (p=0.006, Figure 8.2G). Similarly, exclusively stromal (and not epithelial) expression of CA9 was an indicator of a poorer prognosis in both *BNIP3* methylated and *BNIP3* unmethylated tumors (p=0.0495 and p=0.0725, respectively, Figure 8.2D, H).

This suggests that hypoxic CRCs with stromal expression of HIF-2 α and CA9 have a poorer prognosis, independent of *BNIP3* methylation.

Since *BNIP3* has been reported to induce apoptosis in response to hypoxia, its methylation and associated down regulation might be expected to result in less apoptosis in the HIF-2 α subgroup. However, a low apoptotic activity (low M30 expression) was detected more frequently in *BNIP3* unmethylated CRCs 68% (50/73) compared to *BNIP3* methylated CRCs 46% (42/91, p=0.004, Table 8.3). Although tumors with both methylated *BNIP3* and stromal HIF-2 α expression showed a poorer patient survival compared to HIF-2 α negative tumors, this was not related to apoptosis (data not shown). Furthermore, we did not detect differences in apoptotic levels between tumors with or without CA9 expression, regardless of *BNIP3* methylation status.

Figure 8.2 Survival curves (A) HIF-2 α expression in wild-type *TP53* group, $p=0.001$ ($n=75$). (B) CA9 expression in wild-type *TP53* group, $p=0.0829$ ($n=72$). (C) HIF-2 α expression in *BNIP3* unmethylated group, $p=0.1712$ ($n=83$). (D) CA9 expression in *BNIP3* unmethylated group, $p=0.0725$ ($n=79$). (E) HIF-2 α expression in mutant *TP53* group, $p=0.9312$ ($n=69$). (F) CA9 expression in mutant *TP53* group $p=0.8456$ ($n=71$). (G) HIF-2 α expression in *BNIP3* methylated group $p=0.006$ ($n=98$). (H) CA9 expression in *BNIP3* methylated group $p=0.0495$ ($n=95$). U, Unmethylated; M, Methylated.

Table 8.3 Correlations between *BNIP3* methylation and *TP53* alteration vs. M30 expression

	M30 LOW		M30 HIGH		
	No	%	No	%	p-value
BNIP3					
Unmethylated	50	(68)	23	(32)	.004
Methylated	42	(46)	49	(54)	
TP53					
Wild-type	39	(56)	31	(44)	.703
Mutation	36	(59)	25	(41)	

Discussion

In a previous study on the expression of hypoxia related markers (HIF1 α , HIF2 α , CA9 and GLUT1) in colorectal adenocarcinomas, we found that in all tumors at least one of these proteins is immunohistochemically expressed. This indicates that hypoxia is an all but ubiquitous phenomenon in colorectal tumors. However, only expression of HIF2 α and CA9 in the tumor associated stroma was correlated with a poorer prognosis, suggesting that tumors with this particular phenotype follow a more aggressive course²¹. These results are in contrast to some other studies in colon and rectum tumors, that can be summarized by the observation that HIF1 α and GLUT1 expression in rectal cancer cells is of prognostic significance^{25,38-40}. Our finding was relatively new, in the sense that others have mainly reported the relation between tumor prognosis and expression of hypoxia markers in cancer cells, without paying attention to stromal expression. The results in our study did not differ between tumors of the colon or rectum.

So far, the biological basis of stromal expression of hypoxia related markers in CRC is unclear. It could either be a serendipitous finding, or a genuine indication of altered epithelial-mesenchymal interactions within a subset of tumors. In these tumors, hypoxia driven metabolic and cell biological changes could hypothetically alter the tumor stroma towards an environment that can facilitate cancer progression, along multiple routes, among which an enhanced proliferation or survival of tumor epithelial cells⁴¹. The tumor stroma under hypoxic conditions could select for the propagation of certain subclones of cancer cells that are optimally endowed for tumor progression. A recent study in breast cancer, on how changes in stromal gene expression affect epithelial tumor progression, showed that gene expression profiling of microdissected tumor stroma resulted in a set of stromal genes, that could predict clinical outcome. This set notably included enhanced stromal expression of hypoxia-associated genes⁴².

The current study attempts to pinpoint specific genetic and epigenetic features of colorectal cancer cells, that are both associated with the observed more aggressive presentation of CRCs expressing HIF2α and CA9 in their surrounding stroma, and impact on one of the hallmarks of cancer, namely regulation of apoptosis under hypoxic conditions.

Tumor hypoxia results in the induction of pro-death signals, mediated partly by *TP53* and *BNIP3*⁴³. Therefore it could be envisaged that hypoxia provides a selective environment for outgrowth of cells in which these genes have become mutated or silenced. Loss of pro-death genes such as *TP53* and *BNIP3* may result in increased hypoxia tolerance and cross resistance to other death inducing stimuli associated with metabolic stress or treatment. Immunohistochemical staining showed that *TP53* and *BNIP3* expression are confined to the epithelial cell compartment in CRC, and are not present in surrounding mesenchymal cells, making these two genes interesting candidates to study the hypothesis of cancer cell selection under hypoxia driven modification of tumor-stroma interactions.

Intriguingly, our results indicate that tumors expressing HIF-2α or CA9 in their stroma have a poorer prognosis in *TP53* wild-type tumors compared to mutant tumors. It is unclear how wild-type *TP53* might benefit this tumor subgroup, but several possibilities exist. Firstly, p53 is involved in a metabolic switch to glycolysis when oxidative phosphorylation is impaired during hypoxia⁴⁴. Also, other means of adaptation to metabolic stress, such as an increased fatty acid β oxidation, have been shown to be present in tumor cells with intact p53. With respect to this, increased apoptosis was reported in p53-deficient HCT-116 colorectal cancer cells as compared to wild-type p53 HCT-116 cells, when challenged by metabolic stress⁴⁵. The second possibility is that wild-type *TP53* is not acting directly, but instead simply correlates with defects in another pathway such as the *BNIP3* cell death pathway, that substitutes for *TP53* loss in a similar fashion during carcinogenesis.

With respect to apoptosis and hypoxia driven tumor progression, we did not find important effects related to the mutational status of *TP53*. However, apoptotic levels were lower in *BNIP3* expressing tumors, compared to tumors with epigenetically silenced *BNIP3* (p=0.004), which is somewhat surprising, given the fact that functional *BNIP3* is thought to induce cell death downstream of hypoxia inducible transcription factors. Apparently, things are more complicated. *BNIP3* may not be restricted to regulation of apoptosis, but could also regulate other pathways such as autophagy, in which there is a delicate balance between cell survival and cell death⁴⁶. Conceivably, the lower apoptotic activity in tumors with functional *BNIP3* might be due to autophagic rescue of the tumor cells. Furthermore, *BNIP3* levels appear to modulate cell death not only via apoptosis or autophagy, but also by necrosis. Also, the net effect of *BNIP3* is determined by the level of expression: too high *BNIP3*

expression will lead to autophagic cell death, while lower levels of BNIP3 expression, as in cells where *BNIP3* is silenced, will induce necrosis⁴⁷. In our study we used the immunohistochemical marker M30, which exclusively measures apoptotic cell death, and thus were not able to differentiate between other forms of cell death, such as autophagic death and necrosis^{48,49}.

