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Epigenetic prognostic biomarkers in colorectal cancer

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The research described in this thesis was performed at the department of Surgery of the Leiden University Medical Center, Leiden, The Netherlands and at the department of Molecular Oncology at the John Waynce Cancer Institute, Santa Monica, CA, United States of America.

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Epigenetic prognostic biomarkers in colorectal cancer

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Chapter 1

General introduction and thesis outline

1

Introduction

Colorectal cancer: treatment and prognosis

Colorectal cancer is one of the most common diagnosed cancers worldwide, and is the second most important cause of cancer mortality in Europe (1). About two thirds of colorectal cancer occurs in the colon, and one third occurs in the rectosigmoid or the rectum. The current staging system for both colon and rectal cancer used in clinical practice is based on the tumor, nodes and metastasis (TNM) staging system (2,3).

For colon cancer, surgery is the primary treatment, with adjuvant chemotherapy given as the standard of care in stage III and high-risk stage IIB colon cancer patients (4). Rectal cancer is also primarily treated by surgery, but in contrast to colon cancer, is associated with a high local recurrence rate. As the rectum is fixed in the smaller pelvis, this provides opportunities for radiation therapy. Even though treatment guidelines are updated regularly, the current staging system and treatment regimens are insufficient and result in both over- and undertreatment of many patients. Patients with the same TNM classification present with large differences in patient survival and tumor recurrence, with for example varying 5-year survival rates of 60-80% for stage II and 30-60% for stage III colon cancer (5). In addition, about 30% of colon cancer patients with TNM stage I or II colon cancer, without nodal involvement at the time of diagnosis, will develop distant metastases (6). The implementation of the total mesorectal excision (TME) surgery technique for rectal cancer, combined with preoperative radiotherapy as investigated in the Dutch multicenter TME clinical trial, has resulted in a reduction in local recurrences of 6% (from 11% to 5%), but without an overall survival benefit (7). This also implicates that the majority of rectal cancer patients (at least 94%) is unnecessarily treated with radiotherapy, which can be associated with comorbidities including sexual dysfunction and fecal and urinary incontinence (8,9). Therefore, there is a need for identification of new biomarkers in colorectal cancer in order to identify high-risk patients and to guide treatment decision-making.

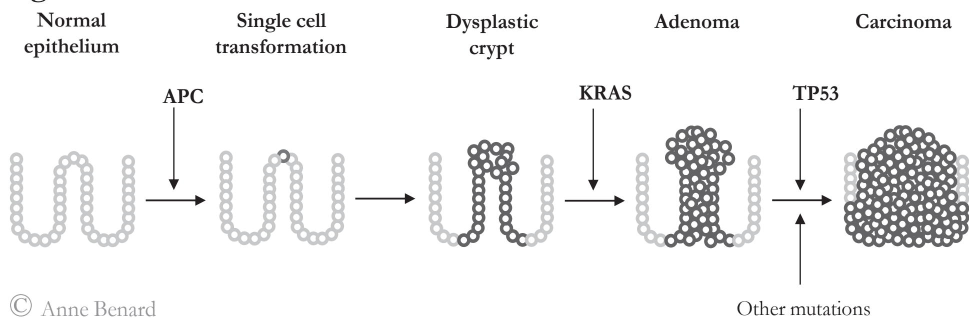
Biomarkers are biological markers that can be measured in for example blood or tumor tissue and can be used as indicators of pathological processes and hence provide information on the likely clinical outcome (prognostic biomarkers) or to measure the response to therapeutic interventions (predictive biomarkers). Many factors have been proposed as clinically prognostic or predictive biomarkers in colorectal cancer. These include measurement of carcinoembryonic antigen (CEA) in blood, determination of MSS (microsatellite stability) status, KRAS mutations, BRAF mutations and p53 mutations, and testing for expression of thymidylate synthase (TS) in colorectal cancer tissues. However, only a few of the numerous proposed prognostic biomarkers have been recommended for clinical use (10). For example, CEA levels in the blood have been approved for postoperative surveillance (although still controversial; 11) and KRAS mutations in tumor cells have been validated as a predictive factor for the response to anti-EGFR therapy in patients with metastatic colorectal cancer (12). At present, insufficient evidence is available for routine implementation of other proposed biomarkers in a clinical setting. Therefore, new clinically prognostic biomarkers are needed in order to better classify patients, to prevent over- and undertreatment, and to advance the field of personalized medicine. Potential new biomarkers can be found in the many pathways involved in tumor development and progression, and can be at the level of DNA (i.e. mutations, single nucleotide polymorphisms, microsatellite instability, copy number changes or translocations), mRNA expression or protein expression. The vast

majority of research in cancer has focused on genetic changes driving tumor development, but in the last decade researchers have also taken an interest in the mechanisms regulating gene expression: epigenetics.

Genetic changes in colorectal cancer – history and current knowledge

The knowledge of the function and changes of the DNA in cancer has increased rapidly over the past century. Already since the 1920s, geneticists advocated the theory that cancer was most likely to originate from "ordinary" cells affected by genetic mutations. In 1953, Nordling proposed a theory that around seven successive mutations that promote cellular division would be necessary for tumor development (13). He also noted that the incidence of cancer seemed to increase with age, which could be explained by the accumulation of mutations, resulting in self-stimulating propagation and ultimately tumor development. In 1971 Knudson posed his well-known "Knudson two-hit hypothesis" based on his findings in retinoblastoma, in which he hypothesized that retinoblastoma is caused by two mutations in the Retinoblastoma (Rb) gene. He proposed that the first mutation would be inherited via germinal cells and was therefore present in all cells in the body. According to this hypothesis, the second mutation would occur in somatic cells in the retina, leaving no functional copies of the Rb gene, which can lead to tumor formation (14). Then, in 1988, just before the start of the Human Genome Project in 1990, Vogelstein proposed a multi-step mutational sequence for colorectal cancer, the adenoma-carcinoma sequence, in which he showed that certain mutations occur early in the carcinogenic process and multiple genetic aberrations accumulate with tumor progression (15). Genome instability– by accumulation of mutational events – expedites the acquisition of capabilities that lead to tumor development, described as the main cancer hallmarks by Hanahan and Weinberg in 2000 (16,17). These capabilities include, among others, evading apoptosis, limitless replicative potential and tissue invasion and metastasis, which allow a somatic cell to transform into a cancer cell. As described in the adenoma-carcinoma sequence by Vogelstein, a multi-step process of successive mutations in tumor suppressor genes takes place during progression from adenoma to carcinoma in the colon (18). New information from genomics and sequencing data has been added since. The most frequently detected mutations in sporadic colorectal cancers are mutations in the adenomatous polyposis coli (APC), KRAS, SMAD2/4 and TP53 genes (Figure 1).

Figure 1



Shown are the various stages of colorectal carcinoma development. Gray cells represent normal epithelial cells, black cells represent transformed cancer cells. Critical mutations occuring at specific stages during adenoma-to-carcinoma transformation are indicated with arrows.

Mutations in the APC gene occur early in tumor development. In patients with hereditary familial adenomatous polyposis (FAP), one of the APC genes harbors a mutation, which predisposes these individuals to development of numerous adenomatous polyps upon a second "hit" (19). Heritable mutations in one of the mismatch repair genes (Lynch syndrome), including MSH2, MSH6 and MLH1 results in microsatellite instability and predisposes the affected individuals to develop colorectal adenocarcinomas at an early age (onset before age 50) (20). Despite the convincing evidence that mutations in the genes mentioned above contribute to tumor development, not all colorectal tumors harbor detectable gene mutations, indicating that other factors than genetic aberrations also play a role in carcinogenesis.

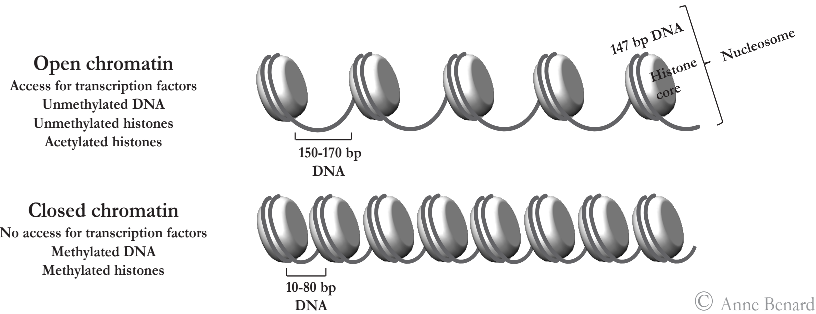
Epigenetic regulation of gene expression

In addition to genetic aberrations, the regulation of gene expression by epigenetic mechanisms has gained interest in the field of cancer research. According to the first definition, as coined by Waddington in 1942, epigenetics involves "the study of the process by which genetic information is translated into the substance and behavior of an organism: specifically, the study of the way in which the expression of heritable traits is modified by environmental influences or other mechanisms without a change to the DNA sequence" (21). In a more contemporary definition, epigenetics refers to the study of heritable changes in an organism by modification of gene expression that are not caused by changes in the underlying DNA sequence. Gene expression is dependent on the local structure of the chromatin, which is the complex of DNA and histone proteins that ensures compaction of the DNA in the cell nucleus. Only an open chromatin structure (euchromatin) allows for transcription factors to bind to gene promoters in order to initiate gene transcription, whereas heterochromatin regions remain densely packed and hence inaccessible for transcription factors (Figure 2). As epigenetic regulation is a dynamic process responsive to environmental stimuli and specific requirements of the cell, these epigenetic mechanisms are attractive targets for anti-cancer therapy, since they are potentially reversible. Driver mutations in epigenetic factors involved in both DNA methylation and histone modifications have been described in several cancers (22,23). Reversion of epigenetic changes might sensitize tumors to other therapeutic agents currently used in the clinic, including chemotherapy and radiotherapy. Epigenetic therapies targeting DNA methylation (24-27) or histone deacetylases (HDACs)(28,29) are currently tested in clinical trials.

DNA methylation

Epigenetic factors that mainly determine chromatin structure are DNA methylation and histone modifications. Although the existence of 5-methylcytosine was already reported in 1948 by Hotchkiss (30), it took several decades to establish its function(s). DNA methylation is involved in many cellular functions, including genomic imprinting of gene regions, X-chromosome inactivation in females, silencing of transcriptionally repressed regions including (peri-) centromeres and telomeres, and regulates gene expression (31). Patterns of DNA methylation are cell-type specific (32,33) and play important roles during embryonic development (34,35). DNA methylation also protects against spontaneous mutagenesis (36). In normal tissue, DNA methylation on CpG dinucleotides in gene promoter regions is usually absent.

Figure 2



Shown are open and closed chromatin structures. In an open chromatin structure, the distance between nucleosomes is about 150-170bp allowing for transcription factors to bind to the linker DNA and initiate transcription. This open chromatin structure is associated with unmethylated DNA and acetylated (and unmethylated) histone proteins. In a closed chromatin structure, nucleosomes are closer together (the distance 10-80bp) and both DNA and histone proteins are methylated, resulting in DNA that is inaccessible for transcription factors.

In contrast, repetitive sequences, generally found outside of gene coding sequences, are methylated in normal tissue. In cancer, aberrant methylation patterns have been observed, with a general hypomethylation of the genome (including repetitive sequences) and local hypermethylation of CpG islands in gene promoter regions (37). These changes in DNA methylation lead to aberrant expression of oncogenes, including APC and MLH1 (38-40), activation of retrotransposon repetitive sequences (41) and genomic instability (42), which can all contribute to the oncogenic transformation of cells. DNA methylation of many genes and non-coding sequences has been described to have prognostic value in cancer (43-46, among others).

Histone modifications

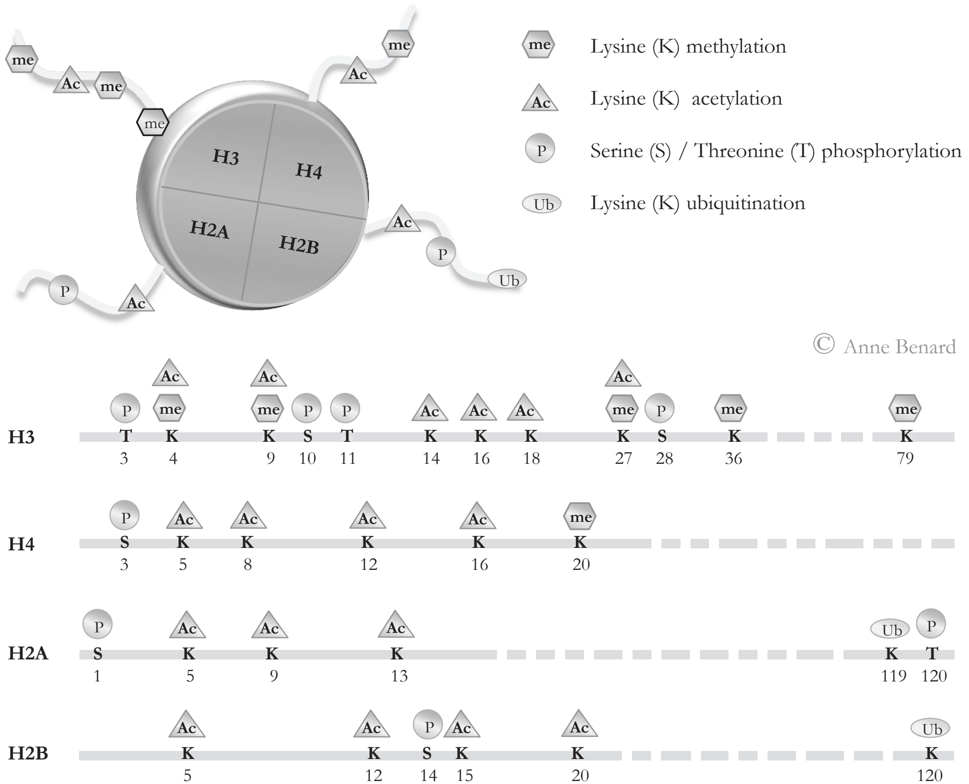
Together with DNA methylation, histone modifications are major factors in determining chromatin structure. Eight histone proteins, in four pairs of histones H2A, H2B, H3 and H4, constitute the core of the nucleosome (Figure 3), around which around 147 basepairs of the DNA are wrapped (47). Protruding histone tails can be modified by acetylation, methylation, phosphorylation and ubiquitination, among others (48). Each of these histone modifications has a specific function (49,50). Generally, histone acetylation is associated with gene activation and an open chromatin structure (Figure 2). Histone methylation can have both gene activating and gene silencing effects, depending on the histone tail residue that is methylated. For example, methylation of lysine residue 4 on histone H3 (H3K4me) is associated with gene activation but methylation of H3K27 is a silencing modification. The different histone modifications are added to or removed from the histone tails by specific histone-modifying enzymes. For example, histone methylases of the Polycomb-group (including EZH2) are responsible for trimethylation of H3K27, and histone demethylase LSD1 is specific for demethylation (removal of methylation) of H3K4me1 and –me2. Histone deacetylases, including HDAC1 and 2 and SIRT1, are responsible for histone deacetylation (removal of acetylation), in the case of SIRT1 preferentially on H3K9 and H4K16.

To add another level of complexity, mono-, di- and trimethylation of lysine residues also have different functions in the cell, on the basis of their position on the histone tails and on different

regions in the genome (51). For example, H3K4me1 is found within transcribed regions, whereas H3K4me2 and H3K4me3 are found in gene promoter regions. Histone core modifications, such as H3K56Ac, regulate the interactions between the histone proteins and DNA (52). Specific histone modifications have different interactions with proteins that specifically bind to certain histone modifications, including DNA binding proteins and chromatin remodelers (53). The complex interplay between the different histone modifications determines the chromatin structure and thereby gene silencing or activation of gene transcription (54,55). In addition, DNA methylation and histone modifications act together during embryonic development and in regulating gene transcription (56,57).

Changes in the expression of histone modifications and histone-modifying enzymes have been implicated in cancer (58,59) and have been shown to have prognostic value in cancer (60-63, among others). These changes in regulatory enzymes and modifications result in altered gene expression patterns, including aberrant expression of oncogenes or silencing of tumor suppressor genes, which could in turn result in enhanced mutagenesis (64).

Figure 3



Shown are histone modifications on the different histone proteins. Histone proteins H3, H4, H2A and H2B all have protruding tails that can be modified by methylation, acetylation, phosphorylation or ubiquitination. Known modification sites with the respective modifications are shown for each of the histone tails (lower part of the figure). Numbers indicate the amino-acid the modification is added to, letters indicate the type of amino-acid (K: lysine, S: serine, T: threonine).

Thesis aim and outline

Thesis aim

The aim of this thesis was to study epigenetic mechanisms, especially DNA methylation and histone modifications, as clinically prognostic biomarkers in colorectal cancer.

Thesis outline

Chapters 2 and 3 describe DNA methylation studies on repetitive sequences and on specific gene promoter regions, respectively. **Chapter 2** describes the prognostic value of DNA methylation of a repetitive retrotransposon sequence, long interspersed element-1 (LINE-1), in rectal cancer tissues from patients enrolled in the Dutch multicenter total mesorectal excision (TME) clinical trial. In **Chapter 3**, DNA methylation was studied on specific apoptosis gene promoter regions in rectal cancer tissues from patients enrolled in the TME trial, using methylation-specific restriction enzymes.

Chapters 4 to 7 are focused on histone modifications in colorectal cancer, both globally and at gene-specific promoter regions. **Chapter 4** shows the prognostic value of nuclear expression of histone deacetylases and correlated acetylated histones in colorectal cancer. In **Chapter 5**, the prognostic value of nuclear expression of Polycomb-group proteins together with their accompanying histone modification H3K27me3 was studied. In **Chapter 6**, histone trimethylation at several histone tail residues was studied in early-stage colon cancer tissues in correlation to patient survival and tumor recurrence. **Chapter 7** reports on the correlation of the transcriptional status of apoptosis genes with sensitivity to treatment regimens including chemotherapy, immunotherapy and radiation.

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Chapter 2

Epigenetic status of LINE-1 predicts clinical outcome in early-stage rectal cancer

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Peter J K Kuppen, Dave S B Hoon

Br J Cancer, Dec 2013; 109(12):3073-3083

2

Abstract

Background: We evaluated the clinical prognostic value of methylation of two non-coding repeat sequences, long interspersed element 1 (LINE-1) and Alu, in rectal tumor tissues. In addition to DNA methylation, expression of histone modifications H3K27me3 and H3K9Ac was studied in this patient cohort.

Methods: LINE-1 and Alu promoter methylation was assessed in DNA extracted from formalin-fixed paraffin-embedded tissues. A pilot (30 tumor and 25 normal tissues) and validation study (189 tumor and 53 normal tissues) were performed. Histone modifications H3K27me3 and H3K9Ac were immunohistochemically stained on tissue microarrays of the study cohort.

Results: In early-stage rectal cancer (TNM stage I-II), hypomethylation of LINE-1 was an independent clinical prognostic factor, showing shorter patient survival (p=0.014; HR 4.6) and a higher chance of tumor recurrence (p=0.001; HR 9.6). Alu methylation did not show any significant correlation with clinical parameters, suggesting an active role of LINE-1 in tumor development. Expression of H3K27me3 (silencing gene expression) and H3K9Ac (activating gene expression) in relation to methylation status of LINE-1 and Alu supported this specific role of LINE-1 methylation.

Conclusion: The epigenetic status of LINE-1, but not of Alu, is prognostic in rectal cancer, indicating an active role for LINE-1 in determining clinical outcome.

Introduction

Under the current TNM (tumor, node, metastasis) staging system (American Joint Committee on Cancer, AJCC (1)) and treatment guidelines (National Cancer Institute, NCI), there is both over- and undertreatment of rectal cancer patients (2). In our search for biomarkers that can complement the current TNM staging system as well as aid in subsequent treatment decisions for rectal cancer patients, we assessed genome-wide DNA methylation using repeat sequences LINE-1 (long interspersed element) and Alu, and global histone modifications in rectal tumor tissues.