Summarizing the results from the current study, levels of apoptosis do not play an important role in determining the poorer prognosis of hypoxic CRC's, as defined by stromal expression of HIF-2 α and CA9. However, the latter phenotype is correlated with the presence of wild-type *TP53* in the tumor cells, and the presence of functional p53 does indeed appear to have an important impact on poorer prognosis. This prognostic effect is not established through regulation of programmed tumor cell death, but may rather be connected to an enhanced capacity for adequate adaptation to metabolic stress. As *TP53* mutations occur in a relatively early stage of colorectal carcinogenesis, the potential deleterious effects of hypoxia on colorectal cancer biology may already be programmed in a very early phase of tumor development.

As opposed to *TP53*, functional or epigenetically silenced *BNIP3* did not turn out to be of influence in determining tumor prognosis. Furthermore, preservation of *BNIP3* function showed to decrease apoptotic activity, and may thus be involved in enhanced cell survival through autophagic rescue, or could be implicated in alternative cell death pathways, such as autophagic cell death or necrosis, which we were unable to measure in our experimental approach.

The findings in this translational study, on the relation between expression patterns of hypoxia related markers in clinical samples of CRC and the functional status of genetically or epigenetically modified proteins involved in regulation of tumor cell death on the one hand and patient outcome on the other, opens up interesting new avenues for more fundamental studies on the mechanisms underlying tumor hypoxia induced changes in epithelial-mesenchymal interactions.

Understanding the mechanisms by which hypoxic tumors can overcome cell death signals and adapt through metabolic changes is critical for our understanding of tumor progression and development of effective therapeutics in CRC patients with adverse prognostic profiles.

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Table 8.1S *TP53* mutation analysis primers and *BNIP3* MSP primer sequences.

<i>TP53</i>	Primer	Sequence	Product size
5 exon	Flank sense	5' CTG TTC ACT TGT GCC CTG AC 3'	268 bp
	Flank antisense	5' AGC CCT GTC GTC TCT CCA G 3'	
	Inside sense	5' TGC CCT GAC TTT CAA CTC TG 3'	
6 exon	Flank sense	5' GGC CTC TGA TTC CTC ACT G 3'	240 bp
	Flank antisense	5' ATA AGC AGC AGG AGA AAG CC 3'	
	Inside antisense	5' GAC CCC AGT TGC AAA CCA G 3'	
7 exon	Flank sense	5' CAT CTT GGG CCT GTG TTA TC 3'	164 bp
	Flank antisense	5' GAT GTG ATG AGA GGT GGA TGG 3'	
	Inside antisense	5' GGA AGA AAT CGG TAA GAG GTG 3'	
8 exon	Flank sense	5' GAT TTC CTT ACT GCC TCT TGC 3'	230 bp
	Flank antisense	5' CAT AAC TGC ACC CTT GGT CTC 3'	
	Inside antisense	5' TCC TCC ACC GCT TCT TGT C 3'	
<i>BNIP3</i>	Primer	Sequence	Product size
	Flank Sense	5'-TTY GTT TTG TTT TGT GAG TTT TTT-3'	129 bp
	Flank antisense	5'-CCR AAC TAC AAA ATA TAC TTC AAC TAC-3'	
	Unmethylated sense	5'-GGT TTT GTT TAG TTT GGG AGT G-3'	74 bp
	Unmethylated antisense	5'-CCT CAA CTA CAA ACA ATA AAA AAA CA-3'	
	Methylated sense	5'-TTT CGT TTA GTT CGG GAG CG-3'	69 bp
	Methylated antisense	5'-CAA CTA CGA ACG ATA AAA AAA CG-3'	

Chapter 9

General discussion

General discussion and future perspectives

Accurate staging of colorectal cancer (CRC) is of crucial importance for optimal management of the disease. The currently used classification system, the American Joint Committee on Cancer (AJCC) TNM classification system is based on three variables: the extent of penetration of tumor into the intestinal wall (T stage), nodal status (N stage) and the presence of distant metastasis (M stage). Although easy to implement, the survival for patients with the same TNM stage varies, which is caused by other factors that influence the prognosis. Therefore, the TNM classification is regularly being revised in order to improve the prediction of outcome. For example, a large study (n=128,000) including readily available patient characteristics such as age, sex, tumor differentiation/grade, and number of regional lymph nodes evaluated, better predicts survival than the currently used seventh edition of the AJCC system¹. This study clearly indicates that adding additional factors influencing prognosis can be helpful in refining the staging system for CRC.

Since the behavior of the tumor is determined by the underlying biology, inclusion of biological aspects of CRC carcinogenesis into the currently used classification system would be the next step. The last two decades it has become clear that CRC is a heterogeneous disease consisting of multiple molecular subtypes differentially influencing prognosis and response to therapy. The molecular subgroups most often used are MSI, CIN and CIMP, however only for MSI CRCs a consistent and accepted positive association with prognosis has been reported². Moreover, in contrast to assessing *KRAS* mutations to predict response to anti-*EGFR* therapy³⁻⁵ and establishing the cause of MLH1 loss⁶ (biallelic *MLH1* promoter CpG island methylation in sporadic CRC or mutations in mismatch repair genes in patients with Lynch syndrome) to determine disease management, this information is currently not being used routinely in the clinic.

In addition to the molecular classification mentioned above, a revolution in the technologies to study the molecular alterations underlying the initiation and development of cancer, such as micro-array- and next generation sequencing approaches enabled a detailed exploration of the genetic and epigenetic basis of CRC. Thus, although the identification of subgroups and key molecular alterations in CRC are rapidly being unraveled, the majority of these alterations have not been confirmed and validated as biomarkers in large validation studies. Multiple reasons account for this. First, independent validation studies confirming the initial observation are often lacking. Second, data analyses are predominantly performed on a mixed group of CRCs, not taking into account specific subgroups. Third, the effect of treatment is not always accounted for.

The aim of this thesis was to assess well characterized genetic and epigenetic alterations in a retrospective study, consisting of patients with CRC who were

entered in two multi-center prospective clinical trials between 1979 and 1981 in the Netherlands and were treated by surgery alone. We studied these alterations in relation with morphological features, hypoxia and prognosis.

First we analysed a set of well characterized molecular parameters associated with CRC, e.g. MSI, CIMP, *APC*-, *KRAS*-, *TP53*-, and *BRAF* mutations in a series of 160 CRC cases. This study was based on a literature analysis by Jass et al.⁷ in 2007, concluding that five distinct molecular subgroups with differential clinicopathological and morphologic features can be identified in CRC. We tested this hypothesis by using computer assisted unsupervised hierarchical clustering of the markers described above. Interestingly, hierarchical clustering of colorectal adenocarcinomas showed that CIMP status is the principal classifier, and that both CIMP+ and CIMP- tumors are further classified on the basis of *TP53* mutational status. Although it has been more than a decade since CIMP was first identified in CRC, controversy still exists on whether this subgroup of CRCs is an etiologically and clinically distinct group within the disease and to date, the cause of CIMP remains unknown⁸. Even more complexity is introduced by the fact that no gold standard with respect to gene panels, marker thresholds or techniques for detection of the altered DNA methylation used to define this phenotype exists⁸. Taking into account these considerations, it is surprising to observe that CIMP was identified as the principal classifier for CRC in this study and although these data need to be replicated, this observation underscores the significance and illustrates the need to establish the group of CRCs characterized by CIMP. Although the subgroups generated by the clustering algorithms did not show strong associations with clinicopathological parameters and therefore could not improve traditional staging with respect to identifying patients with a poorer survival in our first study, we did find an association with CIMP and prognosis in the validation series analysed in Chapter 4. We also observed that, in line with other publications^{7,9,10}, CIMP+ CRCs appear to comprise two subsets, one localized in the right-sided colon, with MSI and mucinous differentiation, the other occurring in the rectum, being MSS^{7,9,10}. This could reflect important differences in the underlying molecular biology of proximal and distal CRCs. For example, it was recently reported that anatomic location is a more significant predictor of progression than the histological type of CRC precursors like hyperplastic polyps, sessile serrated polyp or traditional adenoma¹¹. One explanation for a difference in biology could be a difference in the availability of dietary factors important in promoter CpG island methylation such as folic acid, methionine and related vitamins B2, B6 and B12. Epidemiological evidence suggests that these nutrients are associated with risk of colorectal cancer and CIMP status¹²⁻¹⁵. Notably, methionine has been associated with a decreased risk of proximal colon cancer, whilst among women it was inversely associated with rectal cancer. Conversely, a strong positive association between vitamin

B6 and rectal cancer risk in women has been reported¹⁴. These results underscore that nutrients involved in promoter CpG island methylation are to some extent related to associated phenotypes such as CIMP and tumor location in the large intestine.

Since we observed a strong effect of CIMP on the clustering in this series, we further investigated whether CIMP and 19 other genes reported to be functionally methylated and involved in a diversity of cancer related pathways in CRC, were associated with prognosis. Again, keeping in mind that prognosis is affected by biology of subgroups of cancer and treatment, we specifically analyzed data taking into account defined subgroups (based on MSI and *BRAF*-mutation status, other genetic alterations had no significant effect on prognosis) of CRCs in the population only treated with surgery.

Although for CIMP no clear biological cause or definition exists and numerous methods and definitions are used in CRC to quantify CIMP^{8,16} we chose to use the Weisenberger panel¹⁷ for studying associations with clinicopathological variables. When analyzing all 19 (or 24 if including CIMP) genes, only *APC* and *CHFR* promoter CpG island methylation were associated with a worse prognosis in stage II MSS, *BRAF* wild-type CRCs. For stage III MSS *BRAF* wildtype CRCs, associations with a poorer prognosis were found for promoter CpG island methylation of *RASSF1A*, *THBS1*, *CACNA1G*. When analyzing a second, independent series of CRCs, we could only confirm the prognostic effect of *CHFR* methylation in stage II, MSS, *BRAF* wildtype CRCs. This last observation illustrates the importance of validating findings in independent series. Promoter CpG island methylation of *CHFR* is present in approximately 43% of CRCs and is not a prognostic marker when assessed in a general population of CRCs consisting of all stages and all subgroups. Analyzing this gene in a specific subgroup of CRCs, namely the stage II MSS *BRAF* wildtype CRCs reveals the potential of this marker. Since we only observed this effect in two series of CRCs, we searched the literature for additional evidence for *CHFR* being a clinically relevant gene (Chapter 5). Interestingly, we found multiple lines of evidence for *CHFR* not only having prognostic significance but also predictive significance. *CHFR* is a E3 ubiquitin-protein ligase and functions as a mitotic stress checkpoint protein that delays mitotic entry in response to microtubule damage¹⁸. Although taxanes are not implemented in CRC treatment because they failed to demonstrate a significant clinical benefit in Phase II trials^{19,20}, a specific subgroup of CRCs with *CHFR* promoter methylation might benefit from taxanes. Therefore additional studies comparing the outcome of stage II MSS *BRAF* wildtype *CHFR* methylated CRCs versus unmethylated stage II MSS *BRAF* wildtype CRCs and the effect of taxane treatment in *CHFR* methylated versus unmethylated CRCs is warranted. Although we found multiple lines of evidence, some inconsistent findings have

been reported as well. This clearly shows the need for large, well designed trials to test the clinical relevance of *CHFR* methylation in CRC.

The first chapters of this thesis in detail address the genetic and epigenetic changes that occur during colorectal carcinogenesis, and their role in classification of the disease, notably the importance of promoter CpG island methylation in subcategorizing large intestinal tumors, which can further be characterized by certain genetic alterations, of which *TP53* mutations and microsatellite stability status appear to be the most relevant. These studies also show that CIMP is a more complex entity than was previously anticipated, not only with respect to definition and mode of detection, but CIMP also represents a heterogeneous category of tumors, from which an unequivocal single prognostic or predictive role for the whole group cannot be derived. Rather, it turns out that methylation of individual genes may play specific roles in the process of tumorigenesis and tumor progression, as exemplified by *CHFR*. In the second part of this thesis we therefore continued our search for additional individual candidate methylated genes, which not only could be applied in classification, prognostication and therapy of CRC, but might play a role in tumor biology as well. These studies depart from the notion that promoter CpG island methylation is an event that occurs early in neoplasia, and can even be demonstrated in pre-neoplasia, which in the colorectum is exemplified by gene methylation in adenomas^{21,22}. As promoter CpG island methylation subsequently remains present during later evolutionary stages of malignancy, we presumed that this type of epigenetic change not just accompanies tumor progression, but might exert its influence on tumor progression as well. Apart from accumulation of genetic and epigenetic changes, tumor progression in a biological sense involves the concerted interaction of several important pathways, which have been defined as the so-called “hallmarks of cancer”²³. In chapter 7, we therefore studied promoter CpG island methylation in a selection of multiple genes which individually can be attributed to every one of these tumor progression pathways. Furthermore, as tumors become increasingly dependent on oxygen and nutrients during progression, and upon outgrowing their supply become increasingly hypoxic, we correlated the methylation status of the selected genes with the occurrence of hypoxia in colorectal adenocarcinomas, defined by immunohistochemical expression of hypoxia related markers. We specifically chose to correlate epigenetic changes with such a complex phenomenon as hypoxia, because the latter has a major impact on cancer biology, not only underlying genetic, biochemical and metabolic changes in carcinoma cells, but also in a more integrative way, coordinating cellular alterations and responses in the tumor micro-environment, and influencing the interaction between tumor cells and stroma. With respect to this, in our studies, we did not restrict our observations to the tumor epithelial

compartment, but also specifically addressed stromal changes. Finally, tumor hypoxia is important from a clinical perspective, driving tumor progression towards a more aggressive phenotype, with relative resistance to chemotherapy and radiotherapy.