Changes in epigenetic mechanisms regulating gene expression, including DNA methylation of CpG dinucleotides, are major factors in tumorigenesis (3). We have previously reported that epigenomic aberrations play a significant role in tumor progression and clinical outcome in rectal cancer (4-6). These studies showed that methylation of non-coding regions can be used as prognostic biomarkers in colorectal cancer. Human DNA contains large numbers of non-coding repeat sequences, the most studied sequences being LINE and Alu repeats. LINE-1 repeat sequences constitute about 17% of the total human genome, are present on most of the chromosomes, and comprise about 50 different subfamilies (7). Alu repeats constitute about 11% of the human genome (8). Because of their abundance in the human genome, methylation of LINE-1 and Alu sequences has been used as surrogate for genome-wide DNA methylation status (9). Hypomethylation of both LINE-1 and Alu sequences has been associated with malignancies (10), including sporadic cases of hemophilia (11) and several types of solid tumors (12-18). In addition to DNA methylation, histone modifications play critical roles in regulating gene expression. Gene expression is dependent on the presence of transcription factors, and mostly on the access of these transcription factors to the transcription start sites. The chromatin structure surrounding the transcription start site, determined by the distance between individual nucleosomes comprised of DNA wrapped around histone octamers, is determined by both DNA methylation and histone modifications and determines accessibility of transcription factors to the DNA. DNA methylated at CpG sites in combination with "silencing" histone modifications, including trimethylation of lysine 27 on histone H3 (H3K27me3), is associated with a closed chromatin structure with limited access for transcription factors to the DNA (illustrated in Rodenhiser *et al.*; 19). Unmethylated DNA in combination with "active" histone modifications, including acetylation of lysine 9 on histone H3 (H3K9Ac) is associated with an open chromatin structure, which permits for gene transcription (19).

Given the recognized de-regulation of these epigenetic mechanisms in cancer (3,20), we investigated whether both global DNA methylation and histone modification patterns can be used to predict clinical outcome in rectal cancer patients enrolled in a well-defined, strictly quality-controlled clinical trial (21).

Materials and methods

Patient selection

Formalin-fixed paraffin-embedded (FFPE) specimens were collected from patients enrolled in the Dutch TME multicenter clinical trial (non-irradiated arm) between 1996 and 1999 with

no evidence of disease after surgical resection and a median follow-up of 7 years. Patient characteristics are displayed in Table 1. Informed consent was obtained from all patients enrolled in the TME trial. The study was approved by the Medical Ethical Committee of the Leiden University Medical Center. Western IRB permission was obtained for assessment of patient specimens at John Wayne Cancer Institute. A pilot study was performed using 30 primary rectal tumor FFPE specimens, TNM tumor stages I (n=10), II (n=12) and III (n=8), and 25 normal rectal tissues resected at least 5 cm away from the tumor. TNM tumor stages were defined according to the American Joint Committee on Cancer, AJCC staging system (1).

For the validation study, a patient selection (n=189) representative for the non-irradiated arm of the TME trial was made based on sex, age at randomization, and circumferential margin involvement, with TNM tumor stages I (n=53), II (n=43), III (n=85) and IV (n=8). 53 additional normal rectal tissues were collected, with 45 out of the 53 cases having matching primary tumor specimens included in the study. Histone modifications were assessed on 132 tumor tissues and 50 normal tissues included in the validation cohort of the LINE-1 study and present on a tissue microarray (described below). This study was performed according to the REMARK guidelines (NCI-EORTC, (22)).

DNA extraction from FFPE specimens and quantification of DNA for methylation assays

Tumor areas on H&E-stained sections of FFPE tumor specimens were identified and marked by a pathologist. From each patient block, two 7μm FFPE sections were deparaffinized and stained with hematoxylin, followed by needle microdissection of tumor areas. DNA was extracted using a proteinase K-based protocol as described previously (23) and quantified using a Quant-iT PicoGreen dsDNA kit (Molecular Probes, Eugene, OR, USA).

Bisulfite conversion and quantitative real-time PCR for methylation assays

For each sample, 200ng of DNA was bisulfite-converted using the Epitect Bisulfite Kit (Qiagen; Valencia, CA); converted DNA was eluted in 50μl elution buffer. Both LINE-1 and Alu PCR-based assays have been described previously (24,25). Quantitative real time PCR for LINE-1 sequences (according to the MIQE guidelines; 26) was performed using universal primers and a locked nucleic acid (LNA) probe specific to the bisulfite-converted Methylated or Unmethylated LINE-1 consensus L1.2 sequence. Primer and probe sequences were as follows: LINE-1 Forward 5'-GGGTTTATTTTATTAGGGAGTGTTAGA-3', LINE-1 Reverse 5'-TCACCCCTTTCTT TAACTCAAA-3', LINE-1 FAM-M-Probe (LNA): 5'-TG+CG+CGAGT+CGAAG-3', LINE-1 FAM-U-Probe (LNA): 5'-TG+TG+TGAG+T+T+GAA+GTAG-3'. Thermal cycling reactions were as follows: hot start for 3 minutes at 95°C, followed by 45 cycles of denaturing at 95°C (15 seconds) and annealing/extension at 60°C (1 minute). For Alu repeat sequences, quantitative real time PCR was performed as described previously (27). Primer and probe sequences specific for the Alu consensus sequence were as follows: Alu Forward: 5'-GTTTGGTTAATATGGTGAAATT-3', Alu Reverse: 5'-ATTCTCCTACCTCAACC-3', Alu FAM-M-Probe (LNA): 5'-A+AC+GCGCGCCAC-3', Alu FAM-U-Probe (LNA): 5'-AAC+AC+A+CACCA+CCA-3'. Thermal cycling reactions were as described for LINE-1, but with annealing/extension at 58°C. Quantitative PCR reactions were run on a 384 CFX

TABLE 1. Patient characteristics of the pilot and validation study groups.

		All patients non-irradiated arm TME trial (n=769)		Pilot study (n=30)		Validation study (n=189)	
		n	**(%)**	**n**	**(%)**	**n**	**(%)**
Sex							
	Male	488	63.5	16	53.3	129	68.3
	Female	281	36.5	14	46.7	60	31.7
Age at randomization							
	Mean	64.4		64.1		63.9	
	Standard error	0.405		2.30		0.811	
TNM stage							
	I	224	29.6	10	33.3	53	28
	II	195	25.8	12	40	43	22.8
	III	281	37.2	8	26.7	85	45
	IV	56	7.4			8	4.2
Circumferential margin							
	Negative	621	80.8	29	96.7	152	80.4
	Positive	148	19.2	1	3.3	37	19.6
Tumor location							
	Rectum	679	90.1	26	86.7	161	85.6
	Anal region	54	7.2	3	10	20	10.6
	Other	21	2.8	1	3.3	7	3.7

Patient characteristics are shown for both the pilot and validation study groups. Patient selection for the pilot study was based on TNM stage. Patient selection for the validation study was based on availability of FFPE tissues and all listed variables, i.e. sex, age at randomization, TNM stage, circumferential margin involvement and tumor location. The validation study selection was representative for the entire non-irradiated patient cohort.

thermal cycler (BioRad, Benicia, CA). A serial dilution of plasmids with either the methylated or the unmethylated sequence was used to generate standard curves for quantification. Triplicates of each sample (20ng DNA per reaction) were run on the same plate, and mean copy numbers (CN) were used for statistical analyses. Measurements were repeated when triplicates varied >2 Cq values. Controls used in both LINE-1 and Alu methylation assays were universally methylated DNA (UMC; Millipore, Billerica, MA), universally unmethylated DNA obtained by repeated whole genome amplification of PBL DNA (UUC; Repli-g kit Qiagen, Valencia, CA), and peripheral blood lymphocytes. Specific methylated cell line controls for LINE-1 methylation assays were melanoma cell line M12 and for Alu methylation assays breast cancer cell line MCF-7. Methylation percentages were calculated as follows: methylation percentage = CN methylated / (CN methylated + CN unmethylated) * 100%.

Immunohistochemical staining and semi-automated scoring of histone modifications

Tumor and normal tissue FFPE blocks with enough tissue available were selected from patients enrolled in the non-irradiated arm of the Dutch TME clinical trial. Tumor cores from 496 patients, and normal tissue cores (taken at least 5cm away from the tumor) from 334 patients enrolled in the Dutch TME clinical trial were included to construct a tissue microarray (TMA). From each donor block, three 1-mm2 tissue cores from strictly tumor areas or one tissue core from normal tissue areas (center) as marked by an experienced pathologist were punched and transferred into a recipient paraffin block using a TMA Master (3DHistech, Budapest, Hungary). 4.5µm tissue sections of the TMAs were cut and processed for immunohistochemical staining. TMA sections were stained using mouse anti-H3K27me3 (dilution 1:200; ab6002, Abcam, Cambridge, UK) or rabbit anti-H3K9Ac (dilution 1:600, ab8898, Abcam, Cambridge, UK), using a standard IHC protocol (28). Briefly, antigen retrieval was performed by heating the sections for 10 min at 95°C in a citrate buffer (pH 6.1; pH low Target Retrieval Solution, Dako, Heverlee, Belgium) after deparaffinization. Endogenous peroxidase was blocked by incubating the sections in a 0.3% solution of hydrogen peroxide (in PBS) for 20 min. Sections were incubated with primary antibodies overnight (16 hrs). Immunohistochemical staining was visualized using the Dako REAL™ EnVision™ Detection System, Peroxidase/DAB+, Rabbit/Mouse (Dako, Heverlee, Belgium). Immunohistochemically stained tissue microarrays were scanned at 20x magnification on the Ariol system (Leica Microsystems, Rijswijk, The Netherlands). Expression of histone modifications was scored using the semi-automated Ariol system. Tumor areas were marked on the computer upon visual inspection, followed by training of the system to correctly identify positively stained and negative nuclei in the tissues, for each of the markers separately. Intensity of the staining was measured by the Ariol system and divided into three categories. The Ariol system was trained to count the number of cells in each category and to calculate a mean intensity score for each tissue core. Patients were divided into high and low intensity groups for each histone modification based on the median intensity score.

Statistical analysis

Statistical analysis of the data was performed in consultation with a biostatistician (H.P.) using statistical software SPSS version 20.0 (PASW Statistics, IBM Statistics, New York). Differences in mean LINE-1 methylation between the different TNM tumor stages were calculated using a Student's t-test (data were normally distributed). Univariate Cox Regression analyses were performed to calculate differences in survival time and tumor recurrence between patients with a high or a low methylation index (LINE-1 and Alu) or staining intensity (histone modifications). Cutoff points for the division of patients into the different patient groups were based on median intensity for the histone modifications and on median methylation index for LINE-1 or Alu. For histone modification analyses, only patients included in the LINE-1 validation study were used (tumors n=132, normal tissues n=63). For each patient, three cores (either three tumor or three normal cores) were scored and mean intensity scores were calculated for each patient. Tumor cores with non-specific staining were excluded from the analyses (n=13). Kaplan-Meier survival curves were made to visualize survival differences between high and low methylation groups (LINE-1) and high and low staining intensity (histone modifications) and statistical significance was assessed using the log-rank test. All time-to-event variables were calculated

from the date of surgery. Competing risk analyses were performed for cancer-specific survival and recurrence analyses in order to prevent overestimation of the number of events in each of the categories. Multivariate analyses using LINE-1 methylation or staining intensity of the histone modifications as continuous data were performed using the Cox proportional hazards model. Covariates entered in the multivariate model were age at time of surgery, circumferential margin, TNM-stage and tumor location. For all analyses, a two-sided p-value of 0.05 or less was considered statistically significant. Data were censored when patients were alive or free of recurrence at their last follow-up date.

Results

Methylation assay verification

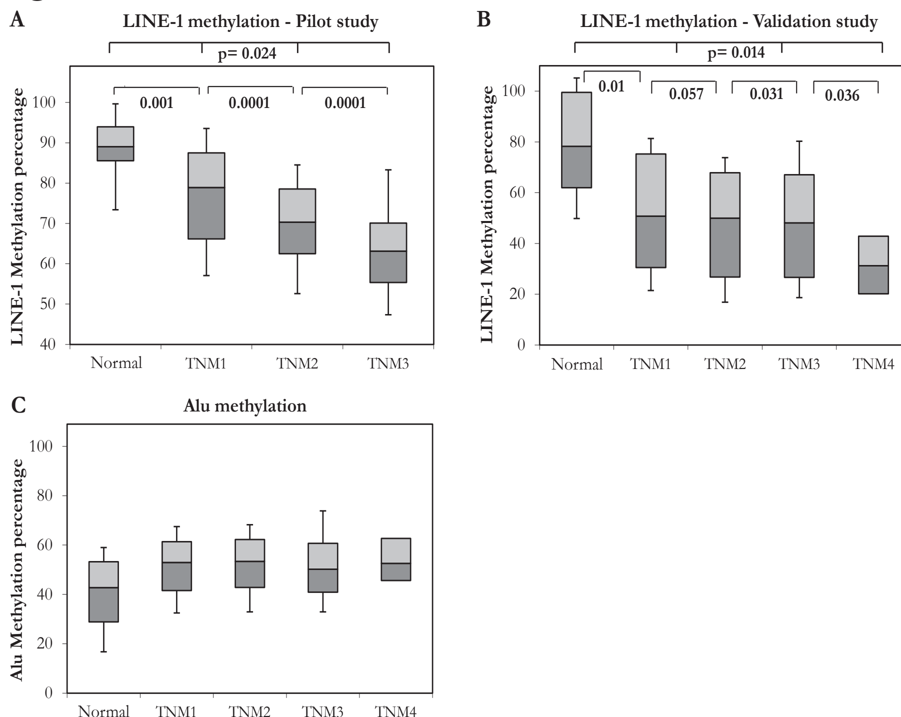
To ensure a good performance and reproducibility of the LINE-1 and Alu methylation assays, we performed several quality checks. To verify the reproducibility of the assays, we ran a test set of 8 patient samples for both Alu and LINE-1 methylation assays, which were independently repeated. Variation in methylation percentages between the two experiments was 0.1-1.6% for LINE-1 and 0.1-1.2% for Alu. Control samples included on every plate showed minimal inter-plate variability (<7%). Standard deviations were 6.6% for UMC and 3.8% for PBL in the LINE-1 methylation assays and 4.3% for MCF-7 and 5.4% for UMC in the Alu methylation assays. The limit of detection of this assay was as low as 100 copies of the respective plasmids in each reaction. Standard curves using plasmids of either the methylated or unmethylated sequences were highly reproducible for all assays, with R^2 values of 0.947 or higher between the duplicates on each of the individual plates. Taken together, the LNA-probe-based real-time PCR method used in this study proved to be very robust and reproducible.

LINE-1 methylation

The pilot study for LINE-1 methylation showed decreasing levels of methylation with increasing TNM tumor stage (Figure 1A). Normal tissues showed the highest LINE-1 methylation percentage as compared to tumor tissues, with an average difference between matching tumor and normal samples (n=8) of 14.2%. Levels of LINE-1 methylation were significantly different across TNM tumor stages and between normal and tumor samples (normal vs T1, p=0.024; across TNM tumor stages, p<0.001).

In the validation study, decreasing levels of methylation were observed with increasing TNM tumor stage (p<0.001) (Figure 1B). The mean difference between the matching normal and tumor samples (45 out of 53) was 21.6%, with a consistently higher methylation percentage in the normal samples. Univariate analyses showed low levels of LINE-1 methylation to be correlated with shorter survival (p=0.006; HR=5.169) and higher chance of distant recurrence (p=0.003; HR=9.943) in stage I and II rectal cancer patients (Table 2). Stage III and IV patients did not show any significant correlation of LINE-1 methylation with survival and recurrence. Subsequent analyses were done with stage I and II patients only. Multivariate analyses of the validation study data showed LINE-1 methylation status to be an independent predictor of survival (p=0.014; HR 4.568) and distant recurrence (p=0.001; HR 9.576) in early-stage rectal

Figure 1



Mean LINE-1 methylation percentages according to TNM tumor stage in rectal cancer patients. *Mean methylation percentages are shown for the different TNM tumor stages. Methylation percentages were calculated as follows: methylated copy number / (methylated copies + unmethylated copies) * 100%.* ***A****. Mean LINE-1 methylation percentages are shown for different TNM tumor stages of the pilot study samples. The different TNM tumor stages are indicated as I, II and III, normal samples are indicated as 'normal'.* ***B****. and* ***C****. Mean LINE-1 and Alu methylation percentages, respectively, are shown for different TNM tumor stages of the validation study samples. The different TNM tumor stages are indicated as I, II, III and IV, normal samples are indicated as 'Normal'.*

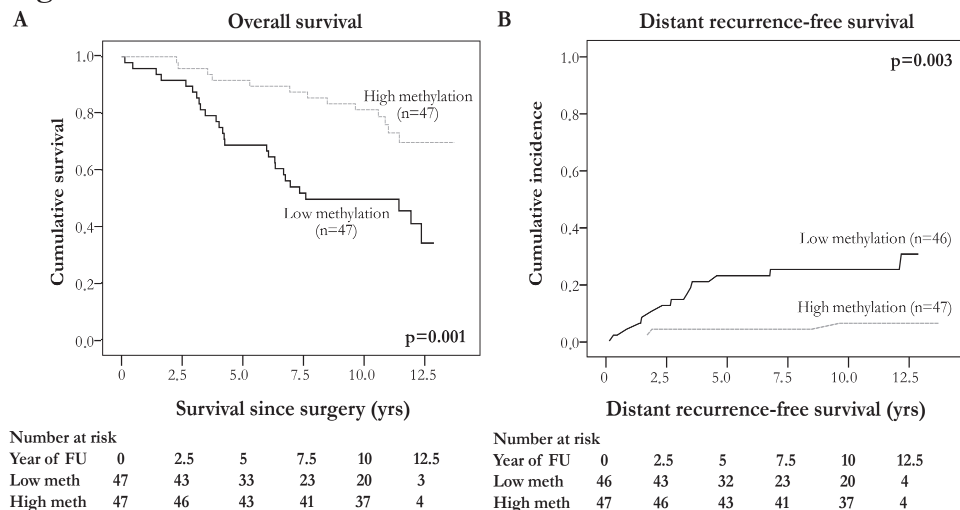
cancer patients (Table 2). Kaplan-Meier survival and cumulative incidence curves were generated (Figures 2A and 2B) to visualize differences in survival and recurrence between high and low LINE-1 methylation groups, based on median methylation percentage (57.4%) of stage I and II patients in the validation study. The log-rank test showed that patients with low LINE-1 methylation have shorter survival times and a higher chance of tumor recurrence than patients with a higher methylation percentage. Significant differences were observed for overall survival (p=0.006), overall recurrence (p=0.0017) and distant recurrence-free survival (p=0.003). No difference was found for local recurrence (p= 0.95), which can be explained by the fact that survival is mainly determined by distant metastases rather than local recurrences. The multivariate analyses showed that patients with a low level of LINE-1 methylation have a 9-fold higher chance of distant recurrence of the tumor and a 5-fold lower chance of survival than patients with a high LINE-1 methylation level (Table 2). No correlation was found between LINE-1 methylation and lymphocyte infiltration (p=0.22), a factor known to impact clinical outcome in colorectal cancer. Our data showed that LINE-1 methylation status was prognostic for disease outcome,

TABLE 2. Univariate and multivariate analyses for both patient survival and tumor recurrence in patients with stage I and II rectal tumors.