In the studies described in chapters 6 and 7 we found an important role for the tumor stroma (as opposed to tumor epithelial cells), with respect to both expression of hypoxia related markers, the relation of this expression pattern with a more aggressive tumor behavior, and the correlation with promoter CpG island methylation of selected genes involved in tumor progression pathways. This points to a potentially important role for the tumor micro-environment during tumorigenesis. It has already been well established that tumor development and -biology cannot be explained exclusively by studying tumor cells, but are modified to a considerable extent through the interaction with surrounding stromal cells. Work by other groups in recent years supports the somewhat underestimated role of the tumor stroma in determining the biology of a malignant tumor. With respect to this, in general, Pietras and Ostman review the role of tumor stroma and specific mesenchymal cell types in governing processes involved in the hallmarks of cancer²⁴, while others describe the role of hypoxia in relation to these hallmarks²⁵. Our results add to these, by linking tumor hypoxia, promoter CpG island methylation of selected genes, and the tumor stroma. Of course our studies are correlative in nature and do not allow any definitive conclusions on the underlying mechanisms. There are however more detailed studies reported in the literature, underpinning the relevance of stromal changes for tumor development and biology. Recently it was revealed that stromal cell signals could lead to silencing of tumor suppressor genes in tumor cells by promoter CpG island methylation²⁶. It was shown that the epigenome of tumor cells is highly plastic and easily modified by the tumor microenvironment²⁶⁻³². Similarly as within tumor cells, stromal cells also harbour unique aberrant epigenomes, relevant to clinical outcome and treatment choices³³. Finak et al.³⁴ supply evidence for the supplementary role of tumor stroma in governing tumor biology. In their study on breast cancer, they addressed stromal gene expression through a microdissection approach, separating tumor stroma from epithelium, and found an interesting set of specific stromally expressed genes, which have an impact on several tumor biological mechanisms, and which they combined into a new stroma derived prognostic predictor set. Interestingly, the stromal genes that were upregulated in their study included genes involved in immune responses, angiogenic and hypoxic responses. Also other studies report genomic alterations in stroma relevant to tumor development³³. An interesting mechanistic and theoretical approach was presented by Lisanti et al.³⁵, describing the role of hypoxia, oxidative stress and autophagy/mitophagy in the tumor microenvironment. In their so-called "Autophagic Tumor Stroma Model of

Cancer”, the theoretical basis is that during tumor growth cancer cells induce oxidative stress in surrounding stroma, which leads to production of reactive oxygen species in the stromal compartment. These subsequently give rise to upregulation of defense mechanisms in the epithelial compartment, which not only protects against oxidative stress, but also serves to prevent tumor cell apoptosis. Moreover, stromal production of reactive oxygen species can induce mutations and genomic instability in the tumor epithelium. Also, stromal autophagy and mitophagy increase, and this process of degradation gives rise to enhanced production of metabolites such as ketones, pyruvate and lactate in the tumor microenvironment, which in turn serves to fuel the tumor cells, enabling tumor growth. Naturally, intricate signaling and sophisticated cross-talk between stromal cells and tumor epithelial cells take place during the course of tumor progression. In this respect, HIF transcription factors and their downstream effects play a central part, but it seems plausible and appealing that methylation and silencing of certain key regulatory genes connected to various tumor progression pathways are involved as well.

As was presented and discussed in Chapter 7, we found an interesting correlation between tumor hypoxia, represented by stromal expression of hypoxia markers, and methylation of certain genes involved in essential tumor progression pathways. Whether the process of methylation is a cause or rather a consequence of hypoxia is still a matter of debate. We were interested in another aspect of gene methylation, namely the ability to protect tumor cells from the deleterious effects of tumor hypoxia. Hypoxia in theory induces cell death, and thus can render selective pressure on a tumor cell population, in which only the most resilient, apoptosis insensitive tumor cells would survive and continue to populate the tumor with the most aggressive cell fractions. We therefore chose to address one of the hallmarks of cancer, the evasion of apoptosis, in an attempt to clarify this issue. For this purpose, in chapter 8, the correlations of *BNIP3* methylation and *TP53* mutational status with levels of apoptosis and tumor behaviour were investigated. The idea being that silencing of pro-apoptotic *BNIP3* in tumor cells could protect them from the occurrence of programmed cell death, and along similar lines, that mutations in *TP53* would result in defective apoptotic machinery, and thus that both events would lead to lower apoptotic activity in a tumor. The hypothesis was that tumors with *BNIP3* methylation and/or *TP53* mutations would have the lowest apoptotic activity, the most accelerated growth, leading to increased hypoxia, and show the most aggressive behaviour, as manifested by the poorest prognosis. However, it turned out that wild-type *TP53* did not have an effect on apoptosis levels but was associated with the more aggressive tumors with stromal hypoxia, whereas conversely, tumors with unmethylated and thus preserved *BNIP3* had lower apoptotic levels, and this on the other hand did not have an impact on tumor prognosis. It was reasoned that functional p53 could act in other ways to

protect cells from the selective pressure exerted by hypoxic stress, such as metabolic adaptation of tumor cells to hypoxia. As *BNIP3* is also known to be involved in autophagy, perhaps alternative causes of cell death come into play in tumors unmethylated for this gene, which could still account for the lack of correlation with tumor aggressiveness and poorer prognosis. These results in Chapter 8 once again show that there is a considerable gap between theoretical models and hypotheses, the data derived from descriptive studies using patient materials, clinical data and statistical analysis on the one hand and the ability to explain important tumor biological processes on the other. Nevertheless, the data presented in the current thesis will hopefully form a theoretical basis for further mechanistic studies to clarify the precise relations between epigenetic changes, tumor hypoxia and the role of tumor stroma during the process of tumor progression.

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Summary

Summary

Colorectal cancer (CRC) is one of the most common cancer types worldwide and approximately 50% of patients with CRC die due to the disease. The histology based Tumor Node Metastasis (TNM) system is currently the best tool to provide information on prognosis and to determine treatment protocols in CRC. Although this anatomical based staging system predicts the survival accurately, variation in the course of the disease and response to treatment among individuals within the same stage exists. Therefore the identification of new prognostic and predictive markers for CRC to determine prognosis and to identify patients that need adjuvant therapy respectively is urgently needed. Understanding the underlying biology of CRC will provide novel molecular markers to accurately predict prognosis and improve treatment options besides the traditional histological staging system.

The **aim** of this thesis was to study genetic and epigenetic alterations in relation to morphological features and hypoxia in CRC, providing insight in the underlying biology of CRC and possibly yielding relevant novel prognostic information.

In chapter 1 we introduce the (epi)-genetic alterations that underlie the process of cancer development and discuss the molecular phenotypes of CRC: chromosomal instability (CIN), microsatellite instability (MSI) and the CpG island methylator phenotype (CIMP). Furthermore, the importance of hypoxia within solid tumors like CRCs is pinpointed, by explaining the cellular responses during hypoxia, with special emphasis on the tumor associated stroma.

In chapter 2, we review the current knowledge on the epidemiology, diagnosis, treatment and the prognostic and predictive value of (epi)genetic markers in CRC.

In the first experimental study (Chapter 3) we classified CRCs by computer assisted unsupervised hierarchical clustering, based upon well described markers in the genesis of CRC including: CIMP, MSI, *APC*-, *KRAS*-, *TP53*-, and *BRAF* mutation status, in a series of 160 CRCs. Subsequently, we investigated in which way this classification was related to patient data, including clinicopathological parameters, morphological features (differentiation grade, mucinous differentiation, dirty necrosis, circumscribed tumor growth, tumor budding and lymphocyte infiltration) and biological behavior in terms of patient prognosis.

These analyses revealed that for molecular clustering of colorectal adenocarcinomas, CIMP status was the principal classifier, and that both

CIMP+ and CIMP- tumors were further classified on the basis of *TP53* mutational status. Hierarchical clustering was only modestly related to morphology and outcome, which appeared to be dependent on additional factors such as microsatellite stability and localization in the intestine. Although molecular clustering provided insight in the underlying biology of CRC, it did not improve traditional staging with respect to prognostic value.

Data on the prognostic significance of promoter CpG island methylation in CRC are conflicting, possibly due to associations between methylation and other factors affecting survival such as genetic alterations and use of adjuvant therapy. Therefore in chapter 4 we examined the prognostic impact of promoter methylation of 19 tumor suppressor- and DNA repair genes and CIMP, as defined by Weisenberger *et al.*, in CRC patients treated with surgery alone in the context of MSI, *BRAF*- and *KRAS* mutations. Our study failed to demonstrate a consistent prognostic effect of CIMP, however we identified promoter CpG island methylation of *CHFR* as a prognostic biomarker in stage II, MSS, *BRAF* wild-type CRCs in two independent populations of CRCs.

Chapter 5 reviews the evidence for *CHFR* as a prognostic and predictive biomarker in a diversity of solid malignancies including CRC.

In CRC, the importance of hypoxia has been demonstrated by clinical studies in which hypoxia predicts for worse outcome and resistance to chemotherapy and radiotherapy. Therefore, in chapter 6 we studied the role of protein expression of hypoxia-inducible factor (HIF)-1 α , HIF-2 α , carbonic anhydrase 9 (CA9) and glucose transporter 1 (GLUT1) in patients with colorectal adenocarcinomas. Expression of these hypoxia markers was detected in the epithelial compartment of the tumor cells as well as in tumor associated stromal cells. Although epithelial tumor cells frequently showed expression of one or more of the investigated hypoxia markers, no correlation among these markers or with clinical response was found. In contrast to this, within the tumor stroma, expression of HIF-2 α and CA9 were both associated with a significantly reduced overall survival. In the Cox proportional hazard model, stromal HIF-2 α expression was an independent prognostic factor for survival. Our study suggested that tumor hypoxia may influence tumor associated stromal cells in a way that ultimately contributes to patient prognosis.

Since epigenetic silencing might play a role in the cellular responses to hypoxia, in chapter 7 we investigated whether hypoxia in CRC is associated with promoter CpG island methylation of genes involved in important tumor biological processes such as: *MGMT*, *CHFR*, *HLTF*, *MLH1*, *RASSF1A* (genomic instability), *p14ARF*, *p16INK4A* (limitless replication potential),

ADAM23, *TFPI2* (tissue invasion and metastasis), *APC* (insensitivity to anti-growth signals), *SOCS1* (self-sufficiency in growth signals), *THBS1* (sustained angiogenesis), and *BNIP3*, *NEURL*, *RUNX3*, *RAB32* (evasion of apoptosis). This study showed that stromal expression of hypoxia regulated proteins HIF-2 α and CA9 was associated with promoter CpG island methylation of genes involved in important tumor biological processes. Tumors with hypoxic features did not show increased overall CpG island promoter methylation, but rather harboured specific methylated cancer biology related genes, which could contribute to the more aggressive behaviour of these tumors.

Finally in chapter 8 we studied whether tumor cell death, regulated by a hypoxic stromal microenvironment could be of importance in the observed poorer prognosis in CRC with stromal expression of HIF-2 α and CA9. Therefore, we correlated apoptosis, *TP53* mutational status and *BNIP3* promoter hypermethylation (both genes involved in regulation of apoptosis) of CRC cells with HIF-2 α and CA9 related poorer outcome. Our study showed that the effect of HIF-2 α as an indicator of a poorer prognoses was dependent on wild-type *TP53* and not methylation status of *BNIP3*. It appeared that wild-type *TP53* in CRC cells favors progression of tumors expressing markers for hypoxia in their stroma, rather than in the epithelial compartment. We also showed that preserved BNIP3 function in CRC cells lowers apoptosis, and may thus be involved in alternative cell death pathways, such as autophagic cell death. Our study suggested that the biology of CRC cells can be modified by alterations in the tumor microenvironment under conditions of tumor hypoxia.

In conclusion, this thesis demonstrated the importance of CIMP in subcategorizing CRC's, which could further be characterized by *TP53* mutations and microsatellite stability status. It turned out that methylation of individual genes may play a specific role in the process of tumorigenesis and tumor progression, as exemplified by the validated prognostic value of *CHFR*. Besides methylation of individual genes, hypoxia related protein expression of HIF-2 α and CA9 in the tumor associated stroma was associated with a poorer patient survival compared to expression of these markers in epithelial tumor cells. We have reasons to believe that promoter CpG island methylation and mutations of crucial cancer genes are involved in the observed more aggressive behaviour of these hypoxic tumors. Future studies are needed to unravel the pathways involved and establish the role of (epi-)genetic markers for treatment stratification of patients in addition to the traditional staging method.

Samenvatting

Samenvatting

Colorectaal carcinoom (CRC), ook wel kanker van de dikke darm en endeldarm genoemd, is een veelvoorkomende vorm van kanker en ongeveer de helft van patiënten met CRC sterft als gevolg van de ziekte. Op basis van het 'Tumor node Metastasis' systeem, een histologisch stadiërings systeem, worden de prognose en de behandelingskeuze van patiënten met CRC bepaald. Ondanks dat dit histologisch stadiërings systeem de overleving van groepen van patiënten accuraat voorspelt, bestaat er binnen eenzelfde stadium van CRC een variatie in het beloop van de ziekte en respons op adjuvante therapie. Dit illustreert dat er een dringende behoefte is aan nieuwe, additionele moleculaire markers, die de respons op adjuvante therapie en de prognose van patiënten met CRC beter kunnen voorspellen. De verwachting is, dat met het verrichten van onderzoek naar de moleculaire 'pathways', die ten grondslag liggen aan deze ziekte, nieuwe moleculaire markers ontdekt kunnen worden. Deze nieuwe, moleculaire markers zouden, naast het huidige histologische stadiërings systeem, de prognose van patiënten beter moeten kunnen voorspellen en mogelijk nieuwe therapeutische opties voor patiënten kunnen identificeren. Het doel van dit proefschrift was het bestuderen van genetische en epigenetische afwijkingen in relatie met morfologische kenmerken en hypoxie in CRC, om met behulp hiervan, nieuwe inzichten in de biologie van CRC te verwerven en mogelijke nieuwe bruikbare prognostische markers voor patiënten met CRC te ontdekken.

In hoofdstuk 1 wordt een introductie gegeven over de bestaande (epi)-genetische afwijkingen die de ziekte CRC in drie verschillende moleculaire subgroepen verdelen: chromosomale instabiliteit (CIN), microsatelliet instabiliteit (MSI) en het CpG-eiland methylerings fenotype (CIMP). Tevens wordt er in dit hoofdstuk een overzicht gegeven van de rol van hypoxie in CRC; de cellulaire paden die hierbij betrokken zijn, en in het bijzonder de rol van het tumor geassocieerde stroma hierin.

Hoofdstuk 2 geeft een overzicht van de epidemiologie, diagnose, behandeling, prognostische en voorspellende waarde van (epi)-genetische markers in CRC.