	Overall Survival		Local recurrence		Distant recurrence	
	HR (95% CI)	**p-value**	**HR (95% CI)**	**p-value**	**HR (95% CI)**	**p-value**
LINE-1 univariate analysis						
LINE-1 methylation	5.239 (1.589-17.268)	**0.006**	0.892 (0.0.23-34.803)	0.951	9.943 (2.476-30.028)	**0.003**
LINE-1 multivariate analysis						
Age	1.028 (0.997-1.061)	*0.080*	0.931 (0.829-1.045)	0.223	0.957 (0.913-1.003)	*0.068*
Circumferential margin	1.352 (0.380-4.817)	0.642	0.513 (0.164-2.839)	0.962	1.778 (0.196-16.168)	0.609
TNM	1.570 (0.814-3.028)	0.178	0.230 (0.096-1.469)	0.964	1.907 (0.607-5.995)	0.269
Tumor location	1.336 (0.643-2.776)	0.437	0.346 (0.123-1.896)	0.974	4.606 (1.932-10.983)	**0.001**
LINE-1 methylation	4.568 (1.359-15.351)	**0.014**	1.438 (0.14-146.4)	0.877	9.576 (4.443-47.131)	**0.001**
Alu univariate analysis						
Alu methylation	1.269 (0.681-2.361)	0.453	1.093 (0.154-7.763)	0.929	1.897 (0.689-5.227)	0.215
Alu multivariate analysis						
Age	1.391 (1.016-1.904)	**0.039**	0.483 (0.156-1.494)	0.207	0.696 (0.451-1.072)	0.100
Circumferential margin	1.135 (0.316-4.077)	0.846	1.978 (0.699-5.604)	*0.080*	0.735 (0.083-6.500)	0.782
TNM	1.209 (0.697-2.097)	0.500	0.245 (0.034-1.780)	0.165	1.462 (0.573-3.731)	0.427
Tumor location	1.199 (0.579-2.484)	0.625	0.203 (0.029-1.543)	0.989	2.332 (1.046-5.201)	**0.038**
Alu methylation	0.996 (0.979-1.014)	0.690	0.985 (0.928-1.045)	0.613	0.980 (0.952-1.010)	0.185

Survival analyses are shown for stage I and II patients only. The total number of patients included in the analyses was n=96. Hazard ratios are displayed for both Cox proportional hazard univariate and multivariate analyses and 95% confidence intervals are given for LINE-1 methylation. In multivariate analyses, age was entered as a continuous variable. For circumferential margin the 'negative' group was used as reference, for TNM the stage I group was used as reference. Tumor location was divided into the categories rectum, anal region, or other, using rectum as the reference category. Statistically ignificant values are indicated in ***bold****, trends in Italic.*

Figure 2



Number at risk

Year of FU	0	2.5	5	7.5	10	12.5
Low meth	47	43	33	23	20	3
High meth	47	46	43	41	37	4

Number at risk

Year of FU	0	2.5	5	7.5	10	12.5
Low meth	46	43	32	23	20	4
High meth	47	46	43	41	37	4

Survival curves of stage I and II rectal cancer patients by LINE-1 methylation status. *Kaplan-Meier curves were made to visualize differences in survival and recurrence between patients with high and low LINE-1 methylation status. Log-rank tests were performed to calculate the difference between the low and high LINE-1 methylation groups. Survival times were calculated as time from surgery till an event (death or recurrence, respectively).* ***A****. Kaplan-Meier curves showing overall survival of the high and low methylation groups based on LINE-1 methylation status. Patients at risk are displayed for each of the groups.* ***B****. Cumulative incidence curves showing distant recurrence in the high and low methylation groups based on LINE-1 methylation status. Patients at risk are displayed for each of the groups.*

but Alu methylation was not. Chi-square (p=0.38) and Pearson's correlation (p= -0.306) analyses confirmed that Alu methylation and LINE-1 methylation indeed did not correlate. Similarly, in multivariate analyses, the interaction between Alu and LINE-1 methylation did not show significant correlation with survival (p=0.23). This confirmed that Alu and LINE-1 methylation did not have a similar effect on patient survival and tumor recurrence in this set of rectal cancer patients, suggesting that LINE-1 may be involved in specific tumor progression events that affect disease outcome in rectal cancer, rather than reflecting a genome-wide methylation status.

Alu methylation

To investigate if LINE-1 methylation did indeed represent genome-wide methylation status, we studied methylation of Alu repeat sequences in the same patient cohort. The levels of Alu methylation were not significantly different between normal and tumor tissues (pilot study; p=0.24) or between patients with or without a recurrence (pilot study; p=0.27). Alu methylation levels did not differ between the different TNM tumor stages in either the pilot (p=0.11) or the validation study (p=0.73; Figure 1C). The mean difference in Alu methylation percentage between normal and matching tumor tissues was 1.03% in the validation series.

Survival analyses on the data in the validation series did not show any significant differences using a cutoff based on median Alu methylation percentage (60%). Neither overall survival nor overall recurrence did show differences between the groups (p=0.65 and p=0.31, respectively).

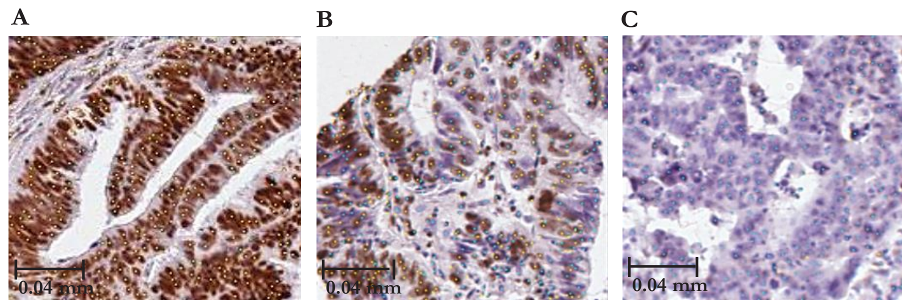
Selection of stage I and II patients only, as described for the LINE-1 methylation studies, did not change outcomes of either univariate or multivariate survival analyses (Table 2).

Histone modifications

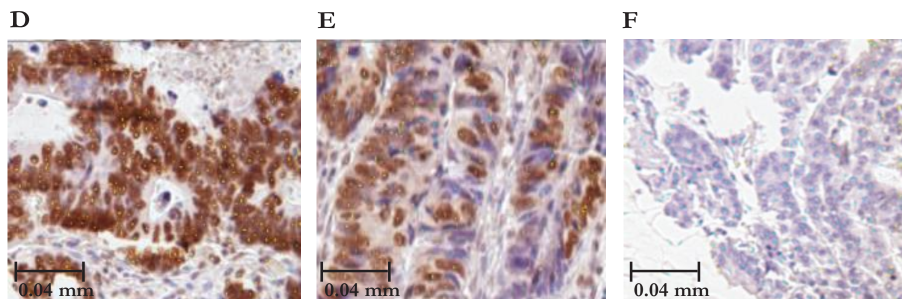
Histone modifications H3K27me3 and H3K9Ac were assessed by IHC on TMA sections. Only patients included in the LINE-1 validation study were used for histone modification analyses (tumors n=132, normal tissues n=63). Semi-automated analyses using the Ariol System yielded intensity scores for each of the individual tumor cores. Correct identification of positive and negative cells by the Ariol system is shown in Figure 3. Mean intensity scores of three tumor tissue cores were calculated for each patient and used for survival analyses. Patients were divided into high and low intensity groups for each histone modification based on the median intensity score. H3K27me3 intensity scores ranged from 0 - 63.14, H3K9Ac intensity scores ranged from 0 - 67.84. Median intensity scores were 50.59 in normal tissues and 48.20 in tumor tissues for H3K9Ac ($p=0.053$) and 49.41 in normal tissues and 51.77 in tumor tissues for H3K27me3 ($p=0.002$). H3K9Ac and H3K27me3 intensities in tumor tissues showed an inverse correlation, with $p=0.024$.

Figure 3

H3K9Ac



H3K27me3



Identification of positively stained and negative tumor cells by the Ariol system. *The Ariol system trainer overlay shows correct identification of positive (yellow dots) and negative (blue dots) cells on tumor cores. TMA slides were scanned using a 20x magnification. Shown for both H3K9Ac and H3K27me3 are highly positive tumor cores (**A** and **D**), tumor cores with both positive and negative cells (**B** and **E**) and negative tumor cores (**C** and **F**). The Ariol system was trained to identify positive and negative cells for each individual marker.*

TABLE 3. Univariate and multivariate analyses for histone modifications in rectal cancer patients.

	H3K27me3		H3K27me3		H3K9Ac	
	LINE-1 methylation low		*LINE-1 methylation low*		*LINE-1 methylation high*	
	Overall Survival		Local recurrence-free survival		Local recurrence-free survival	
	HR (95% CI)	**p-value**	**HR (95% CI)**	**p-value**	**HR (95% CI)**	**p-value**
Univariate analysis						
Intensity histone modification staining	0.461 (0.236-0.901)	**0.024**	0.118 (0.014-0.960)	**0.046**	1.066 (0.974-1.261)	0.163
Multivariate analysis						
Age	1.070 (1.030-1.113)	**0.001**	1.044 (0.948-1.134)	0.274	1.012 (0.937-1.088)	0.739
Circumferential margin	2.003 (0.696-5.768)	0.198	1.187 (0.845-6.775)	0.852	1.150 (0.912-1.136)	0.309
TNM	3.826 (1.034-5.082)	**0.000**	9.846 (0.364-20.682)	0.159	0.318 (0.116-1.863)	0.582
Tumor location	3.329 (0.782-5.552)	0.115	0.249 (0.036-0.905)	0.968	0.415 (0.051-3.915)	0.979
Intensity histone modification staining	0.249 (0.107-0.582)	**0.001**	0.067 (0.007-0.599)	**0.016**	1.069 (0.973-1.274)	0.235

Survival analyses are shown for H3K27me3 and H3K9Ac staining intensity. The total number of patients included in the analyses was n=63 for H3K27me3 analyses and n=61 for H3K9Ac analyses, corresponding to LINE-1 low and high methylation categories, respectively. Hazard ratios are displayed for both Cox proportional hazard univariate and multivariate analyses and 95% confidence intervals are given for the histone modification intensity entered as a continuous variable. In multivariate analyses, age was entered as a continuous variable. For circumferential margin the 'negative' group was used as reference, for TNM stage I was used as reference. Tumor location is divided into the categories rectum, anal region, or other, using rectum as the reference category. Statistically ignificant values are indicated in ***bold****.*

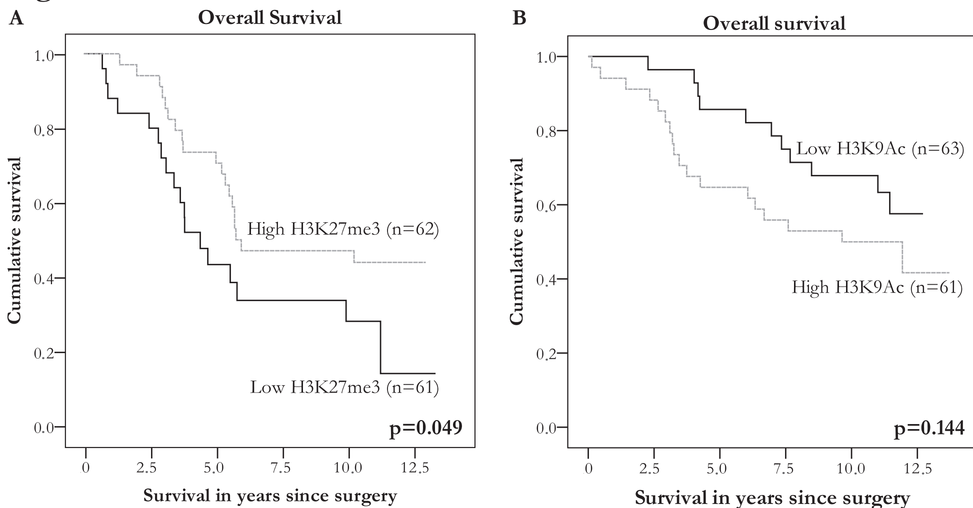
Both H3K27me3 (p=0.049) and H3K9Ac (p=0.14) show differences in overall survival between high and low intensity groups (Figures 4A and 4B, respectively). For H3K27me3, low intensity scores are correlated with worse overall survival, whereas for H3K9Ac, high intensity is correlated with worse overall survival. As for tumor recurrence, histone modification intensities were correlated with local recurrence with H3K27me3 (p=0.001) and H3K9Ac (p=0.084), but not with distant recurrence with H3K27me3 (p=0.172) and H3K9Ac (p=0.291). Multivariate analyses including LINE-1 methylation and histone modification intensities as continuous variables showed that LINE-1 methylation is a dominant factor in determining clinical outcome, as adding histone modification intensities did not improve the multivariate model. LINE-1

methylation showed correlation with overall survival (with addition of H3K27me3 p=0.062 and H3K9Ac p=0.15) and distant recurrence-free survival (with addition of H3K27me3 p=0.019 and H3K9Ac p=0.018), but not with local recurrence-free survival (with addition of H3K27me3 p=0.82 and H3K9Ac p=0.97).

In order to further study the relationship between LINE-1 methylation and histone modifications, we stratified histone modifications according to LINE-1 methylation levels. H3K27me3 intensity was found to significantly correlate with overall survival (p=0.020) and local recurrence-free survival (p=0.017) only when LINE-1 methylation was low (Figures 5A and 5C, respectively). Within this patient subset with low LINE-1 methylation, patients with high H3K27me3 intensity scores show better survival rates than patients with low presence of H3K27me3. High H3K27 methylation could have a "protective" function in the cells, preventing deregulated gene expression when DNA methylation is absent. H3K27me3 did not show any differences between high and low intensity groups when LINE-1 methylation was high, with overall survival (p=0.37) and local recurrence-free survival (p=0.28), again indicating that LINE-1 methylation is a dominant factor determining clinical outcome in this patient group. Uni- and multivariate analyses for H3K27me3 also showed statistically significant differences between high and low staining intensity groups when LINE-1 methylation was low, both for overall survival (univariate p=0.024, multivariate p=0.001) and for local recurrence-free survival (univariate p=0.046, multivariate p=0.016) (Table 3). Stratifying H3K27me3 intensity scores according to Alu methylation status did not show any significant differences (Figures 5B and 5D).

H3K9Ac intensity correlated with local recurrence-free survival (p=0.030) only when LINE-1 methylation was high (Figure 5E). The presence of H3K9 acetylation in combination with high

Figure 4



Survival curves of histone modifications H3K27me3 and H3K9Ac by expression levels. *Survival curves were made to visualize differences in survival and recurrence between patients with high and low expression of H3K27me3 or H3K9Ac. Log-rank tests were performed to calculate the difference between the low and high H3K27me3 expression groups. Survival times were calculated as time from surgery till an event (death).* ***A****. Cumulative incidence curves showing overall survival of the high and low methylation groups based on H3K27me3 expression.* ***B.*** *Cumulative incidence curves showing overall survival in the high and low methylation groups based on H3K9Ac expression.*

Figure 5

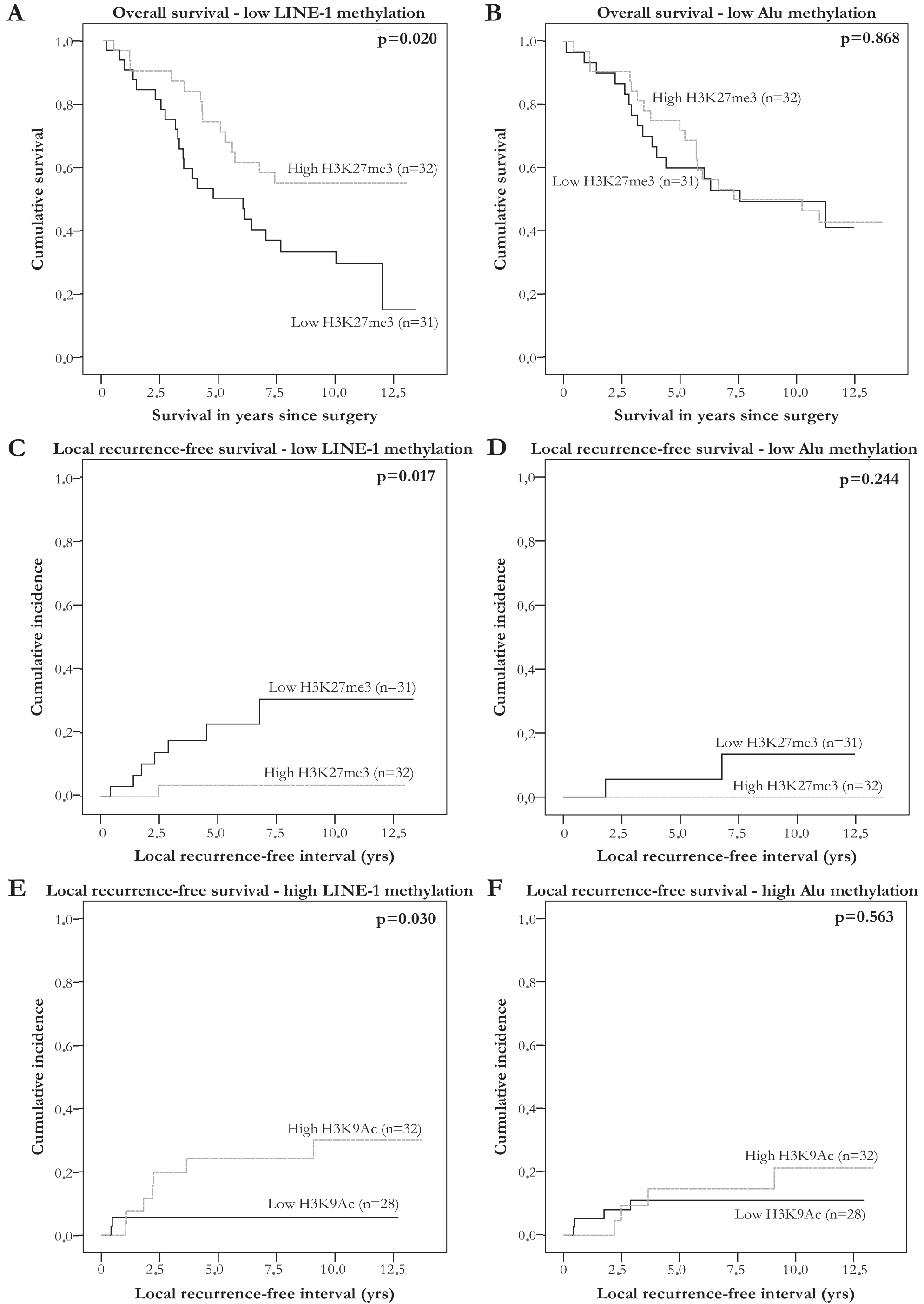


Figure 5 (see previous page) Survival curves of histone modifications H3K27me3 and H3K9Ac according to LINE-1 or Alu methylation levels. *Survival curves were made to visualize differences in survival and recurrence between patients with high and low expression of H3K27me3 and H3K9Ac. Log-rank tests were performed to calculate the difference between the low and high H3K27me3 or H3K9Ac expression groups, respectively. Survival times were calculated as time from surgery till an event (death or recurrence).* ***A****. Overall survival based on H3K27me3 expression in the LINE-1 methylation low group.* ***B****. Overall survival based on H3K27me3 expression in the Alu methylation low group.* ***C****. Local recurrence-free survival based on H3K27me3 expression in the LINE-1 methylation low group.* ***D****. Local recurrence-free survival based on H3K27me3 expression in the Alu methylation low group.* ***E****. Local recurrence-free survival based on H3K9Ac expression in the LINE-1 methylation high group.* ***F****. Local recurrence-free survival based on H3K9Ac expression in the Alu methylation high group.*

levels of DNA methylation could affect disease outcome through activation of aberrant gene expression. H3K9Ac did not show any differences between high and low intensity groups when LINE-1 methylation was low, with overall survival (p=0.83) and local recurrence-free survival (p=0.68). Univariate analyses showed a trend for H3K9Ac, when LINE-1 methylation is high, with local recurrence-free survival (p=0.066) (Table3). Unfortunately, in multivariate analyses, this trend was no longer observed. H3K9Ac did not show a significant correlation with overall survival in either uni- or multivariate analyses. Stratifying H3K9Ac intensity according to Alu methylation status did not show any significant differences (Figure 5F).

Discussion

The results presented in this paper showed that LINE-1 methylation should be regarded as an independent prognostic biomarker in early-stage rectal cancer. LINE-1 and Alu methylation were assessed using quantitative PCR after needle microdissection of tumor tissue areas, which were carefully marked by an experienced pathologist. Tumor tissue extracted using needle microdissection, in contrast to laser capture microdissection, may include more stromal tissue, including inflammatory cells. We therefore selected only tumor areas with >80% tumor epithelial cells, thereby minimizing the amount of stroma included in these assays. Although previous reports showed an association of lymphocytic infiltrates with clinical outcome (29) in colorectal cancer, in our rectal cancer study cohort we did not find a correlation between the amount of lymphocytic infiltrate and LINE-1 methylation that could influence our analyses. Therefore, it is not likely that our findings are influenced by stroma or infiltrating lymphocytes. In addition, Irahara *et al.* have shown, by direct comparison, that LINE-1 methylation levels are comparable between needle microdissected and laser capture microdissected tumor tissues, indicating that the effect of contaminating cells on LINE-1 methylation is indeed minimal (30). BRAF mutation and MSI status were not determined in this study cohort, as the prevalence of BRAF mutations (around 2%; 31,32) and MSI (around 2%; 33) is very low in rectal cancer. The impact of these markers on clinical outcome in multivariate analyses would therefore be negligible.

Because we were looking for a rapid, robust, high-throughput and highly sensitive assay, we chose to use a quantitative real-time PCR assay instead of pyrosequencing to measure LINE-1 methylation. Pyrosequencing relies on PCR amplification of the region of interest, followed by sequencing of this PCR-amplified product. According to Nelson *et al.* (34) and Irahara *et al.* (30) pyrosequencing of LINE-1 sequences typically includes 3-6 CpG sites at the 5' end of LINE-1 sequences, which is comparable to the quantitative real-time PCR assay conducted in this study. Also, as suggested in Figure 1 of the pyrosequencing analysis by Irahara *et al.*, the methylation percentages of the individual CpG sites measured with the LINE-1 pyrosequencing assay seem

very similar within one sample. Therefore, measuring the three CpG sites at once using the qPCR method, instead of measuring individual CpG sites by pyrosequencing, is representative. In addition, for survival analyses conducted in this study, the median methylation percentage was used to divide the patients into high- and low-risk groups. As described by Irahara *et al.*, the average of the relative amounts of methylated cytosines found using pyrosequencing was used as overall LINE-1 methylation level in each sample, which is a similar approach as we used with our qPCR data. Pyrosequencing information would probably result in a lower median methylation percentage, as hemimethylated CpGs can be accurately measured. However, this would yield a lower LINE-1 methylation percentage for every patient in our cohort and we expect this will not affect the distribution of the patients into the two groups based on the median LINE-1 methylation percentage. Therefore, we are confident the qPCR method used in this study was a valid method to study LINE-1 methylation.

In our study cohort, stage III and IV patients did not show a significant correlation of LINE-1 methylation with survival and recurrence, most likely because other clinical factors such as nodal status and circumferential resection margin involvement have a significant role in advanced stage cancers, overpowering the effect of an earlier event in tumorigenesis. Epigenomic aberrations in tumors of stage I and II patients without invasive tumor characteristics, however, may be associated with the development of aggressive tumors and have clinical relevance for risk stratification. LINE-1 methylation has been described as an early-stage tumor marker in previous studies (18,35).

Methylation of LINE-1 and Alu repeats have both been used as surrogate markers for genome-wide methylation status (9). Based on the general hypothesis that tumorigenesis is associated with genome-wide DNA hypomethylation and locus-specific hypermethylation of individual CpG islands (15,36), we expected to see a decrease in both Alu and LINE-1 methylation levels with increasing TNM tumor stage in rectal cancer tissues. However, as we showed here that Alu methylation, in contrast with LINE-1, did not change with tumor progression and that methylation levels of LINE-1 and Alu did not correlate, our results suggest a more specific effect of LINE-1 methylation in early rectal tumors instead of reflecting a genome-wide methylation status. Other studies also suggest a specific role for LINE-1 methylation in tumorigenesis, while other repetitive sequences such as Alu repeats remain equally methylated (37). Active LINE-1 sequences, an estimated 30-60 copies per cell, have retrotranspositional activity (38) and can relocate to other (non-)coding regions (7,13,39), thereby contributing to (epi)genetic variation (40,41). In normal cells, retrotransposition of repeat sequence elements is repressed by methylation of cytosine residues (42). Reintegration of LINE-1 retrotransposons can disrupt genes and regulate their expression (43,44), as described for APC in colon cancer (16). Using the genome vicinity information of the full-length active LINE-1 sequences provided in the L1base database (Max-Planck Institute Berlin and University of Würzburg; 45), we found that several LINE-1 sequences are located near or in (intronic) gene regions. As neighbouring DNA methylation patterns can influence nearby promoter regions (46,47) or longer stretches of DNA, up to 2kb distant (48), demethylation of LINE-1 sequences might therefore represent demethylation events of the coding gene promoters in their vicinity and hence influence expression of genes in these regions, translating into worse clinical outcome for the patient. This hypothesis was further supported by Hur *et al.*, who show that specific LINE-1 sequences residing in intronic regions of several proto-oncogenes were hypomethylated in metastatic tissues compared to the

corresponding primary tumors (49), indicating that differentially methylated LINE-1 sequences can indeed influence progression of colorectal tumors.

Recent articles by Goel *et al.* and Ogino *et al.* describe that LINE-1 hypomethylation is correlated with shorter survival, independent of clinical or molecular features, in non-MSI (microsatellite instability) familial tumors (50,51). Mechanisms predisposing patients with a family history of (and thus early-onset) colorectal cancer, but with no evidence of MSI or other molecular features, for the development of a tumor are still unknown. The authors of both articles conclude that LINE-1 might be an important factor promoting tumor growth in familial cases of (MSS) colorectal cancers. In our study, consisting of MSS rectal cancer patients, we also show a significant prognostic role for LINE-1 hypomethylation in determining clinical outcome. Together, these studies suggest that LINE-1 methylation might represent a frequently deregulated regulatory epigenetic mechanism that might promote primary tumor progression and metastasis of rectal tumors. Hence, LINE-1 methylation may potentially serve as a new biomarker to identify high-risk patients. Future studies will have to be performed to validate the clinical utility of LINE-1 in rectal cancer.

The histone modifications reported in this study support the clinical impact of genome-wide changes in epigenetic modifications in rectal cancer patients. On the basis of our current knowledge about the interplay between histone modifications and DNA methylation, we hypothesized that silencing modification H3K27me3 should follow the same pattern as LINE-1 methylation, as both epigenetic mechanisms are involved in silencing of gene expression. Indeed, low expression of H3K27me3 was associated with worse survival. The finding that H3K27me3 was associated with clinical outcome only when LINE-1 methylation is low, supports the generally accepted hypothesis that DNA methylation and histone modifications together control gene expression and thereby define the cellular phenotype. Activating histone modification H3K9Ac was expected to show opposite results, as the presence of this histone modification will lead to (aberrant) activation of gene expression. Indeed, high expression of H3K9Ac was associated with a shorter survival and higher chance of recurrence compared to patients with low H3K9Ac expression. As both H3K27me3 and H3K9Ac expression only correlate with LINE-1 methylation and not with Alu methylation, we again conclude that LINE-1 methylation may be involved in specific tumor progression events in rectal cancer, rather than reflecting genome-wide methylation status in these tumors. Histone modifications H3K27me3 and H3K9Ac intensity scores can further subdivide rectal cancer patients into high- or low risk groups when stratified according to LINE-1 methylation status. This indicates that LINE-1 methylation and histone modifications work closely together in determining gene expression and hence tumor progression and ultimately clinical outcome.

In conclusion, we have shown in this study that LINE-1 methylation is an independent predictor of survival and recurrence in early-stage rectal cancer. Expression of histone modifications H3K9Ac and H3K27me3 further supports an active role in rectal cancer progression of LINE-1. Further research should be conducted to investigate the exact function of LINE-1 elements, and their influence on neighbouring genes, in order to better understand the complex nature of the rectal tumorigenic process. We show here that methylation of LINE-1 repeat sequences can be used as a biomarker to distinguish rectal cancer patients with a high risk of distant recurrence at an early stage, and therefore, has potential to complement the current TNM staging system.

2

Acknowledgements

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Chapter 3

DNA methylation of apoptosis genes in rectal cancer predicts patient survival and tumor recurrence

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Abstract

Deregulation of the apoptotic pathway, one of the hallmarks of tumor growth and -progression, has been shown to have prognostic value for tumor recurrence in rectal cancer. In order to develop clinically relevant biomarkers, we studied the methylation status of promoter regions of key apoptosis genes in rectal cancer patients, using methylation-sensitive restriction enzymes. DNA was extracted from fresh-frozen tumor tissues of 49 stage I-III rectal cancer patients and 10 normal rectal tissues. The results of this pilot study were validated in 88 stage III tumor tissues and 18 normal rectal tissues. We found that methylation of the intrinsic apoptotic pathway genes Apaf1, Bcl2 and p53 correlated with the apoptotic status (M30) of the tumor. Combined survival analyses of these three genes, on the basis of the number of genes showing high methylation (all low, 1 high, 2 high or all high), showed shorter patient survival and recurrence-free periods with an increasing number of methylated markers. Multivariate analyses showed significant differences for overall survival (p=0.01; HR=0.28 (0.09-0.83)), cancer-specific survival (p=0.004; HR=0.13 (0.03-0.67)) and distant recurrence-free survival (p=0.001; HR=0.22 (0.05-0.94)). The shortest survival was observed for patients showing low methylation of all markers, which – as was expected - correlated with high apoptosis (M30), but also with high proliferation (Ki-67). The study of epigenetic regulation of apoptosis genes provides more insight in the tumorigenic process in rectal cancer and might be helpful in further refining treatment regimens for individual patients.

Introduction

Apoptosis is one of the major pathways frequently deregulated in cancer (1-3). Deregulation of this pathway provides the tumor cell with a survival advantage and thereby promotes tumor growth and -progression. The apoptotic process is complicated and can be activated by stress or damage to the cell (intrinsic pathway), or by external factors (initiation by the immune system; extrinsic pathway), with both pathways converging at the level of the caspase cascade. This cascade eventually leads to cleavage of key proteins for cell structure and -function, causing fragmentation of the DNA, membrane blebbing and ultimately removal of the destructed cell by macrophages (4). Previous studies have demonstrated that high levels of apoptosis in rectal cancer specimens correlated with low levels of local tumor recurrence (5-7). A malfunctioning apoptotic pathway could also explain a poor response to anti-cancer treatment strategies such as pre-operative radiotherapy (RT).

Current treatment regimens of rectal cancer patients include radical removal of the primary tumor including all regional tumor cell deposits according to the Total Mesorectal Excision (TME) technique. In addition to TME surgery, the majority of stage I and II patients and all stage III patients receive pre-operative RT in order to reduce the local recurrence rate (8), on the basis of data provided by several large randomized clinical trials (9-11). Unfortunately, treating all rectal cancer patients with pre-operative RT results in overtreatment of many individuals, as only a small number of patients - those who would develop a local recurrence - potentially benefit from this treatment and not all of these patients will respond to the therapy. Most of the patients treated with pre-operative RT will suffer from the side-effects such as increased risks of poor anal and sexual function, small bowel toxicity with obstruction and secondary malignancies (12-14).

The goal of this study was to investigate the regulation of the apoptotic pathway through DNA methylation, in order to better understand the biological processes underlying tumor growth and -progression in rectal cancer. Epigenetic mechanisms, responsible for regulation of gene transcription, have been shown to be deregulated in many cancers (15-18), thereby altering the expression levels of many genes in tumor cells. We aim to develop biomarkers that will assist in treatment decisions in rectal cancer patients. For this study we chose to focus on the apoptosis genes Apaf1, Bcl2, p53, Fas (CD95), and TrailR2, as a review of the current literature indicated these apoptotic proteins to have prognostic value in cancer (19-27). We hypothesized that methylation of the promoter region of these genes would represent deregulation of the apoptotic pathway and, therefore, would correlate with patient survival and tumor recurrence in rectal cancer. Methylation assays were performed on DNA extracted from frozen tumor tissues of patients enrolled in the Dutch Total Mesorectal Excision (TME) trial (28,29) using a methylation-sensitive restriction enzyme-based approach (30). We also assessed tumor cell proliferation status in this study, as a delicate balance may exist between apoptosis and proliferation in determining clinical outcome.

Materials and methods

Patient selection

Patients were selected from the study population of the non-irradiated (TME surgery only) arm

of the Dutch TME trial (28,29). We selected patients with no evidence of disease after surgical resection of the tumor and of whom frozen tissue blocks were available (n=137) (28,29,31). Samples were collected between 1996 and 1999 and stored at -80ºC. The median follow-up time in this study cohort was 6 years. Trial eligibility criteria and follow-up protocols have been described previously (31-33). Informed consent for the use of tumor specimens was obtained from all patients enrolled in the TME trial and the study has been approved by the Medical Ethical Committee of the Leiden University Medical Center. For the pilot study, frozen tumor tissues of 49 patients with stage I, II, or III rectal cancer were collected. In addition, normal colorectal tissues, taken at least 5 cm away from the tumor, were collected from 10 patients included in this study. The validation study consisted of a set of 88 frozen tumor tissues of patients with stage III rectal cancer of whom sufficient frozen tissue was available, and 18 normal rectal tissue samples. Clinicopathological parameters of all patients included in this study have been summarized in Table 1. This study was performed according to the REMARK criteria (34).

DNA extraction and enzyme digestion

DNA was extracted from frozen tissues using a Trizol-based protocol according to manufacturer's recommendations (Life Technologies Corp, Bleiswijk, the Netherlands). DNA was dissolved in Tris-EDTA buffer pH 8.0 and quantity was measured using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE). To analyze the methylation status of specific promoter regions, we used methylation-sensitive restriction isoschizomers *MspI* (New England Biolabs, Ipswich, MA; R0106L) and *HpaII* (New England Biolabs, Ipswich, MA; R0171L). The isoschizomeric restriction enzymes share restriction site C^CGG but whereas *MspI* cuts the DNA irrespective of DNA methylation, *HpaII* is blocked by methyl groups at CpG dinucleotides. Reactions were optimized according to the manufacturer's procotol (New England Biolabs, protocol "Optimizing restriction endonuclease reactions"). 250 ng of DNA was used for each digestion reaction. Per reaction, 25 units of *HpaII* and 100 units of *MspI* were used in combination with their respective buffers (5µl) in a total reaction volume of 50 µl. Mock digestions were included for every sample, substituting the restriction enzymes with 5 µl 50% glycerol. Overnight incubation for 16 hours at 37°C of all reaction mixtures was followed by heat inactivation for 20 min at 65°C, and subsequent cooling down of the samples to 4°C. Incubation times and conditions were optimized using the active (non-methylated) housekeeping gene β2-microglobulin (B2m) and the silenced (methylated) gene Myogenic differentiation 1 (MYOD1) that is only activated in muscle tissue. Methylated (universally methylated DNA, UMC; Millipore, Billerica, MA) and unmethylated (DNA from peripheral blood leukocytes, PBL, 5 different patients) controls were included in every digestion assay and on every PCR plate. We verified for every digestion procedure that >75% of the DNA participated in the digestion reactions, measured as Ct>2 between the *MspI*- and mock-treated samples, and that the PCR product after digestion with *HpaII* was less than the product in the mock-treated samples.

Real-time PCR

PCR was performed in duplicate for apoptosis markers Apaf1, Bcl2, TrailR2, p53 and Fas (CD95) using genomic DNA primers surrounding (but not including) at least two enzyme restriction sites per amplicon. PCR was performed using 20 ng DNA, 2 pmol/µl primers

and PerfeCTa® SYBR® Green SuperMix for iQ™ (Quanta Biosciences, Gaithersburg, MD, USA) in a final volume of 10 µl. Melting curves were used to ensure a single PCR product for each of the markers, and PCR products were run on a 1% agarose gel to ensure for correct size of the products. Quantitative PCR reactions were run on a 96 well CFX thermal cycler (BioRad, Benicia, CA). Methylated (UMC) and unmethylated controls (DNA from PBL) were included on every PCR plate. Thermal cycling reactions were as follows: hotstart for 3 min at 95°C, followed by 40 cycles of denaturing for 10 sec at 95°C and annealing/extension for 30 sec at the optimal melting temperatures for each primer set, as indicated below. Primer sequences and melting temperatures per primer set were as follows: Apaf1 Forward 5'- TTGACTGCTCCGCTGTC -3'; Apaf1 Reverse 5'- TCCCCACCTCTGGTTCT -3' (Tm 63°C); Fas Forward 5'- CCAACTTCCCAGGTTGAA -3'; Fas Reverse 5'- GCACAAATGGGCATTCC -3' (Tm 63°C); p53 Forward 5'- GTATCTACGGCACCAGGTC -3'; p53 Reverse 5'- CATGACAAGTAAGGGCAACT -3' (Tm 62°C); Bcl2 Forward 5'- GGTCCCGTGGATAGAGAT -3'; Bcl2 Reverse 5'- GCAGATGAATTACAATTTTCAG -3' (Tm 56°C); TrailR2 Forward 5'- CCTGGGAAGGGGAGAAGAT -3'; TrailR2 Reverse 5'- AGTTGAGGGAGGCACTTGG -3' (Tm 60°C). Primer sequences for methylation controls B2m and MYOD1 were as follows: B2m Forward 5'- GCCTTCTTAAACATCACGAG -3'; B2m Reverse 5'- CCAGCCAATCAGGACAA -3' (Tm 58°C); MYOD1 Forward 5'- TACAGCCGCTCTACCCAT -3'; MYOD1 Reverse 5'- CTCCAACACCCGACTGC -3' (Tm 60°C). Methylation percentages were calculated as follows: methylation percentage = 2^- (Ct *HpaII*-treated samples – Ct mock-treated samples) x 100%. The amount of product detected after digestion with *MspI* was used to calculate the percentage of the DNA that was digested by the restriction enzymes, using the following formula: 2^-(Ct *MspI*-treated samples – Ct mock-treated samples) x 100%.

Immunohistochemical staining and scoring

Whole tumor tissue sections (4µm) of 117 patients of whom enough paraffin-embedded tumor tissue was available were IHC stained using a primary M30 antibody (Roche Diagnostics, Germany), staining for caspase-cleaved cytokeratin 18 (7). Whole tissue sections (4µm) of 40 patients in the validation study, representative of the complete series of stage III patients, were IHC stained at predetermined optimal concentrations using anti-Apaf1 (ab53152; Abcam, Cambridge, UK), anti-Bcl2 (ab*7973*; Abcam, Cambridge, UK), or anti-p53 (M7001; Dako, Glostrup, Denmark). A tissue microarray (TMA) was constructed including 495 patients from the non-irradiated arm of the Dutch TME trial. Three 1mm^2 tumor tissue cores were punched from each tumor block and transfered to a recipient block using a TMA Master (3D Histech, Budapest, Hungary). TMA sections, including 119 patients of our study cohort, were IHC stained at a predetermined optimal concentration with primary Ki-67 antibodies (Dako, Glostrup, Denmark; clone MIB-1). For all IHC stainings, tissue sections were incubated with the respective primary antibodies overnight (16 hrs). IHC staining was visualized using the Dako REAL™ EnVision™ Detection System, Peroxidase/DAB+, Rabbit/Mouse (Dako, Glostrup, Denmark). The level of apoptosis was scored as the number of M30-positive cells per mm^2, as described previously (5). Patients were classified into high or low apoptosis groups, on the basis of the median number of M30-positive cells. The level of proliferation (Ki-67) was scored as the percentage of Ki-67-positive tumor cells. The average percentage of positive cells per patient

(3 tumor cores) was used to classify patients into either high or low proliferation groups, on the basis of the median percentage of Ki-67-positive tumor cells. For Apaf1, Bcl2 and p53, the percentage of positive tumor cells was scored in three different randomly chosen fields within the tumor tissue (similar to three tissues cores on a tissue microarray). For each tumor field, the percentages of negative, weak, moderate and strong staining in tumor cells was scored. For each of these categories, an average percentage was calculated over the three tumor fields. For each marker and for each patient separately, a histoscore – as a measure of marker expression - was calculated as follows: histoscore = (0 x mean percentage negative tumor cells) + (1 x mean percentage weak positive tumor cells) + (2 x mean percentage moderate positive tumor cells) + (3 x mean percentage strong positive tumor cells).