De eerste experimentele studie (hoofdstuk 3) beschrijft de resultaten die gevonden werden met het 'unsupervised' hiërarchisch clusteren van 160 CRCs op basis van genetische en (epi)-genetische afwijkingen waaronder: CIMP, MSI, *APC*-, *KRAS*-, *TP53*-, en *BRAF* mutaties. Vervolgens werd er onderzocht in hoeverre de verkregen moleculaire clusters, correleerden met klinisch relevante patiënten gegevens, morfologische tumorkenmerken (differentiatie graad, mucineuze differentiatie, necrose, circumscripte tumorgroei, tumor

budding en lymfocyttaire infiltratie) en overleving van patiënten. Uit deze analyse bleek dat CRCs in eerste instantie op basis van CIMP onderverdeeld werden in CIMP+ en CIMP- tumoren en vervolgens op basis van *TP53* mutaties verder onderverdeeld werden. Hiërarchisch clusteren was slechts in geringe mate gecorreleerd aan morfologische tumorkenmerken en patiënten overleving. Deze correlaties bleken afhankelijk te zijn van andere factoren zoals microsatelliet instabiliteit en de locatie van de tumor in de darm. Onze moleculaire cluster analyse geeft nieuwe inzichten in de onderliggende biologie van CRC, echter de clusters bezitten geen toegevoegde prognostische waarde in vergelijking met het traditionele, histologisch gebaseerde stadiëringssysteem.

Hoofdstuk 4 richt zich op het bestuderen van de prognostische waarde van CIMP, (gedefinieerd door Weisenberger *et al.*) en promotor CpG-eiland methylering van 19 andere tumor suppressor- en DNA reparatie genen in patiënten met CRC. Omdat dit soort studies vaak wordt verstoord door factoren die een invloed hebben op het ziektebeloop, zoals genetische afwijkingen en chemotherapie, werd in onze studie de prognostische waarde bestudeerd onafhankelijk van MSI, *BRAF*, *KRAS* mutaties en tumor stadium in een populatie van patiënten met CRC die uitsluitend behandeld werden middels chirurgie. Uit deze analyse kwam naar voren dat niet CIMP, maar promotor CpG-eiland methylering van een individueel gen, het *CHFR* gen, een prognostisch effect toonde in stadium II, microsatelliet stabiele, *BRAF* wildtype CRC; in twee onafhankelijke studie populaties van CRCs.

Hoofdstuk 5 geeft een literatuur overzicht over veranderingen die optreden in de expressie van het *CHFR*-gen in verschillende kanker soorten waaronder CRC en de mogelijke rol van *CHFR* als een biomerker.

Een belangrijke rol voor hypoxie in CRCs is gebleken uit studies die laten zien dat hypoxie gerelateerd is aan een slechtere overleving van patiënten met CRC en resistentie voor chemo- en radiotherapie. Vandaar dat we in hoofdstuk 6 de expressie bestuderen van de hypoxie gereguleerde eiwitten HIF-1 alpha, HIF-2 alpha, CA9 en GLUT1 in patiënten met CRC. Hieruit bleek dat in CRC de expressie van deze hypoxie gereguleerde eiwitten zowel in de epitheliale tumorcellen als in de tumor geassocieerde stromale cellen voorkwam. Echter, in tegenstelling tot expressie in epitheliale tumorcellen, was de expressie van hypoxie gerelateerde eiwitten HIF-2 alpha en CA9 in stromale cellen gerelateerd aan een slechtere overleving van patiënten met CRC. Dit onderzoek suggereert dat hypoxie in tumoren stromale cellen dusdanig beïnvloedt dat dit uiteindelijk resulteert in een slechtere prognose voor CRC patiënten.

Het is bekend dat promoter CpG-eiland methylering betrokken is bij de cellulaire respons van tumoren tijdens hypoxie. Vandaar dat er in hoofdstuk 7 onderzocht is of de expressie van hypoxie eiwitten in CRC geassocieerd is met promoter CpG-eiland methylering van genen die een rol spelen in belangrijke kanker gerelateerde processen waaronder: *MGMT*, *CHFR*, *HLTF*, *MLH1*, *RASSF1A* (genoom instabiliteit), *p14ARF*, *p16INK4A* (ongelimiteerde celdeling), *ADAM23*, *TFPI2* (invasieve groei en metastasering), *APC* (ongevoeligheid voor groeiremmende signalen), *SOC1* (zelfvoorzienend in groeifactoren), *THBS1* (onbeperkte angiogenese), en *BNIP3*, *NEURL*, *RUNX3*, *RAB32* (voorkomen van apoptose). Onze resultaten toonden aan dat niet de expressie van hypoxie eiwitten in de epitheliale tumorcellen, maar juist in de stromale cellen geassocieerd was met promoter CpG-eiland methylering van diverse genen die een belangrijke rol spelen in kanker gerelateerde processen. Dit onderzoek suggereert dat genmethylering in hypoxische tumoren niet een willekeurig proces betreft, maar juist gekenmerkt wordt door methylering van specifieke kanker gerelateerde genen, waardoor deze hypoxische tumoren in staat zijn zich agressiever te gedragen.

Ten slotte is in hoofdstuk 8 onderzocht of tumorcel dood en hieraan gerelateerde (epi-)genetische afwijkingen in de epitheliale tumorcellen, geassocieerd zijn met de geobserveerde slechtere overleving van CRC patiënten met expressie van hypoxie eiwitten in stromale cellen. Hiervoor werd er in epitheliale tumorcellen gekeken naar afwijkingen in apoptose regulerende genen zoals *TP53* en *BNIP3*, en onderzocht of deze gen afwijkingen gecorreleerd waren met de slechtere overleving van CRC patiënten met stromale expressie van hypoxie eiwitten HIF-2 α en CA9. Uit dit onderzoek bleek dat de geobserveerde slechtere prognose van CRC patiënten met expressie van HIF-2 α in stromale cellen, gecorreleerd was met wild-type *TP53* en onafhankelijk van de methylerings status van *BNIP3*. Kennelijk draagt ongemuteerd *TP53* ertoe bij, dat tumoren met stromale expressie van hypoxie eiwitten progressie laten zien, in tegenstelling tot tumoren met expressie van hypoxie eiwitten in de epitheliale tumorcellen. Verder bleek dat een behouden functie van het *BNIP3* gen resulteerde in minder apoptose, en dus mogelijk betrokken is bij een alternatief tumorcel dood mechanisme, zoals 'autofagie' geregleerde tumorcel dood. Onze studie laat zien dat de biologie van CRC cellen gemodificeerd wordt door hypoxie in het tumor geassocieerde micro-milieu.

Concluderend leveren de resultaten van dit proefschrift nieuwe inzichten in de ontstaanswijze van colorectale tumoren. Men kan op basis van (epi-)genetische afwijkingen zoals CIMP, *TP53* mutaties en microsatelliet

instabiliteit, CRCs onderverdelen in aparte subgroepen, waarbij individuele epigenetische markers zoals *CHFR* methylering, een onafhankelijke en gevalideerde prognostische marker bleken te zijn. Naast DNA promotor methylering van individuele genen, was de expressie van hypoxie eiwitten HIF-2 α en CA9 in stromale cellen gerelateerd aan een slechtere overleving van CRC patiënten en aan methylering van specifieke kanker gerelateerde genen. Toekomstige studies zullen de betrokken celbiologische processen moeten ophelderen en het potentiële belang van epigenetische markers voor het bepalen van de therapie in CRC patiënten moeten aantonen.