Statistical analysis

Only samples with a difference of ≥2 Ct between MspI- and mock-treated samples and < 1 Ct difference between the supplicate PCR reactions were considered for statistical analyses. The distribution of data of each individual methylation marker was tested for normality using the Shapiro-Wilk test (35). Methylation levels of each marker were defined as high or low methylation based on the median methylation percentage. Univariate and multivariate Cox proportional hazard models were used to statistically test the differences between the groups. *Chi*-square tests were performed to compare the level of apoptosis (M30 staining) and the methylation percentage of individual markers. Interpolation plots were made to visualize the correlation between methylation and protein expression (IHC staining) data for Apaf1, Bcl2 and p53. We then studied combinations of two markers, both of the intrinsic and the extrinsic apoptosis pathways (Apaf1 and Bcl2, Apaf1 and p53, Bcl2 and p53, Fas and TrailR2). The patient groups were divided into three groups: 2 high (both markers showing high methylation), 1 high and 0 high (both markers showing low methylation). To obtain more power for statistical analyses, markers Apaf1, Bcl-2 and p53 were combined into a new variable. Data of all three markers were available for 78 patients in the validation cohort. The combined-marker set was divided into four groups: all low (low methylation in all 3 markers), 1 high (1 out of 3 markers high methylation), 2 high (2 out of 3 markers high methylation) and all high (high methylation in all 3 markers). Univariate and multivariate Cox proportional hazard analyses were performed to assess the correlation of the combined marker patient groups with overall survival (OS), cancer-specific survival (CSS), local recurrence-free survival (LRFS) and distant recurrence-free survival (DRFS). Multivariate analyses included covariates age, gender, circumferential margin and tumor location. Kaplan-Meier curves were used to visualize differences between the combined marker patient groups. Cumulative incidence curves were calculated for CSS, LRFS and DRFS, accounting for death due to other causes (36). For all survival analyses we used a pre-established patient group, the patient group with the expected shortest survival and recurrence-free periods (the “all high” patient group), as the reference group. We assessed both apoptosis (M30) and proliferation (Ki-67) in the tumor specimens of the validation cohort. Only patients with both M30 and Ki-67 data available (n=76) were included in these analyses. Combining both apoptosis and proliferation based on high versus low level of IHC staining resulted in four patient groups: low apoptosis and low proliferation (n=16), low apoptosis and high proliferation (n=13), high apoptosis and low proliferation (n=16) and high apoptosis and high proliferation (n=31). Kaplan-Meier curves were used to visualize differences between the patients groups.

Results

Enzyme digestion assay

Using methylation-sensitive restriction enzymes, we investigated the methylation status of key apoptosis genes Apaf1, Bcl2, p53, Fas and TRAILR2 in DNA extracted from frozen rectal cancer tissues. For quality control purposes, we verified performance of the enzyme assays using control genes B2m and MYOD1. Based on optimization of the assays using active housekeeping gene B2m and silenced gene MYOD1, incubation of enzyme reactions was set to 16 hrs (overnight) at 37°C, followed by 20 min heat inactivation at 65°C and subsequently cooling the samples at 4°C for one hour. The mean methylation percentages were 86% (78-100%) for MYOD1 and 38% (18-68%) for B2m. Standard deviations for the controls included in every digestion and on every PCR plate ranged from 7.5-8.2% for UMC DNA, and from 0.14-1.71% for PBL DNA. The methylation percentages of these controls ranged from 65-100% for UMC DNA, and from 1-6.5% for PBL DNA. For every digestion procedure, we verified that >75% of the DNA was actually digested in the digestion reactions, measured as Ct ≥2 between the *MspI*- and mock-treated samples, and a lower Ct value after digestion with *HpaI* as compared to the product in the mock-treated samples. Correct PCR product sizes were confirmed by running the products on a 1% agarose gel. Based on these results, we continued with statistical analyses of the patient data.

Pilot study results for individual markers

The patient cohort of the pilot study was representative of the complete non-irradiated patient cohort of the Dutch TME trial with respect to the main clinicopathological parameters (Table 1). Methylation percentages in the tumor samples were significantly different from the normal samples analyzed for Apaf1, Bcl2, TrailR2 and p53 (Figure 1). No significant differences were observed for Fas. *Chi*-square tests showed significant correlation of a high methylation of two of the markers with a low level of apoptosis based on M30 staining, with Apaf1 (p=0.03) and p53 (p=0.04). No significant correlation with apoptosis status was found for the markers Fas, Bcl2 or TrailR2 using *Chi*-square analyses. However, correlation analyses did show a negative correlation for these markers between protein expression and methylation index, indicating a decreasing amount of methylation with a higher number of apoptotic cells, with values between -0.2 and -0.3. No significant difference in methylation of the individual markers, apoptosis (M30) or proliferation (Ki-67) was observed between the tumor stages (data not shown). Therefore, we continued with stage III patients for the validation study.

Validation study results for individual markers

In the validation study, we included only stage III rectal cancer patients, as large differences in patient survival and tumor recurrence are observed within this specific patient group (37,38), and these patients will likely benefit the most from finding new biomarkers that could complement the current TNM staging system. Patients included in the validation study were representative of the non-irradiated patient cohort of the Dutch TME trial with respect to the main clinicopathological parameters (Table 1). Mean methylation percentages in the tumor tissues in the validation study were similar to those found in the pilot study for all markers. The mean methylation percentages in the tumor samples were significantly different from the

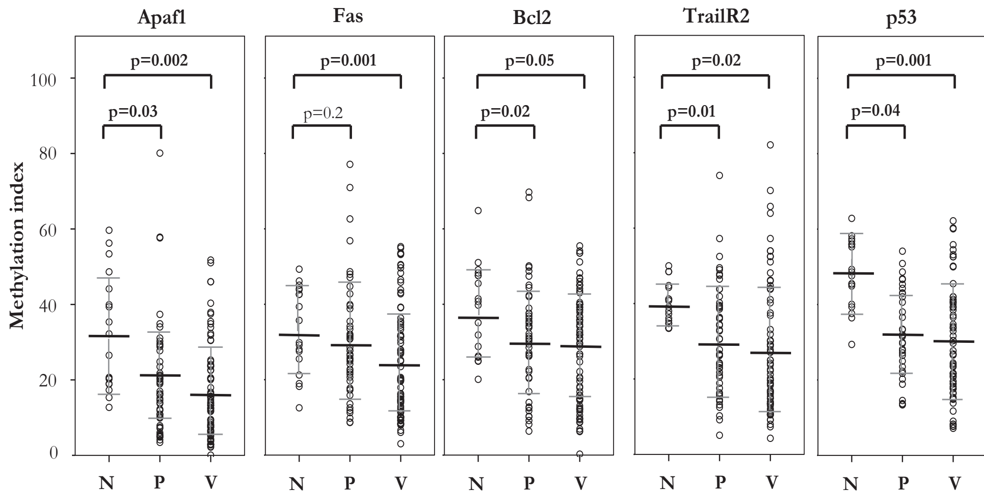
TABLE 1. Patient characteristics of the pilot and validation study cohort

		Stage I-III patients TME trial (n=685)		**Pilot study** *(n=49)*			*Stage III patients TME trial (n=281)*		**Validation study** *(n=88)*		
		n	**(%)**	**n**	**(%)**	p-value	**n**	**(%)**	**n**	**(%)**	p-value
Gender											
	Male	432	63.1	35	71.4	0.24	181	64.4	62	70.5	0.3
	Female	253	36.9	14	28.6		100	35.6	26	29.5	
Age at randomization											
	Mean	64.3		66		0.31	63.37		62.59		0.58
	Standard error	0.43		1.58			0.67		1.3		
TNM stage											
	I	209	30.5	14	28.6	0.13					*n.a.*
	II	195	28.5	25	51						
	III	281	41	10	20.4		281	100	88	100	
Tumor location											
	Rectum	607	88.6	41	83.3	0.45	246	89.5	84	95.4	0.06
	Anal region	48	12.4	8	16.7		21	10.5	4	4.5	
Circumferential margin											
	Negative	555	81	36	73.5	0.34	187	66.5	67	76.1	0.1
	Positive	129	19	13	26.5		93	33.5	21	23.9	

Patient characteristics are shown for both the pilot and validation study groups. The pilot study included stage I-III rectal cancer patients (n=49), the validation study stage III patients only (n=88). Variables listed are standard clinicopathological factors used in multivariate analyses, i.e. gender, age at randomization, TNM stage, tumor location, and circumferential margin involvement. P-values representing the difference between the TME trial patients and the pilot or validation study cohort, respectively, were calculated using a Student's t-test.

methylation percentages in the normal samples included in the validation study (Figure 1). DNA methylation percentages of the individual markers were not normally distributed. Therefore, the median methylation percentage for each individual marker was used as a cut-off value to divide patients into low and high methylation groups. To verify that a lower methylation status indeed corresponded with higher apoptosis levels in the tumor, we compared the methylation percentage of each of the different markers to the known apoptotic status (based on M30 IHC data) in each of the tumors. A representative example of IHC staining results of M30 staining is shown in Supplementary Figure 1. *Chi*-square tests showed significant correlation of a high methylation of Apaf1 (p=0.05) and Fas (p=0.01) with a low level of apoptosis (Supplementary Figure 2A). Methylation of the other markers (Bcl2, p53 and TrailR2) did show a similar correlation with M30 apoptosis levels, although not statistically significant. Linear regression using the number of

Figure 1



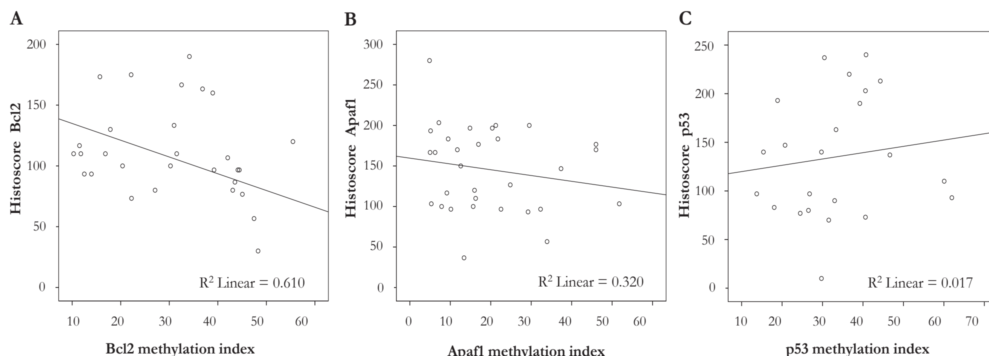
Methylation values and means of individual markers in normal, pilot and validation study tissues. *Shown are methylation percentages for normal and tumor tissues (in both pilot and validation studies) for each of the apoptosis markers separately. Mean methylation percentages are indicated with horizontal bars. Methylation percentages were calculated as follows: methylation percentage = 2^- (Ct HpaII treated samples – Ct mock treated samples) * 100%. P-values comparing the pilot and validation tumor samples with their normal counterparts were calculated using paired samples t-tests. N= normal, P=pilot series, V=validation series.*

M30-positive (apoptotic) cells per mm^2 in each of the tumors and the methylation percentages of each of the individual markers as continuous variables showed a significant correlation between the methylation status and apoptosis status for Apaf1 (p=0.03), Bcl2 (p=0.01) and p53 (p=0.04) (Supplementary Figure 2B). No significant correlation was found for markers Fas and TrailR2. Subsequently, we analyzed if methylation of the three markers Apaf1, Bcl2 and p53, correlated to protein expression (representative IHC staining results are shown in Supplementary Figure 1). As can be observed in the interpolation plots in Figure 2, Bcl2 and Apaf1 methylation correlated well with protein expression, with R^2 values of 0.610 (Bcl2, Figure 2A) and 0.320 (Apaf1, Figure 2B). For p53, methylation did not directly correlate with protein expression (Figure 2C), which might be explained by differences in p53 mutation status. Unfortunately, p53 mutation status was not known for these patients. Survival analyses did not yield any significant difference between samples with high or low methylation for any of the individual markers, based on the median methylation percentage. Using Kaplan-Meier curves, we did observe that for Apaf1, Bcl2 and p53, high methylation in the tumor tissues was associated with shorter patient survival and higher probability of tumor recurrence, although not statistically significant. We hypothesized that combining the intrinsic apoptotic pathway markers might result in better classification of rectal cancer patients in our study cohort.

Combined marker analyses

First, we studied combinations of two markers within the intrinsic or the extrinsic apoptosis pathway. A trend towards statistically significant differences between the patient groups was

Figure 2



Methylation compared to protein expression for Apaf1, Bcl2 and p53. *Interpolation plots show methylation versus protein expression for Apaf1, Bcl2 and p53. Protein expression was scored as percentage of tumor cells showing negative, weak, moderate or strong IHC staining. The histoscore was calculated as follows: histoscore = (0 * percentage negative) + (1 * percentage weak) + (2 * percentage moderate) + (3 * percentage strong). R-squared values indicate the degree of correlation between methylation and protein expression for each of the markers.*

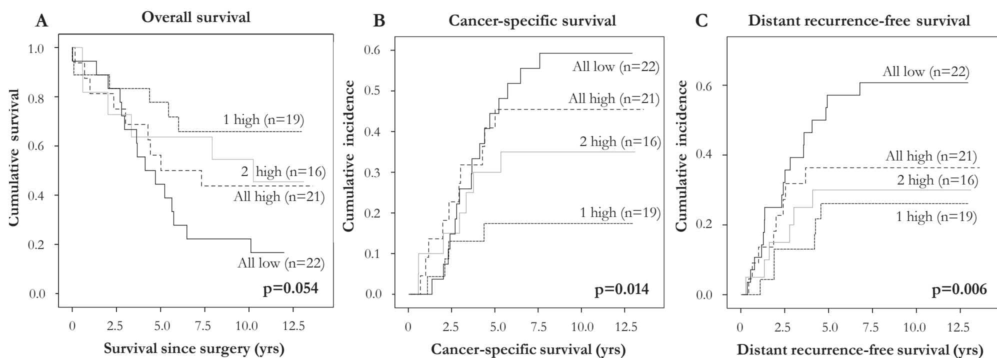
TABLE 2. Univariate and multivariate analyses of combined markers Apaf1, Bcl2 and p53 in the validation series.

	Overall survival		Cancer-specific survival		Distant recurrence-free survival		Local recurrence- free survival	
	HR (95% CI)	**p-value**	**HR (95% CI)**	**p-value**	**HR (95% CI)**	**p-value**	**HR (95% CI)**	**p-value**
Univariate analyses								
Combined marker group	0.44 (0.24-0.89)	**0.05**	0.19 (0.04-0.68)	**0.01**	0.28 (0.16-0.87)	**0.006**	0.14 (0.04-1.04)	0.21
Multivariate analysis								
Age	1.94	0.5	1.46	0.85	1.89	0.43	1.69	0.68
Gender	0.68	0.32	0.72	0.47	0.84	0.69	1.29	0.75
Circumferential margin	2.1	0.1	3.04	0.03	4.62	**0.005**	6.29	**0.03**
Tumor location	2.1	0.11	2.93	0.07	2.78	0.12	5.37	0.12
Combined marker group	0.28 (0.09-0.83)	**0.01**	0.13 (0.03-0.67)	**0.004**	0.22 (0.05-0.84)	**0.001**	0.07 (0.01-1.06)	0.17

Univariate and multivariate analyses are shown for combined markers Apaf1, Bcl2 and p53. The "all high" group (with high methylation of all three markers) was used as the reference group. Combined marker groups were defined as follows: all high (high methylation in all 3 markers), 2 high (2 out of 3 markers high methylation), 1 high (1 out of 3 markers high methylation) and all low (low methylation in all 3 markers). Hazard ratios are shown for both the univarite and the multivariate analyses, with a 95% confidence interval for the combined marker group. Statistically significant values (p<0.05) are indicated in ***bold****.*

observed for the combination of Apaf1 and Bcl2 for LRFS, with p = 0.09 (HR 0.59, 95% CI 0.33-1.09) in univariate and p = 0.1 (HR 0.58; 95% CI 0.28-1.17) in multivariate analyses. Also for the combination of Bcl2 and p53 a trend was observed in multivariate analyses for DRFS, with p = 0.07 (HR 1.51; 95% CI 0.96-2.38). For the other combinations, no significant differences were observed. To obtain more power for statistical analyses, all three intrinsic pathway markers (Apaf1, Bcl-2 and p53) were combined into a new variable. Methylation of markers Apaf1, Bcl2 and p53 was combined into one variable, on the basis of the number of markers showing high methylation (all low, 1 high, 2 high, or all high). Methylation of the three combined markers showed a correlation to apoptosis status as measured by M30 (p=0.07). Cox proportional hazard models were used to assess the differences in patient survival and tumor recurrence between the groups in both univariate and multivariate analyses (Table 2). In univariate analyses, significant differences were observed for overall survival (p=0.05), cancer-specific survival (p=0.01) and distant recurrence-free survival (p=0.006). Multivariate analyses included covariates gender, age at the time of surgery, circumferential margin and distance to the anal verge. Significant differences were observed for OS (p=0.01), CSS (p=0.004), and DRFS (p=0.001). No significant differences were observed for LRFS, in neither univariate nor multivariate analyses. The differences in patient survival and tumor recurrence between the combined marker groups were visualized using Kaplan-Meier survival curves (for OS) and cumulative incidence curves (for CSS and DRFS) (Figure 3). The curves indicated that the more markers show high methylation, the shorter the survival and recurrence-free periods. The patient group with high methylation for only one out of the three markers (“1 high” group) showed the best survival, directly followed by the patient group with two out of three markers showing high methylation (“2 high” group). The patient group with high methylation of all three markers (“all high” group) showed even shorter survival and recurrence-free periods, but the shortest survival

Figure 3



Survival curves of combined markers Apaf1, Bcl2 and p53. *Intrinsic apoptotic pathway markers Apaf1, Bcl2 and p53 were combined based on the number of markers showing high methylation. Only patient with data for all three markers available were included in the survival analyses (n=78). The resulting combined marker groups were: all low (low methylation in all 3 markers), 1 high (1 out of 3 markers high methylation), 2 high (2 out of 3 markers high methylation) and all high (high methylation in all 3 markers).* ***A****. Kaplan-Meier curves were made to visualize differences in patient survival and tumor recurrence between the different methylation groups for OS. Survival times were calculated as the time from surgery till an event (death or recurrence, resp.). Cumulative incidence curves were calculated for CSS (**B**) and DRFS (**C**).*

and disease-free periods were observed for the patient group with low methylation on all three markers ("all low" group). In addition to low methylation of Apaf1, Bcl2 and p53 in the "all low" combined marker patient group, low methylation of Fas and TRAILR2 was observed for 96% and 81% of the patients in this group, respectively. No difference in patient characteristics was observed between the combined marker groups that could explain these results, suggesting that other tumor-intrinsic factors might be responsible for the shorter patient survival and recurrence-free periods.

Proliferation and apoptosis analyses

In order to explain the survival and recurrence data observed for the "all low" combined marker patient group, we also assessed tumor proliferation (as measured by Ki-67 IHC staining, see Supplementary Figure 1) for patients in the validation study. Only patients with both M30 and

Figure 4

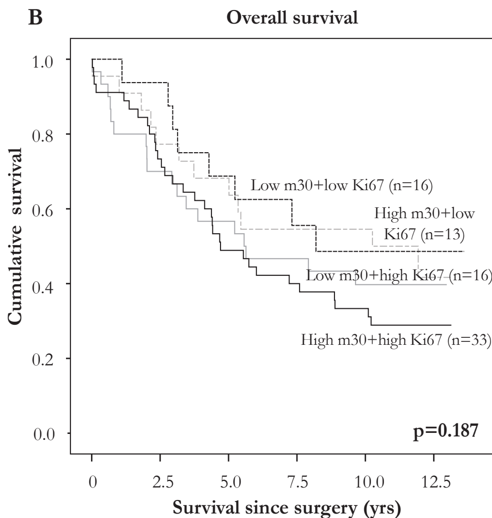
A **Numbers of patients in each of the combined apoptosis-proliferation groups**

Combined marker group "All low"		Apoptosis (M30) Low	High
Proliferation (Ki-67)	Low	0	4
	High	3	15

Combined marker group "1 high"		Apoptosis (M30) Low	High
Proliferation (Ki-67)	Low	4	3
	High	6	6

Combined marker group "2 high"		Apoptosis (M30) Low	High
Proliferation (Ki-67)	Low	6	2
	High	3	5

Combined marker group "All high"		Apoptosis (M30) Low	High
Proliferation (Ki-67)	Low	6	4
	High	4	7



Apoptosis and proliferation for combined markers Apaf1, Bcl2 and p53. *Apoptosis (M30, IHC) and proliferation (Ki-67, IHC) were combined and compared to the methylation percentages of the markers Apaf1, Bcl2 and p53. Patients with data for all markers available (n=78) were classified into high or low apoptosis or proliferation groups based on the median number or percentage of positive tumor cells, respectively.* ***A****. For all combined marker groups, the number of patients in each of the combined apoptosis-proliferation categories was determined.* ***B****. Kaplan-Meier curves were made to visualize survival differences for the combined apoptosis-proliferation categories.*

Ki-67 data available (n=76) were included in the analyses. Missing data were due to unavailability of tumor specimens or missing punches on the TMA sections. In the "all low" group showing low methylation of Apaf1, Bcl2 and p53, 15 out of 22 patients (68%) showed both high apoptosis and high proliferation (Figure 4A). For the other combined marker groups (1 high, 2 high and all high), the same numbers of patients were observed in each of the combined apoptosis-proliferation groups. As shown in Figure 4B, this also translated into differences in survival for each of the combined apoptosis-proliferation patient groups, although not statistically significant (p=0.19). The patient group with both high proliferation and high apoptosis indeed showed the shortest survival, which corresponds to the poor survival observed for patients with low methylation of all three apoptosis markers.

Discussion

Changes in the regulation of the apoptotic process, one of the hallmarks of cancer (1,2), provides tumor cells with a survival advantage and could hence promote tumorigenesis. Epigenetic aberrations have been shown to contribute to the process of tumorigenesis in many ways (16). As the outcome of the apoptotic pathway has been proven to correlate with patient outcome parameters in rectal cancer (6), we hypothesized that studying the epigenetic regulation of the apoptotic process might provide more insight in this crucial but complicated cellular process. Furthermore, it may bring us one step closer to the discovery of new clinically relevant prognostic biomarkers in rectal cancer.

In this study, methylation of key apoptosis genes using a restriction enzyme-based protocol was correlated to patient survival and tumor recurrence. We showed that combining multiple markers (intrinsic pathway markers Apaf1, Bcl2 and p53) resulted in better patient stratification and therefore better prognostication as compared to the individual markers or combinations of only two markers. With little modifications, including varying amounts of enzyme and DNA added to the digestion reactions, the enzyme-based protocol presented in this paper can be used to analyze small amounts of DNA without any loss of DNA due to prior processing steps, which is a major advantage compared to the current bisulfite modification-based methods used to detect DNA methylation. In a clinical setting this approach will be useful, as the amount of DNA available for analyses, usually derived from tumor biopsies, is limited and should be used with great care.

Current literature indicates that methylation of apoptosis proteins Apaf1, Bcl2 and p53 have prognostic value in various cancers (26,39-42). In colorectal cancer, reduced Apaf1 expression was found to be associated with tumor progression and adverse prognosis (25,43). High co-expression of Bcl2 and p53 proteins was found to be associated with poor prognosis in colorectal cancer (44), gastric MALT lymphoma (45) and B-cell lymphoma (45,46). Piris *et al.* suggested aberrant expression of both p53 and Bcl2 to be part of a multistep process of dysregulation of the apoptotic machinery critical for progression of tumors (46). Abnormal expression of p53 itself has been related to a poor patient prognosis in colorectal cancer (47). Methylation of the investigated apoptosis markers Apaf1, Bcl2 and p53 has been reported in various cancers, including lung cancer (Bcl2; 39), renal cell carcinoma (Apaf1; 40), melanoma (Apaf1; 41) and ovarian cancer (p53; 42).

To our best knowledge, the study presented here is the first study combining methylation data of several apoptosis genes at the same time, on the basis of their functions in the intrinsic apoptotic process (48). The methylation percentages of the combined markers Apaf1, Bcl2 and p53 correlated significantly with the apoptotic status of the tumors (M30 IHC staining) and disease outcome (patient survival and disease recurrence). We therefore conclude that gene promoter methylation status can be a useful surrogate marker for the apoptotic status of individual tumors, but also provides additional information about specific apoptosis-related genes as compared to a 'general' apoptotic status as determined by M30 staining.

Previous research of our group has shown that the risk of a local recurrence is lower when tumor intrinsic levels of apoptosis are high (6). In this study we showed that low methylation is correlated with higher levels of apoptosis, indeed correlating with better survival and longer recurrence-free periods. This finding is supported in literature, where high expression of Apaf1 and Bcl2 was reported to be significantly correlated with better overall survival (49). Although mean methylation levels were significantly lower in tumor tissues as compared to normal tissues for all of the markers suggesting higher apoptotic activity, in individual tumors we found that higher levels of methylation of the combined markers Apaf1, Bcl2 and p53 correlated to shorter patient survival and recurrence-free periods as compared to the patients showing low expression of one or two of the markers. The patient group showing low methylation of all three markers, however, did not comply with our expectations, as this patient group showed the shortest survival and recurrence-free periods. To explain this phenomenon, we studied cell proliferation in addition to apoptosis. We observed that patients showing both high apoptosis and high proliferation, of which 68% of the patients corresponded to the "all low" combined marker group, showed the shortest survival. This finding is supported by literature, in which both increased apoptosis and increased proliferation were reported in rectal tumors with lymph node metastases as compared to non-metastatic tumors (50). The distorted balance between the apoptosis pathways and cellular proliferation in the "all low" combined marker group hence might explain the observed survival data. Dysfunctioning of the apoptotic pathway could not be demonstrated in this patient group based on the results presented for the studied apoptosis genes, as methylation was reported to be low for all five markers. Of course, there might be other apoptotic factors involved that could compromise proper functioning of the apoptotic pathway in this patient group, which have not been investigated in this study. Epigenetic mechanisms other than DNA methylation at gene promoters could also be involved in the regulation of apoptosis gene expression in these tumors, as was suggested by the work of Hinoue *et al.* (51). Using a genome-wide approach, cancer-specific methylation of multiple gene regions has been described in CpG island methylator phenotype (CIMP)-high colorectal cancers, with characteristic genetic and clinical features. In addition, 48 of 112 genes were also found to be transcriptionally downregulated in non-CIMP tumors, but this could not be correlated to higher DNA methylation at these specific regions, suggesting involvement of other (epigenetic) mechanisms. In contrast to these genome-wide studies, a more pathway-focused approach was used in our study that facilitated the identification of new biomarkers in rectal cancer prognosis.

In conclusion, in this study we found that methylation of apoptotic genes is correlated with the overall apoptotic status of a tumor, and that this status can be used to assess clinical outcome in terms of patient survival and tumor recurrence. The methylation analysis presented using methylation-sensitive restriction enzyme digestion provides a biological explanation for the

differences in apoptotic status in individual tumors. High methylation of combined intrinsic apoptosis pathway markers Apaf1, Bcl2 and p53, suggesting deregulation of the apoptotic pathway, was associated with poor prognosis in our study cohort of colorectal cancer patients. High proliferation and high apoptosis were observed when methylation of the intrinsic apoptosis pathway markers was low. The study of epigenetic regulation of apoptosis genes will provide more insight in the tumorigenic process in rectal cancer and might be helpful in further refining treatment regimens for individual patients.

Acknowledgements

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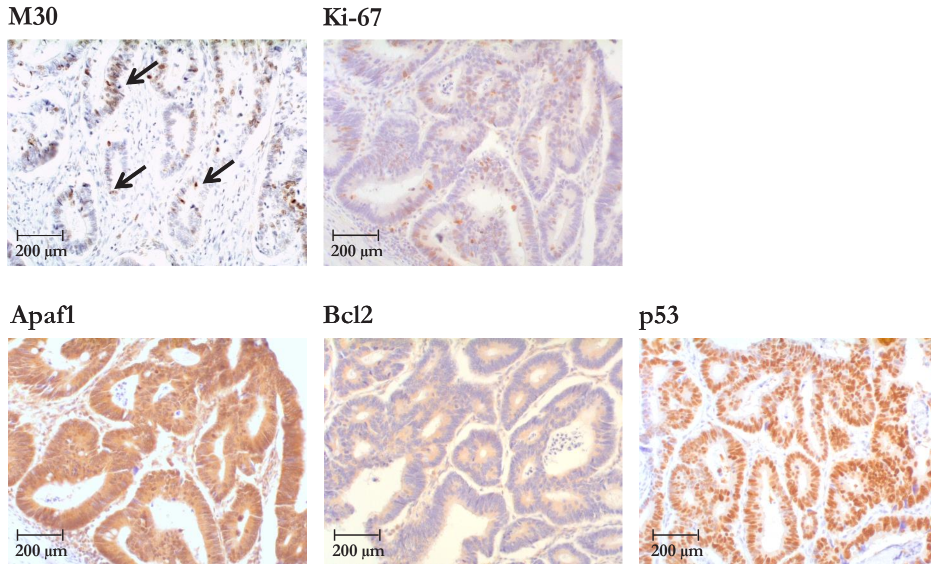
Supplementary files

Supplementary Figure 1



Representative IHC staining results of individual markers. *Shown are representative IHC staining results of the individual markers M30, Ki-67, Apaf1, Bcl2 and p53 on the tumor of one patient included in our study. Images were taken at a 20x magnification and scales are indicated in the figures. Apoptosis using M30 was scored as the number of positive cells (indicated with arrows), proliferation using Ki-67 was scored as the percentage of positively stained tumor cells. For Apaf1, Bcl2 and p53 a histoscore was calculated on the basis of staining intensity and the percentages of positive cells (as discussed in Materials and Methods).*

Supplementary Figure 2

A Chi-squared analyses validation study

Apaf1

M30	Low	High	Total
Low	9	21	30
High	19	15	34
Total	28	36	64

χ^2: p=0.05

Fas

M30	Low	High	Total
Low	9	25	34
High	18	13	31
Total	27	28	65

χ^2: p=0.01

Bcl2

M30	Low	High	Total
Low	10	18	28
High	14	19	33
Total	24	37	61

χ^2: p=0.6

TrailR2

M30	Low	High	Total
Low	11	17	28
High	14	17	31
Total	25	34	59

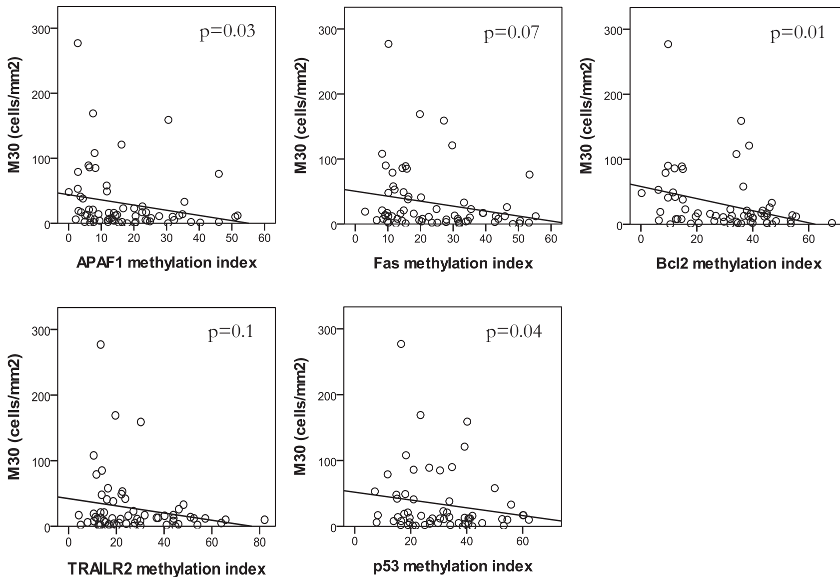
χ^2: p=0.7

p53

M30	Low	High	Total
Low	9	17	26
High	13	19	32
Total	22	36	58

χ^2: p=0.8

B Linear regression analyses validation study



Chi-squared and linear regression analyses validation study. A*. Chi-squared analyses comparing M30 staining with the methylation status of all individual markers in the validation study (high or low based on median expression) are shown, including the χ2 p-values.* ***B****. Scatter plots of the linear regression models are shown for each of the individual markers in the validation study are shown in panel, including the linear regression p-values.*

Supplementary TABLE 1 (next page)

Univariate and multivariate analyses are shown for the combinations of two markers. Hazard ratios and 95% confidence intervals (95% CI) are shown for both the univariate and multivariate analyses, as well as the p-values. Patient groups for the combined marker analyses were defined as follows: 2 high (both markers showing high methylation, 1 high and 0 high (both markers showing low methylation). OS= overall survival; CSS= cancer-specific survival; LRFS= local recurrence-free survival; DRFS= distant recurrence-free survival. Trends ($p \leq 0.1$) are indicated in Italic.

Supplementary TABLE 1. Univariate and multivariate analyses of combinations of 2 markers

Apaf1 and Bcl2	OS		CSS		LRFS		DRFS	
	HR (95% CI)	**p-value**	**HR (95% CI)**	**p-value**	**HR (95% CI)**	**p-value**	**HR (95% CI)**	**p-value**
Univariate analyses								
Combined markers	0.89	0.4	0.83	0.3	0.59	*0.09*	1.01	0.9
	(0.66-1.19)		(0.58-1.18)		(0.33-1.09)		(0.70-1.44)	
Multivariate analysis								
Combined markers	0.92	0.6	0.95	0.8	0.58	*0.1*	1.12	0.6
	(0.66-1.27)		(0.64-1.39)		(0.28-1.17)		(0.76-1.66)	

Apaf1 and p53	OS		CSS		LRFS		DRFS	
	HR (95% CI)	**p-value**	**HR (95% CI)**	**p-value**	**HR (95% CI)**	**p-value**	**HR (95% CI)**	**p-value**
Univariate analyses								
Combined markers	1.13	0.4	1.09	0.6	0.89	0.7	1.17	0.4
	(0.84-1.53)		(0.76-1.57)		(0.50-1.61)		(0.81-1.69)	
Multivariate analysis								
Combined markers	1.14	0.5	1.16	0.5	0.83	0.6	1.26	0.2
	(0.81-1.59)		(0.78-1.70)		(0.43-1.61)		(0.85-1.88)	

Bcl2 and p53	OS		CSS		LRFS		DRFS	
	HR (95% CI)	**p-value**	**HR (95% CI)**	**p-value**	**HR (95% CI)**	**p-value**	**HR (95% CI)**	**p-value**
Univariate analyses								
Combined markers	1.13	0.4	1.11	0.6	0.82	0.5	1.3	0.2
	(0.82-1.55)		(0.76-1.64)		(0.46-1.46)		(0.89-1.96)	
Multivariate analysis								
Combined markers	1.15	0.4	1.17	0.5	0.75	0.4	1.51	*0.07*
	(0.79-1.67)		(0.76-1.79)		(0.38-1.49)		(0.96-2.38)	

Fas and TRAILR2	OS		CSS		LRFS		DRFS	
	HR (95% CI)	**p-value**	**HR (95% CI)**	**p-value**	**HR (95% CI)**	**p-value**	**HR (95% CI)**	**p-value**
Univariate analyses								
Combined markers	0.97	0.8	0.79	0.2	0.92	0.8	0.85	0.3
	(0.74-1.28)		(0.58-1.09)		(0.56-1.53)		(0.62-1.16)	
Multivariate analysis								
Combined markers	0.92	0.5	0.79	0.2	0.87	0.6	0.85	0.3
	(0.69-1.22)		(0.57-1.11)		(0.49-1.52)		(0.61-1.18)	

Chapter 4

Nuclear expression of histone deacetylases and their histone modifications predicts clinical outcome in colorectal cancer

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Abstract

Aims: Epigenetic changes are of crucial importance in cancer development and are potentially reversible, thus presenting as interesting targets for anti-cancer therapy. We investigated the clinical prognostic value of histone deacetylases SIRT1, HDAC1 and HDAC2 and histone modifications H4K16Ac and H3K56Ac in colorectal cancer.

Methods and results: The epigenetic markers were immunohistochemically stained on tissue microarrays containing colorectal tumor (n=254) and normal colorectal tissues (n=50). Nuclear expression was assessed on the semi-automated Ariol system. Multivariate trend survival analyses of the combined markers showed better patient survival and less tumor recurrence when more markers showed high nuclear expression. For the combination of the histone deacetylases and H3K56Ac, the hazard ratio (HR) for overall survival (OS) was 0.82 (0.72-0.94; p=0.005) and for distant recurrence-free survival (DRFS) was 0.77 (0.64-0.92; p=0.003) per additional marker showing high expression. Similarly, for the combination of histone deactylases and H4K16Ac, a HR of 0.86 (0.76-0.97; p=0.01) for OS and 0.79 (0.68-0.93; p=0.006) for DRFS were observed per additional marker showing high expression.

Conclusions: The studied epigenetic markers showed clinical prognostic value in colorectal cancer, both as individual markers and when combined into multi-marker analyses. These results indicate that epigenetic mechanisms play an important role in colorectal carcinogenesis.

Introduction

There is a need to identify new biomarkers in colorectal cancer in order to better stratify patients for treatment based on their individual tumor characteristics. For TNM stage I-III colorectal tumors, patient survival and tumor recurrence vary widely among patients, indicating that the current TNM staging system needs further refinement. New biomarkers may be found by unraveling the underlying biology of individual tumors. Epigenetics is a promising field for biomarker research, since changes in epigenetic status have been frequently reported in tumor tissues compared to their normal counterparts (1). In addition, epigenetic mechanisms are potentially reversible, which makes them suitable targets for the development of new therapies (1).

Epigenetic mechanisms include DNA methylation and histone modifications, which directly influence chromatin structure and thereby accessibility of the DNA for transcription factors (2). Several research groups have found global expression of histone modifications to have prognostic value in different cancers, including prostate (3), lung and kidney (4), breast cancer (5), and colorectal cancer (6). In addition to histone modifications, expression of histone-modifying enzymes, including histone deacetylases (7), have also been shown to have prognostic value in colorectal cancer.

Specific histone modification patterns are associated with regions of the genome that are either actively transcribed or repressed (8). One of the histone modifications that is strongly linked to gene activation and can by itself prevent chromatin compaction is H4K16Ac (9). The major H4K16 deacetylase in mammalian cells is SIRT1 (Sirtuin 1), a class III histone deacetylase (10). Human SIRT1 has been shown to be involved in many (disease) processes (11) and altered expression of SIRT1 has been described in several cancers, including colorectal cancer (12). Global levels of H4K16Ac are dependent on the balance between SIRT1 and class I histone deacetylases HDAC1 and HDAC2. Both HDACs have been shown to contribute to the process of non-homologous end-joining (13), which is important for error-free repair of DNA double strand breaks, through deacetylation of histone modification H3K56Ac. Dysregulation in these cellular processes could facilitate carcinogenesis.

Using immunohistochemistry (IHC), we analyzed nuclear expression of histone deacetylases SIRT1, HDAC1 and HDAC2 and histone modifications H3K56Ac and H4K16Ac in tumor specimens of 254 TNM stage I-III colorectal cancer patients from a consecutive patient cohort with extensive clinical follow-up data. We analysed the correlations of expression of the individual markers and combinations of the histone deacetylases with each of the respective histone modifications with clinical outcome.

Materials and methods

Patient selection

Tumor tissues were collected from a consecutive series of 409 (TNM-stage I-IV) patients who underwent surgery at the Leiden University Medical Center (LUMC) of their primary tumor

between 1991 and 2001 and of whom tumor tissue was available. All specimens were handled with a standard protocol for fixation, dissection and histopathological reporting. Patients with pre-operative treatment, multifocal tumors, or a history of cancer (other than basal cell carcinoma or *in situ* tumors) were excluded from analyses. We included only patients with a histologically proven colorectal adenocarcinoma and TNM tumor stage I-III cancer, as determined by an experienced pathologist. Complete clinicopathological data were available for 259 TNM stage I-III patients, and complete covariate and study marker data were available for 254 patients (Table 1), with a mean follow-up of 8.6 years. Clinicopathological parameters of patients in the study cohort were representative for the complete patient cohort. Data were censored when patients were alive or free of recurrence at their last follow-up date. Patient records information was anonymized and de-identified prior to analysis according to national ethical guidelines ("Code for Proper Secondary Use of Human Tissue", Dutch Federation of Medical Scientific Societies), and approved by the Medical Ethical Committee of the Leiden University Medical Center (LUMC). MSI status was tested using the PCR-based MSI Analysis System, Version 1.2 (Promega, Mannheim, Germany), as described previously (14). This study was performed according to the REMARK guidelines (NCI-EORTC) (15).