Dankwoord

Dankwoord

Het dankwoord is het meest frequent gelezen hoofdstuk van een proefschrift.

Gek genoeg bedankt de gemiddelde promovendus in dit dankwoord zijn vrienden, opa, oma en de burens, en nog net niet zijn cavia of diepzeeschildpad. Waarvoor? Hetzelfde principe dat je op je eigen verjaardagsfeest rond rent met bitterballen, mosterd en lege flesjes bier voor vrienden, opa, oma en de burens! Daarbij komt dat deze vrienden, opa, oma en de burens, je voor gek verklaren tijdens je promotie en de wederkerende vraag stellen: "Wanneer is het nu eens af?" of deze: "Waarom maak je het niet gewoon af?" of deze: "Loopt alles volgens de planning?"

Dat is een goede in wetenschapsland waar het principe 'Tomorrow never dies!' zegeviert. Tijdens een promotietraject zit je in een spannende, eeuwig durende treinreis op je werk, na je werk, op vakanties, ga zo maar door. Als je wilt, kun je ook aan je onderzoek werken tijdens 30 min. fietsen in de fitnessclub. Terwijl andere zwetende medeburgers tijdschriften lezen zoals: Privé, Linda en de Telegraaf, lukte het mij om gemiddeld 1 artikel uit: JAMA, JNCI, CCR of JCO te lezen. Uiteindelijk na al het gefitness zegt de conducteur: "Meneer Cleven, u heeft uw bestemming bereikt." Je stapt na alle hoogtepunten en ontberingen uit te trein waarin je jaren hebt gezeten; een tijd waarin relaties werden gewisseld, baby's geboren, banen gewisseld, er vielen zelfs doden! Ik heb nog net geen oorlog meegemaakt. Plotseling sta je dus op het pad in de Minderbroedersberg 4-6, en dat doet me denken aan een scène uit 'Monty Python and the Holy Grail'. Een ridder ligt op een eenzaam zandpad met afgehakte onderste en bovenste extremiteiten en schreeuwt vervolgens tegen zijn opponent: "Come on, tis but a scratch- A scratch? Your arm's off.- I've had worse, come here I'll bite your legs off!"

Na deze korte introductie m.b.t. het promotietraject, moet ik toegeven dat onze volksheld Lee Towers gelijk heeft met zijn lied vanaf de Rotterdamse haven 'You never walk alone!'.

Vol enthousiasme en een hoop wetenschappelijke bagage, dacht ik, startte ik aan een nieuw onderzoeksproject met als doel uiteindelijk te promoveren. Ik klopte aan bij mijn promotor prof. dr. Adriaan de Bruïne.

Beste Adriaan, hoe kan ik nu in godsnaam formuleren wat jouw rol is geweest in het tot stand komen van dit proefschrift? Aangezien god niet bestaat, zal ik proberen het chronologisch in verhaalvorm uit te leggen.

Na ons eerste gesprek op de afdeling, nam je mij op sleeptouw in de wetenschappelijke wereld van de afdeling pathologie te Maastricht. Bij het eerste onderzoeksvoorstel dat ik schreef, kan ik mij nog herinneren dat je zei: "The sky is the limit meneer Cleven".

Na een jaar *in vitro* werk met cellijnen onder hypoxische omstandigheden, zonder bruikbare resultaten, bleek er inderdaad een 'sky' te zijn. Jij maakte snel de beslissing om het onderzoek te concentreren op *in vivo* werk in een serie van dikke darm tumoren dat uiteindelijk geresulteerd heeft in het huidige proefschrift. Tijdens deze lange periode nam je mij mee naar verschillende congressen waarbij ik nieuwe onderzoeksdata mocht presenteren. Naast het leerzame aspect hiervan, was het altijd ontzettend gezellig: je regelde leuke hotels, heerlijke etentjes en minstens een 'City-tour' per vreemde stad. Deze gezelligheid resulteerde in de beruchte: 'The flying burrito with a lot of sour cream' op het nieuwe driedelig pak van een perifere patholoog in Brighton; tot de bratwurst met curry om zes uur 's ochtends in hartje Berlijn. We waren om vier uur vertrokken vanuit Maastricht en eenmaal aangekomen in Berlijn hadden we geen muntgeld voor een treinkaartje. Het kopen van een bratwurst resulteerde in muntgeld, maar je was volgens mij blij en ik overigens ook, nadat de bratwurst met curry om zes uur in de ochtend in de prullenbak belandde.

Naast het leren om belangrijke details te signaleren uit een wereld van resultaten, heb ik door jou geleerd snel te lezen, met behulp van het boek 'gebruik je hersenen'. Dit boek lag op een ochtend op de rijderstoel in onze carpool sessie naar Venlo. Een stille hint!

Af en toe was ik ongeduldig, ik vuurde dan simpelweg een kruisraket met kernkop af. Bij de aanblik van dit projectiel aan de horizon, brommelde jij je tot een kolonel in het kaliber van 'Apocalypse now' William Kilgore: "Charlie don't surf, but I love the smell of napalm in the morning". Het resultaat was, dat de kernkop terugkwam op mijn eigen grondgebied en vervolgens ontplofte. Dat was verhelderend, aangezien ik na zo'n Hiroshima momentje, weer door de weinige bomen het bos kon zien!

Adriaan, ik denk dat ik mijn achternaam 'Cleven' waar heb gemaakt!

Jij bent de grondlegger van dit boekje en ik wil je bedanken, niet alleen voor de goede en langdurige begeleiding, maar ook voor de inhoudelijke vrijheid die je mij gegeven hebt en de ontzettend gezellige tijd die hiermee gepaard ging.

Voor mijn promotietraject had ik mijn hobo al lang ingewisseld voor een stevige elektrische gitaar met versterker. Dit heeft mijn promotor prof. dr. Manon van Engeland vaak moeten ervaren.

Beste Manon, laat me beginnen te zeggen dat het niet raar is dat wij elkaar nog ontmoet hebben in de prachtige wereld van de wetenschap. Jaren geleden mocht ik, als broertje samen met mijn vader mijn zus en haar discovriendinnetje 's avonds met de auto ophalen. Jij bevond je ook onder deze discovriendinnetjes. Op de terugweg naar city Susteren zagen alle inzittenden rondom middernacht een schotelvormig object met rode lampen in een verlaten Susters weiland. We dachten allemaal dan nu eindelijk een UFO gespot te hebben in de achtertuin van de Hommele Hei. Slechts één

discovriendinnetje trok deze hypothese in twijfel en ging op onderzoek uit. Bij thuiskomst volgde er een telefoontje: “Ja, hier met Manon. Neen, het is geen UFO maar een bietenwagen van een boer die daar zo stond te blinken.” Jaren later kwam ik je weer tegen in het onderzoekslaboratorium waar jij leiding geeft. Ideeën en plannen waren er genoeg, maar wat zonder onderzoeksgeld? Manon, jij hebt meerdere bietenwagens vol met euro's in mijn onderzoek gepompt. De reagentia, een analist, high throughput sequencing; zonder jouw investering had er geen letter geschreven kunnen worden. Je hebt me alle do's en dont's uitgelegd in de wereld van de epigenetica, het stappenplan bij het schrijven van een helder hoofdstuk en als vrouw heb je mij ook weten te overtuigen dat een lay-out essentieel is. Terugkomend op de gitaar, Jimi Hendrix introduceerde zichzelf tijdens zijn eerste concert in Europa met het doormidden breken van zijn gitaar en het vervolgens in brand steken van zijn schitterend solo-instrument. Dit soort sessies hield ik af en toe ook in jouw Headquarter. Je keek ernaar, je luisterde ernaar en met een symbolische emmer koud water bluste je het vuurtje, waardoor ik onbeschadigd verder kon gaan met mijn wetenschappelijke carrière. Manon, bedankt voor alles.