Immunohistochemistry and scoring

Formalin-fixed paraffin-embedded (FFPE) tissues from each of the patients in this retrospective study were used to construct a tissue microarray (TMA) with 0.6 mm tissue cores, as described previously (16). Sections of 4µm were cut from each of the TMA blocks including 254 colorectal tumor tissues and 50 histopathologically normal colorectal tissues and used for IHC (manual protocol). TMA sections were incubated overnight (16 hours) using primary antibodies at predetermined optimal dilutions. Antibodies used in this study were: anti-H3K56Ac (ab76307, Abcam, Cambridge, UK), anti-H4K16Ac (ab61240, Abcam, Cambridge, UK), anti-SIRT1 (ab32441, Abcam, Cambridge, UK), anti-HDAC1 (ab19845, Abcam, Cambridge, UK) and anti-HDAC2 (ab39669, Abcam, Cambridge, UK), using a standard IHC protocol (17). Briefly, antigen retrieval was performed by heating the sections for 10 min at 95°C in a citrate buffer (pH 6.1; pH Low Target Retrieval Solution, Dako, Glostrup, Denmark) after deparaffinization. Endogenous peroxidase was blocked by incubating the sections in a 0.3% solution of hydrogen peroxide (in PBS) for 20 min. Staining was visualized using the Dako REAL™ EnVision™ Detection System, Peroxidase/DAB+, Rabbit/Mouse (Dako, Glostrup, Denmark). In each TMA block, control tissues (colon, spleen and liver) were included serving as positive controls across TMA sections for nuclear staining. A no-antibody control section was used as negative control. Stained tissue microarrays were scanned using a 20x magnification and nuclear expression of all markers was assessed using the semi-automated Ariol system (Leica Microsystems, Wetzlar, Germany). Tumor areas (tumor tissues) and colon epithelium (normal tissues) were marked on the computer screen upon visual inspection. The semi-automated Ariol system is specifically designed to recognize cells, nuclei, cell membranes and pixel intensity and was trained carefully for each individual staining. For each TMA section, several random cores were evaluated by visual inspection after automatic analysis in order to verify correct identification of positively stained nuclei.

TABLE 1. Patient characteristics of the study cohort (n=254)

		n	(%)
Age at operation			
	<50	32	12.6
	50-75	161	63.4
	>75	61	24
Gender			
	Male	128	50.4
	Female	126	49.6
TNM stage			
	I	53	20.9
	II	113	44.5
	III	88	34.6
pT stage			
	T1	19	7.5
	T2	38	15.0
	T3	166	65.4
	T4	31	12.2
pN stage			
	N0	168	66.1
	N1	54	21.3
	N2	32	12.6
Histological Subtype			
	Adenocarcinoma	190	74.9
	Mucinous	34	13.6
	Cribriform	14	5.5
	Tubulovillous	5	2.0
	Undifferentiated	10	3.9
	Signet ring cell	1	0.1

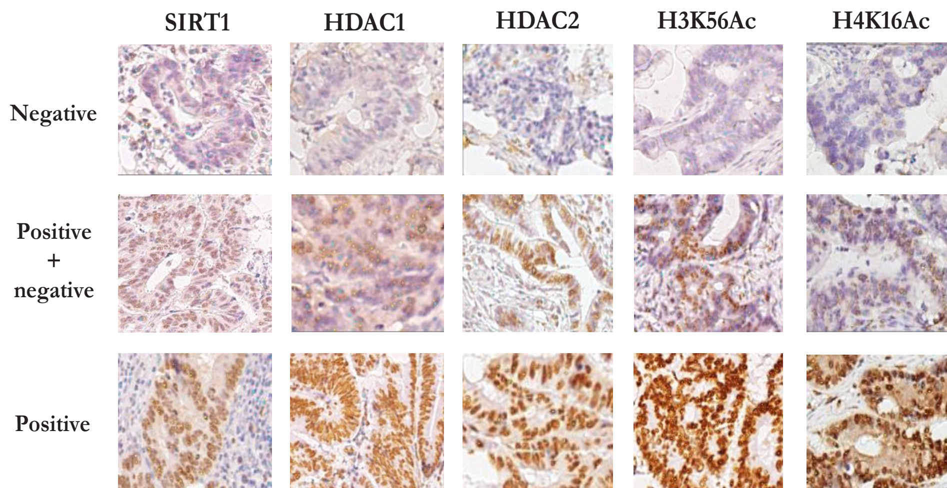
		n	(%)
MSS status			
	MSI	34	13.4
	MSS	175	68.9
	Unknown	45	17.7
Tumor location			
	Colon	187	73.6
	Rectum	67	26.4
Tumor size			
	Mean	4.69	
	Standard error	2.32	
Number of lymph nodes retrieved			
	Mean	8.09	
	Median	8	
	<12	250	98.4
	≥12	4	1.6
Location in the colon			
	Proximal	94	37.0
	Distal	160	63.0
Tumor in follow-up *			
	No	215	84.6
	Yes	39	15.4
Adjuvant therapy			
	No	206	81.1
	Yes	48	18.9

*Patient characteristics are shown for the study cohort (n=254). Patients with unknown status for any of the covariates are not reported in this table, except for MSS status. * = second primary tumor during follow-up period.*

Statistical analysis

Data were analyzed in consultation with a statistician (H.P.) using SPSS 20.0 for Windows (SPSS Inc, Chicago, USA). The Cox proportional hazard model was used for univariate and multivariate survival analysis. Covariates included in all multivariate analyses were age at operation, gender,

Figure 1

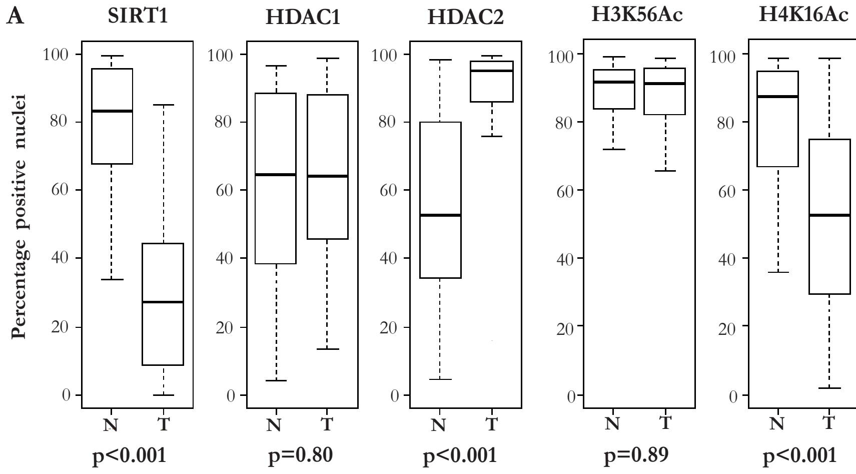


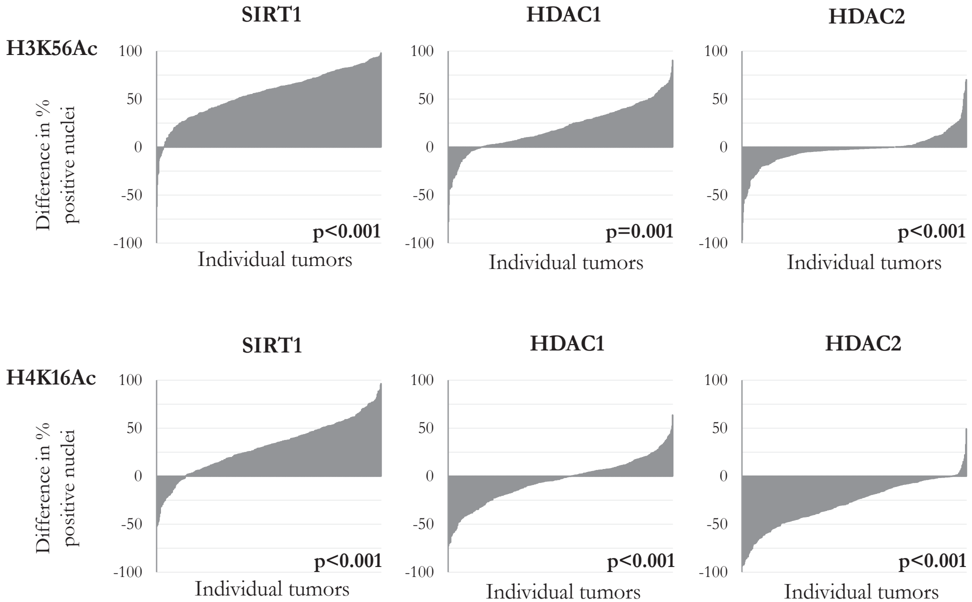
Identification of positively stained and negative nuclei by the Ariol system. *The Ariol system trainer overlay shows correct identification of positive (indicated by yellow dots) and negative (blue dots) nuclei in tumor tissues using immunohistochemistry. TMA slides were scanned using a 20x magnification. Shown for all markers are negative tumor cores (top row), tumor cores with both positive and negative cells (middle row) and highly positive tumor cores (bottom row). The Ariol system was trained to identify positive and negative cells for each marker individually.*

TNM tumor stage (tumor stages I-III), tumor location, tumor size and microsatellite stability (MSS) status. Covariates "tumor in the follow-up" (second primary tumor) and "adjuvant therapy" were entered as time-dependent covariates. Normality of the data was tested using the Shapiro-Wilk test, and non-parametric Wilcoxon signed rank tests were performed to test for statistical differences in expression between normal and tumor samples and between the expression levels of histone modifications and histone deacetylases in individual tumors. Spearman's rank correlation coefficient was used to test the correlation between the expression of histone deacetylases and the respective histone modifications. Median expression was used as a cut-off value to divide patients into high or low expression groups. Kaplan-Meier curves or cumulative incidence plots were generated to visualize the differences in patient survival or tumor recurrence. We performed trend analyses using combined markers with the group numbers as continuous variables. Cox regression analyses were performed using the combined markers as categorical variables to assess the hazard ratios for each of the individual patient

***Figure 2 (see next page) Paired analyses of normal versus tumor and histone deacetylases versus histone modifications**. **A**. Boxplots showing mean expression (indicated as the percentage of immunohistochemically stained positive nuclei). Normal samples (n=50) are shown on the left of each figure (labeled "N") and mean expression of the tumor samples (n=254) is shown on the right (labeled "T"). P-values indicate the results of the non-parametric Wilcoxon signed rank test. **B**. Histograms showing the difference in expression (percentage of positive nuclei as determined after immunohistochemistry) between the histone modifications and histone deacetylases are displayed for H3K56Ac and H4K16Ac against each of the individual histone deacetylases. The difference in expression (y-axis) was calculated for each individual patient (x-axis), according to the following formula: expression difference = expression histone modification – expression histone deacetylase. Negative values indicate higher expression of the histone deacetylase, positive values indicate higher expression of the histone modification. P-values represent the results of the Spearman's correlation analysis.*

Figure 2





groups. Competing risk analyses were performed to assess disease-specific survival. Kaplan-Meier curves or cumulative incidence plots were generated to visualize the differences in patient survival and tumor recurrence between the five groups. For individual marker analyses, the low expression group was used as the reference group, and for combined analyses group 1 (all low)

was used as reference group. Overall survival (OS) was defined as the time from surgery until death (by any cause). Disease-specific survival (DSS) was defined as the time from surgery until death by colorectal cancer, and was based on autopsy reports, where available, and otherwise on death certification. Loco-regional recurrence-free survival (LRRFS) was defined as the time from surgery until the occurrence of a (loco)regional recurrence or death by cancer. Distant recurrence-free survival (DRFS) was defined as the time from surgery until the occurrence of a distant recurrence or death by cancer. For all statistical analyses, a two-sided p-value of 0.05 or less was considered statistically significant.

Results

Expression in normal versus tumor tissues

To minimize observer bias, nuclear expression of all markers in both tumor cells (tumor tissues) and colon epithelium (normal tissues) was scored using the semi-automated Ariol system (Figure 1). We analyzed expression of SIRT1, HDAC1, HDAC2, H3K56Ac and H4K16Ac in normal and tumor tissues. The expression data were not normally distributed for any of the markers (data not shown). SIRT1 and H4K16Ac showed lower nuclear expression in tumor samples compared to normal tissue samples (both $p<0.001$), whereas HDAC2 showed higher nuclear expression in tumor samples compared to normal tissue samples ($p<0.001$) (Figure 2A). The expression of HDAC1 ($p=0.80$) and H3K56Ac ($p=0.89$) did not differ between normal and tumor samples when analyzing the whole patient cohort (Figure 2A). However, within individual tumors, differences between normal and tumor samples were observed for both HDAC1 and H3K56Ac (Figure 3A). For both markers, approximately 50% of the tumor tissues showed higher expression compared to paired normal tissues, and the other 50% showed lower expression in tumor tissues compared to normal tissues. This also translated into survival differences between patients with higher expression and patients with lower expression in the tumor as compared to the normal tissues (Figure 3B).

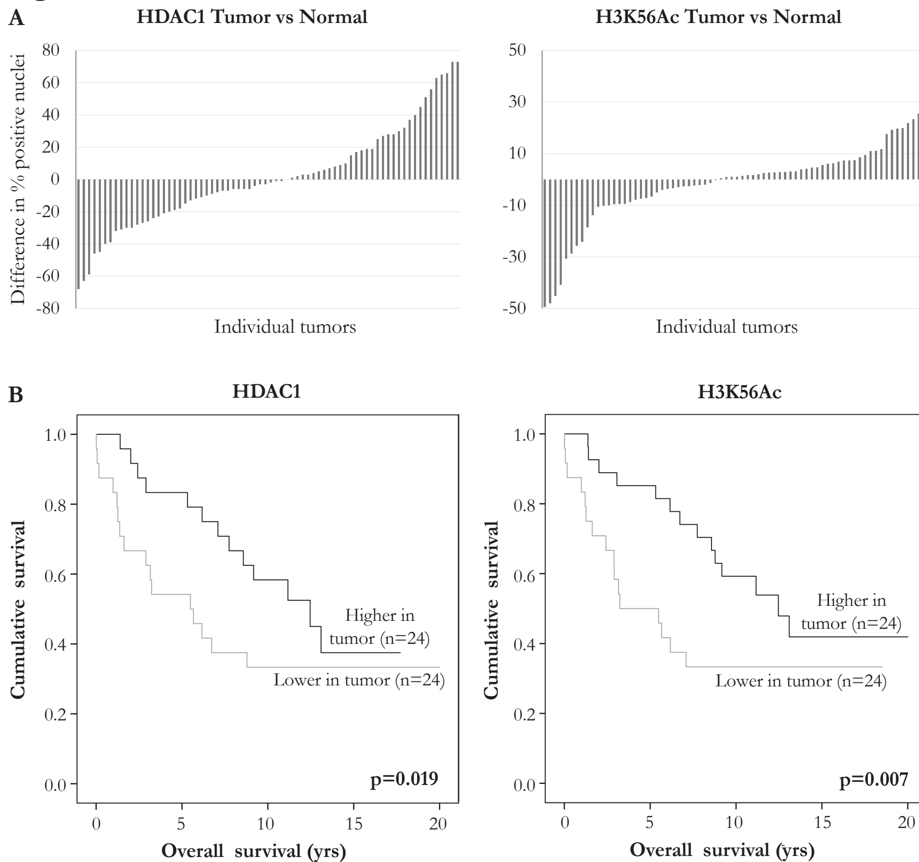
Paired analyses of expression of histone deacetylases and histone modifications

The differences in expression levels between each of the histone deacetylases and either of the histone modifications were plotted for the whole study cohort (Supplementary Figure 1) and for each individual tumor (Figure 2B). In individual tumors, high expression of the histone modifications correlated to low expression of the histone deacetylases (positive values), and *vice versa* (negative values). Spearman's correlation analyses showed that for all three histone deacetylases, there was a significant inverse correlation with the respective histone modifications ($p\leq0.001$).

Survival analyses of individual markers

The median expression for each of the markers, used to divide patients into high and low expression groups, was as follows: SIRT1 (30%), HDAC1 (68%), HDAC2 (95%), H3K56Ac (93%) and H4K16Ac (63%). Median survival was 9.3 years (for both OS and DSS), median recurrence-free survival was 8.8 years for LRRFS and 9.2 years for DRFS. All markers showed

Figure 3



***Expression of HDAC1 and H3K56Ac in tumor and normal tissues of individual patients. A**. Histograms showing the difference in expression (indicated as the percentage of immunohistochemically stained positive nuclei) between paired normal and tumor tissues are displayed for HDAC1 and H3K56Ac. The difference in expression (y-axis) was calculated for each individual patient (x-axis), according to the following formula: expression difference = expression in tumor tissue – expression in normal tissue. Negative values indicate higher expression in normal tissues, positive values indicate higher expression in tumor tissues. **B**. Kaplan-Meier curves were made to visualize differences in overall survival between patients with higher expression and patients with lower expression in tumor tissues as compared to normal tissues. P-values represent the results of multivariate Cox proportional hazards survival analyses.*

highly significant correlations with patient survival and tumor recurrence, in both univariate and multivariate analyses (Table 2). Patients with high nuclear expression of either of the markers showed better survival and a lower chance of tumor recurrence, which was confirmed by plotting Kaplan-Meier curves or cumulative incidence plots for both patient survival (OS and DSS) and tumor recurrence-free survival (LRRFS and DRFS) (data not shown).

Survival analyses of combined markers

As we know that most of these markers work together in multi-protein complexes in order to remodel the (local) chromatin structure, we performed combined analyses of histone

TABLE 2. Univariate and multivariate survival analyses individual markers

			SIRT1	HDAC1	HDAC2	H3K56Ac	H4K16Ac
OS	Univariate	p-value	0.8	0.07	0.3	**0.004**	**0.03**
		HR	1.0	0.7	0.8	0.6	0.7
		(95% CI)	(0.69-1.33)	(0.53-1.02)	(0.60-1.16)	(0.45-0.86)	(0.50-0.97)
	Multivariate	p-value	0.2	**0.03**	0.1	**0.02**	**0.02**
		HR	0.8	0.7	0.7	0.7	0.7
		(95% CI)	(0.58-1.15)	(0.49-0.97)	(0.54-1.07)	(0.47-0.94)	(0.47-0.94)
DSS	Univariate	p-value	**0.05**	**0.02**	**0.05**	0.1	**0.009**
		HR	0.6	0.6	0.6	0.7	0.5
		(95% CI)	(0.36-0.99)	(0.34-0.92)	(0.36-1.003)	(0.42-1.11)	(0.31-0.84)
	Multivariate	p-value	**0.01**	**0.009**	**0.03**	0.2	**0.02**
		HR	0.5	0.5	0.6	0.7	0.5
		(95% CI)	(0.29-0.87)	(0.29-0.84)	(0.33-0.95)	(0.44-1.21)	(0.31-0.89)
LRRFS	Univariate	p-value	0.2	**0.01**	**0.03**	0.2	**0.03**
		HR	0.7	0.6	0.6	0.7	0.6
		(95% CI)	(0.48-1.15)	(0.37-0.88)	(0.39-0.95)	(0.49-1.16)	(0.40-0.95)
	Multivariate	p-value	**0.05**	**0.008**	**0.009**	*0.07*	**0.03**
		HR	0.6	0.5	0.5	0.6	0.6
		(95% CI)	(0.41-1.01)	(0.34-0.85)	(0.34-0.86)	(0.41-1.03)	(0.38-0.95)
DRFS	Univariate	p-value	0.1	**0.03**	*0.09*	0.2	*0.06*
		HR	0.7	0.6	0.7	0.8	0.7
		(95% CI)	(0.45-1.10)	(0.39-0.94)	(0.43-1.06)	(0.50-1.19)	(0.42-1.01)
	Multivariate	p-value	**0.04**	**0.02**	**0.04**	0.1	*0.07*
		HR	0.6	0.6	0.6	0.7	0.7
		(95% CI)	(0.38-0.98)	(0.35-0.89)	(0.38-0.98)	(0.44-1.11)	(0.41-1.04)

Shown are the results of the univariate and multivariate analyses of all individual markers, with all p-values and hazard ratios (HR) with their 95% confidence intervals (95% CI). OS = overall survival, DSS = disease-specific survival, LRRFS = locoregional recurrence-free survival, DRFS = distant recurrence-free survival. For each marker, the low expression group (below median expression) was used as reference group. Differences in clinical outcome between patient groups are presented as hazard ratios compared to the reference group. Significant p-values are indicated in ***bold****, p-values showing a trend (between 0.05 and 0.1) in Italic.*

deacetylases SIRT1, HDAC1 and HDAC2 together with either H3K56Ac or H4K16Ac. We divided the patients into five groups, based on the number of markers with "high expression" for this specific group of patients, i.e. all low (group 1), one high (group 2), two high (group 3), three high (group 4) and all high (group 5). All multivariate trend analyses showed significant

differences in patient survival and tumor recurrence (Table 3). For the combined analyses of the histone deacetylases and H3K56Ac, each increase of one unit (one additional marker showing high expression) compared to the “all low” patient group resulted in a hazard ratio (HR) of 0.82 (0.72-0.94; p=0.005) for OS, 0.72 (0.59-0.88; p=0.001) for DSS, 0.74 (0.62-0.88; p=0.001) for LRRFS and 0.77 (0.64-0.92; p=0.003) for DRFS. Similarly, for the combination of the histone deactylases and H4K16Ac, a HR of 0.86 (0.76-0.97; p=0.01) for OS, 0.73 (0.60-0.88; p=0.001) for DSS, 0.77 (0.66-0.90; p=0.001) for LRRFS and 0.79 (0.68-0.93; p=0.006) for DRFS were observed per unit of increase. Competing risk analyses showed that the more markers showed high expression, the lower the cumulative incidence (Figures 4A and 4B). For each of the individual patient groups, a decrease in hazard ratio was observed when more markers showed high expression (Figure 4C and Supplementary Table 1). The lowest hazard ratio was observed for patients with high expression of all markers (group 5) as compared to the reference group with low expression of all markers (group 1). A similar stratification of patient groups was observed for overall survival.