Een lotgenoot! Net zo goed als moeder Theresa de lotgenoot is van Gandhi, had ik tijdens mijn promotietraject ook een lotgenoot.

Beste dr. Sarah Derks, mijn co-promotor. Sarah, wat ik bedoel met lotgenoot is dat het prettig was te weten dat ik niet de enige idioot was die op een vrije dag een agarose gel stond op te dweilen. Dit nadat deze omslachtig voorbereide gel had besloten na de eerste sequence run, de polonaise te nemen tussen de glasplaten door tot op de grond; terwijl ander vrije collega's verkleed de polonaise liepen op het Vrijthof. Beiden waren we zeer kritisch naar elkaar toe, wat vaak verhitte discussies opleverden die meestal eindigden met: “Ja, ik begrijp wat je bedoelt, maar ik heb gelijk!” Daarnaast konden we als burensamen goed dineren, met Joep en mij als wijn- en whiskydeskundigen: prachtige tijden!

Laat me eindigen te zeggen dat er meer artsen zouden moeten zijn zoals jij, die hun wetenschappelijke ideeën zo plannen, dat ze een klinisch toepasbare relevantie hebben. Ik ga ervan uit dat ik op de hoogte blijf van je carrière en eventueel een Tweede Kamerdebat met je voer! Sarah, bedankt voor de hulp en het fantastische stuk over *CHFR*.

Het lab: Ingrid, bedankt voor het uitleggen van de IHC en cellijn basisprincipes, jij hebt mij mijn eerste labboek gegeven, dat ik kon vullen met allerlei experimenten. De Mark en de Mork, bedankt Margriet en Leentje voor het openstellen van jullie IHC faciliteiten. De eerste *TP53* en *APC* mutatieanalyses, ga zo maar door, een DNA vrije kamer, soep en de ingrediënten. Bedankt Guido, Andrea en Angela. Rik, de verloren zoon,

bedankt voor de HIF-1-alpha oplossing, de pH-waarde van de wasbuffer was zeer essentieel! Peter en Joep, bedankt voor jullie mannelijkheid!

Charlies Angels: Sandra, Kim en Kathleen, mega bedankt voor al die primer sequencies, epjes vol met reagentia, pipetpunten en pipetten. Onderzoek is onmogelijk zonder jullie voortdurende back-up!

Beste Angela, je hebt veel werk uit mijn handen weggenomen en je hebt me uitgelegd wat een logische sample codering is. Ik heb je uitgelegd waar "Der, Die, Das Party-zone is in Maastricht", en welke mannelijke tube "Der, Der, Der Falsche ist!"

Andere lotgenoten: Veerle, Ingrid, Iris, Femke, Coen, Kim (Survival of the Fittest) en Debbie. We zijn allemaal goed terecht gekomen, maar Debbie de meeste dromen zijn bedrog!

Nu aangekomen bij een ander lotgenoot, paranimf en patho-collega: Marjolein Lentjes-Beer. Beste Marjolein, wat heb ik vaak met jou kunnen lachen op de wetenschapskamer. We hadden op vrijdagmiddag ons verdiende you-tube halfuurtje: "Hi my name is Veronique, but recently I changed my name in REFLEX, REFLEX, I like to put the tip of my tong into your earhole, you sick b....", Gunter. En nu op zijn Duits!

Over patho-land gesproken, daar zal ik zeer zeker een aantal zwaargewichten van moeten bedanken. Collega arts, u bent gewaarschuwd: zodra er tegen u gezegd wordt dat uw proefschrift klaar moet zijn voordat de opleiding begint, is het al te laat! Vergelijk het met het medisch anticipatie krediet van de ABN-AMRO na het behalen van uw propedeusediploma!

Het geweldige team uit Venlo, ik kon bij jullie een half jaar van mijn perifere stage lopen. Naast het leren van het vak op zijn pragmatisch en degelijk perifeers, gaven jullie meteen aan dat er tijd was voor onderzoek. De dieselmotor van Venlo, ook wel Siep Wouda genaamd, hield in zijn eentje de fabriek in Venlo draaiende terwijl Adriaan en ik in een afgesloten kamer zonder telefoontjes van de kliniek, werkten aan een wetenschappelijk hoogtepunt. Jij beste Siep, kwam om half vijf met je jas aan polsen hoe het was in wetenschapsland, 80 coupes lichter en 2 obducties rijker verliet je tevreden het pand! Bedankt voor de tijd die jij letterlijk voor mij en Adriaan creëerde. Dat geldt tevens voor het andere teamlid uit Venlo, Ariënne van Marion. Ariënne, bedankt voor de uitleg over het beenmerg, de kleine lymfkliercelletjes en de fles Champagne. Tja, ik zal hem zelf moeten betalen, hopelijk kom je naar de Domkeller in Aken, dan toosten we samen!

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zonder immuno's (Paul Theunissen), "een microscoop is ouderwets", "dicteer wat je ziet en concludeer ondubbelzinnig" (Marius Nap). Ik begrijp alle afkortingen in de gynaecologiewereld: CIN, VIN, VAIN, EIN, CGIN (Nathalie Vandevijver). En ja, ik weet dat Bier belangrijk is, dat de kassa rinkelt na een send rapport en wat het verschil is tussen boven en onder, voor en achter, als je op de aardbol van de prostaat staat (Ruud Clarijs). Jullie alle vier, enorm bedankt voor de goede opleiding in de baas zijn tijd, wat dus inhield dat ik mijn boekje in de weekenden en avonduren eindelijk eens kon gaan afschrijven!

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Arjen Cleven
Aachen, maart 2012

Curriculum vitae

Curriculum vitae

Arjen Cleven was born in Rinteln in Germany on January 13th, 1977. After completing Athenaeum at the Serviam Lyceum in Sittard in 1997, he started studying Biological Health Sciences (1997) and Medicine (2000). He performed research for his master thesis at the department of General Practise, Epidemiology and Statistics at Maastricht University under supervision of Dr. H.E.J.H. Stoffers. In 2002 he received his master degree in Biological Health Sciences on the topic: Prediction of the outcome of peripheral arterial disease using the ankle-brachial pressure index. After his graduation in 2002 he started as a research assistant in the cancer pathology laboratory of Prof. dr. M. van Engeland and Prof. dr. A.P. de Bruijne at the department of Pathology of Maastricht University Medical Center. In 2003 he continued his research and started his Ph.D. program by studying the prognostic implications of hypoxia and promoter CpG island methylation in colorectal cancer, which resulted in this dissertation.

In 2006 he completed Medical School and in 2008 he started his residency training Pathology at the Maastricht University Medical Center, Maastricht.

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