Discussion

It is becoming increasingly clear that epigenetics plays an important role in tumor development. Increasing knowledge about the role of epigenetic mechanisms in cancer has guided the development of new epigenetic anti-cancer therapies, often combined with existing therapies (1). To date, however, such epigenetic therapies have only been proven effective in hematological diseases and treatment of solid cancers has proven challenging. For solid tumors, epigenetic therapies may require the development of therapies that for example target multi-protein complexes. In ongoing research an increasing number of such multi-protein complexes are being identified (18). In this study, we investigated three histone deacetylases that act together to remodel the chromatin in response to DNA damage and are important regulators of gene expression during embryonic development (19) and play a role in tumor initiation and progression (20). Deregulation of these histone deacetylases could result in tumor development and progression (12,21). In this study, we demonstrated an increased nuclear expression of HDAC2, and decreased nuclear expression of SIRT1 and H4K16Ac in tumor cells as compared to normal cells. Other groups also reported similar changes in expression between normal and tumor tissues in literature.(7,12) Loss of H4K16Ac has been described to be a common hallmark of human cancers (22), which was mostly linked to hypomethylation of DNA repetitive sequences during tumor progression. This might be correlated to LINE-1 hypomethylation, which we previously showed to correlate with shorter patient survival and higher chances of tumor recurrence in early-stage rectal cancer (23).

Table 3 (see next two pages). *Shown are the results of the univariate and multivariate trend analyses of the combined markers using the group numbers as continuous variables, with all p-values and hazard ratios (HR) with their 95% confidence intervals (95% CI). Displayed hazard ratios reflect the hazard ratio with an increase of 1 unit, meaning an increase in the number of markers showing high expression (reflected in a higher group number). OS = overall survival, DSS = disease-specific survival, LRRFS = locoregional recurrence-free survival, DRFS = distant recurrence-free survival. Significant values are shown in* **bold**, *trends in Italic. * = second primary tumor during follow-up period.*

TABLE 3. Univariate and multivariate trend analyses combined markers SIRT1, HDAC1, HDAC2 and H3K56Ac

	OS			DSS		
Univariate	p-value	HR	(95% CI)	p-value	HR	(95% CI)
Combined markers	**0.02**	0.86	(0.75-0.98)	**0.003**	0.75	(0.62-0.91)
Multivariate						
Combined markers	**0.005**	0.82	(0.72-0.94)	**0.001**	0.72	(0.59-0.88)
Age at operation	**<0.001**	1.86	(1.55-2.23)	**0.009**	1.37	(1.08-1.73)
Gender	0.86	1.03	(0.73-1.46)	0.9	1	(0.59-1.68)
TNM stage *1*	**0.005**			**<0.001**		
2	0.13	1.5	(0.89-2.55)	*0.07*	2.47	(0.91-6.68)
3	**0.002**	2.43	(1.38-4.29)	**<0.001**	8.02	(2.93-21.98)
Tumor location	0.24	1.26	(0.85-1.87)	*0.08*	1.67	(0.94-2.94)
Tumor size	**0.01**	1.09	(1.02-1.18)	**0.05**	1.11	(1.00-1.24)
MSS status *MSS*	0.79			0.3		
MSI	0.5	0.84	(0.49-1.41)	0.2	0.55	(0.23-1.32)
Unknown	0.89	0.97	(0.59-1.58)	0.6	1.2	(0.59-2.41)
Tumor in follow-up *	**0.002**	2.18	(1.32-3.59)	0.2	1.71	(0.69-4.20)
Adjuvant therapy	0.69	1.1	(0.67-1.81)	0.1	0.57	(0.29-1.13)

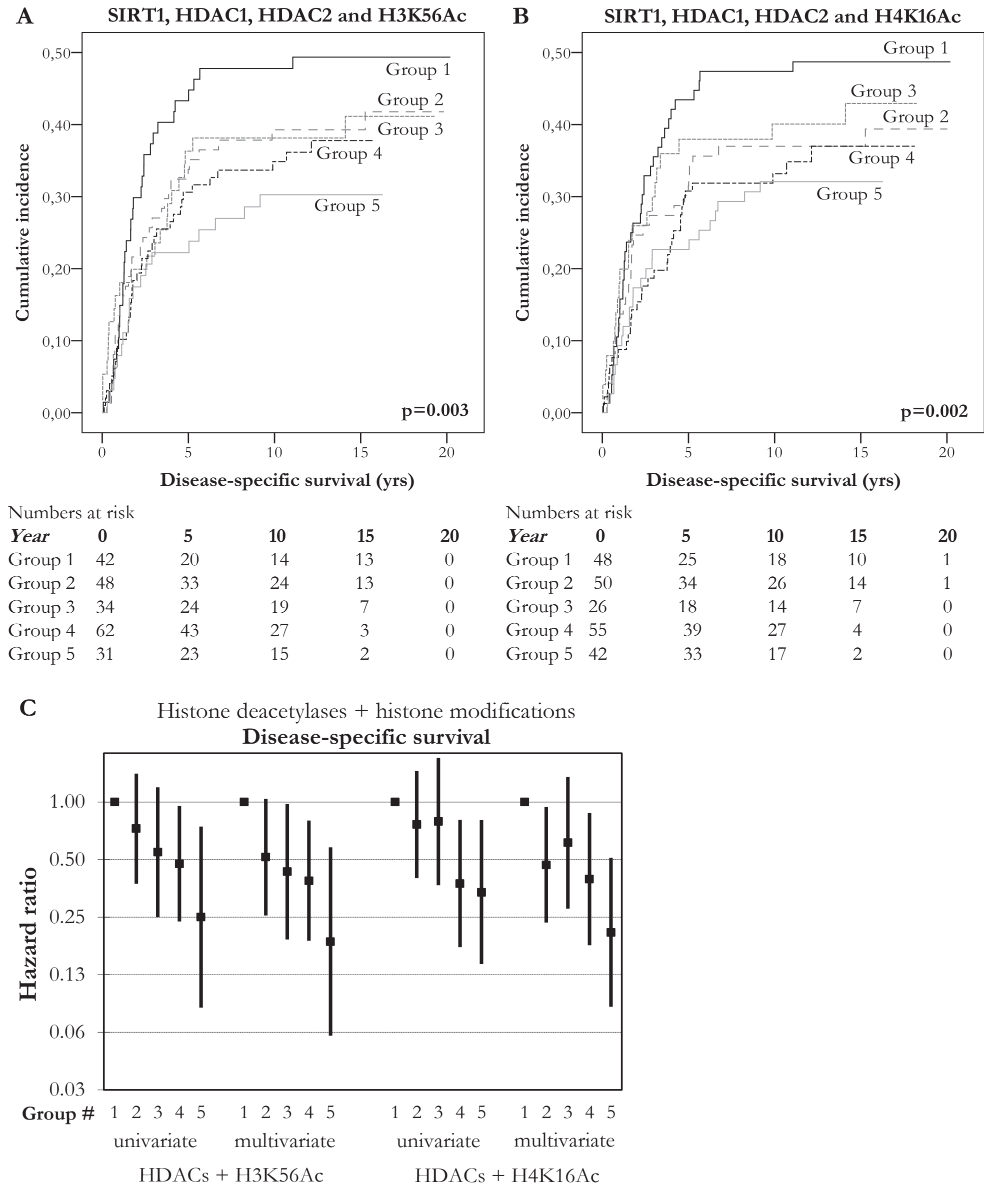
	LRRFS			DRFS		
Univariate	p-value	HR	(95% CI)	p-value	HR	(95% CI)
Combined markers	**0.004**	0.78	(0.66-0.93)	**0.01**	0.81	(0.68-0.95)
Multivariate						
Combined markers	**0.001**	0.74	(0.62-0.88)	**0.003**	0.77	(0.64-0.92)
Age at operation	**0.05**	1.23	(1.00-1.50)	**0.02**	1.29	(1.04-1.59)
Gender	0.67	0.91	(0.58-1.42)	0.7	0.93	(0.58-1.48)
TNM stage *1*	**<0.001**			**<0.001**		
2	0.3	1.51	(0.72-3.18)	0.2	1.62	(0.72-3.67)
3	**0.001**	3.63	(1.69-7.80)	**0.001**	3.99	(1.73-9.21)
Tumor location	**0.03**	1.71	(1.05-2.78)	0.2	1.4	(0.84-2.35)
Tumor size	**0.004**	1.14	(1.04-1.25)	**0.004**	1.15	(1.04-1.26)
MSS status *MSS*	0.36			0.6		
MSI	0.33	0.69	(0.34-1.44)	0.4	0.71	(0.34-1.48)
Unknown	0.37	1.32	(0.72-2.39)	0.8	1.07	(0.56-2.02)
Tumor in follow-up *	**<0.001**	3.39	(1.84-6.27)	**<0.001**	3.89	(2.07-7.30)
Adjuvant therapy	0.13	0.62	(0.33-1.16)	0.3	0.69	(0.36-1.32)

SIRT1, HDAC1, HDAC2 and H4K16Ac

	OS			DSS		
Univariate	p-value	HR	(95% CI)	p-value	HR	(95% CI)
Combined markers	**0.06**	0.89	(0.79-1.003)	**0.002**	0.75	(0.63-0.90)
Multivariate						
Combined markers	**0.01**	0.86	(0.76-0.97)	**0.001**	0.73	(0.60-0.88)
Age at operation	**<0.001**	1.91	(1.59-2.29)	**0.006**	1.39	(1.01-1.77)
Gender	0.8	1.05	(0.74-1.47)	0.8	0.93	(0.56-1.55)
TNM stage *1*	**0.005**			**<0.001**		
2	*0.07*	1.61	(0.95-2.73)	*0.07*	2.54	(0.94-6.86)
3	**0.002**	2.6	(1.42-4.14)	**<0.001**	7.72	(2.82-21.13)
Tumor location	0.3	1.26	(0.85-1.86)	*0.09*	1.62	(0.92-2.88)
Tumor size	**0.01**	1.1	(1.02-1.18)	**0.02**	1.14	(1.03-1.27)
MSS status *MSS*	0.9			0.4		
MSI	0.6	0.88	(0.53-1.47)	0.2	0.59	(0.24-1.42)
Unknown	0.9	0.96	(0.59-1.56)	0.6	1.23	(0.61-2.48)
Tumor in follow-up *	**0.005**	2.02	(1.24-3.29)	0.2	1.72	(0.80-4.24)
Adjuvant therapy	0.8	1.06	(0.65-1.73)	0.1	0.57	(0.29-1.13)

	LRRFS			DRFS		
Univariate	p-value	HR	(95% CI)	p-value	HR	(95% CI)
Combined markers	**0.003**	0.79	(0.68-0.93)	**0.01**	0.81	(0.69-0.95)
Multivariate						
Combined markers	**0.001**	0.77	(0.66-0.90)	**0.006**	0.79	(0.68-0.93)
Age at operation	**0.03**	1.25	(1.02-1.54)	0.01	1.32	(1.06-1.63)
Gender	0.5	0.85	(0.54-1.33)	0.6	0.87	(0.54-1.39)
TNM stage *1*	**0.001**			0.001		
2	0.2	1.57	(0.75-3.29)	0.2	1.66	(0.73-3.76)
3	**0.001**	3.49	(1.62-7.53)	0.001	3.94	(1.70-9.12)
Tumor location	**0.04**	1.67	(1.02-2.72)	0.2	1.37	(0.81-2.31)
Tumor size	**0.001**	1.16	(1.06-1.27)	0.002	1.17	(1.06-1.28)
MSS status *MSS*	0.4			0.7		
MSI	0.4	0.76	(0.36-1.57)	0.5	0.76	(0.36-1.58)
Unknown	0.3	1.34	(0.74-2.45)	0.8	1.08	(0.57-2.06)
Tumor in follow-up *	**<0.001**	3.22	(1.76-5.90)	<0.001	3.75	(2.01-6.99)
Adjuvant therapy	0.1	0.62	(0.33-1.17)	0.3	0.69	(0.36-1.32)

Figure 4



Numbers at risk (A)

Year	0	5	10	15	20
Group 1	42	20	14	13	0
Group 2	48	33	24	13	0
Group 3	34	24	19	7	0
Group 4	62	43	27	3	0
Group 5	31	23	15	2	0

Numbers at risk (B)

Year	0	5	10	15	20
Group 1	48	25	18	10	1
Group 2	50	34	26	14	1
Group 3	26	18	14	7	0
Group 4	55	39	27	4	0
Group 5	42	33	17	2	0

Survival analyses of the combined marker groups. *Shown are cumulative incidence curves after competing risk analyses for multi-marker analyses using histone deacetylases SIRT1, HDAC1 and HDAC2 combined with either H3K56Ac (**A**) or H4K16Ac (**B**). Group numbers 1-5 indicate the patient groups based on the number of markers showing high expression, with group 1 (all low), group 2 (one high), group 3 (two high), group 4 (three high), and group 5 (all high). In panel **C**, the hazard ratios (HR; y-axis) related to disease-specific survival (DSS) are shown for the combined HDACs (SIRT1, HDAC1 and HDAC2) with each of the histone modifications (H3K56Ac or H4K16Ac) compared to the reference group (group 1) for both univariate and multivariate analyses. HRs are indicated with ■, the corresponding 95% confidence intervals are indicated with protruding black lines.*

It seems paradoxical that although both HDAC1 and H3K56Ac showed no significantly different overall nuclear expression levels in normal versus tumor tissues in the whole study cohort, within individual tumors an inverse correlation was observed. Several studies have suggested different roles for HDAC1 in early stage versus advanced tumors (21,24), which suggests a qualitative difference rather than a quantitative difference between normal and tumor tissues. For both markers, survival differences were observed between patients with high and low nuclear expression in tumor cells of the respective markers, indicating that in individual tumors, aberrant expression of these markers could contribute to the tumorigenic process.

We have shown in this study that by combining multiple histone-modifying enzymes and histone modifications, distinct patient groups can be identified, stressing the importance of analyzing multi-protein complexes together. A higher number of markers showing high nuclear expression correlated with better patient survival and a lower chance of tumor recurrence. This finding can be explained by regarding the cellular functions of the histone deacetylases and the histone modifications. Higher expression of the histone deacetylases might prevent aberrant activation of oncogenes and DNA repetitive sequences. Higher levels of H4K16Ac, as discussed above, could be associated with silenced (methylated) repetitive sequences, which may result in less genomic events such as retrotransposition (LINE-1), translocations, or DNA double strand breaks. High levels of H3K56Ac are necessary for proper non-homologous end-joining, resulting in less error-prone repairs of double strand breaks and hence lower chances of developing rapidly mutating and aggressive tumors.

The immunohistochemical stainings presented in this study can be easily implemented in a clinical setting, as all stainings are performed on paraffin-embedded tissues. With the present-day technological advances using computer-based recognition software, the semi-automated scoring we used might be a first step towards automated scoring of nuclear staining in a clinical setting, thereby reducing the influence of subjectivity of human interpretation of color and color-intensity. Future studies could address the differences in epigenetic regulation between the tumor center and the tumor invasive front on whole tumor sections, as many studies have already reported differential expression of various proteins at different sites within the tumor (25). In addition, comparing tumor cells and the tumor microenvironment might provide useful information in understanding the role of epigenetic changes in colorectal cancer development and/or progression.

In conclusion, we have shown in this study that global nuclear expression of histone modifications and histone deacetylases were correlated to clinical outcome in colorectal cancers. Combining multiple markers gives us more insight into the complex interplay between histone modifiers and histone modifications. These results are a first indication that combining multiple epigenetic markers results in identification of distinct patient groups, and provide insight in the involvement of epigenetic mechanisms in colorectal cancer growth. More research is needed to study the exact functions of the studied histone deacetylases and their associated histone modifications, and to identify other combinations of epigenetic markers that play a role in colorectal cancer.

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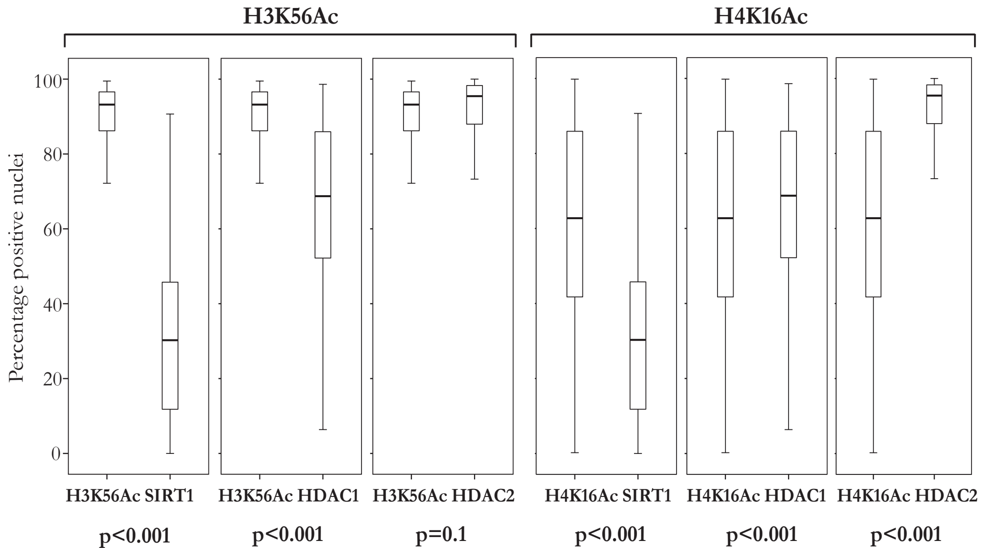
Supplementary files

Supplementary Figure 1



Expression of histone modifications versus histone deacetylases. *Shown are boxplots indicating mean nuclear expression levels (as determined using immunohistochemistry) in tumor cells of the histone modifications versus each of the individual enzymes. P-values represent paired students t-test results.*

Supplementary TABLE 1 (see next two pages)

Shown are the results of the univariate and multivariate analyses of the combined markers using the patient groups as categorical variables, with all p-values and hazard ratios (HR) with their 95% confidence intervals (95% CI). OS = overall survival, DSS = disease-specific survival, LRRFS = locoregional recurrence-free survival, DRFS = distant recurrence-free survival. Patients groups were made based on the number of markers showing high (above-median) expression: all low (group 1), 1 high (group 2), 2 high (group 3), 3 high (group 4) and all high (group 5). Significant values are shown in ***bold****, p-values showing a trend (between p=0.05 and p=0.1) in Italic. * =second primary tumor during follow-up period.*

Supplementary TABLE 1. Univariate and multivariate survival analyses individual groups combined markers

SIRT1, HDAC1, HDAC2 and **H3K56Ac**		**OS**			**DSS**			**LRRFS**			**DRFS**		
		Univariate			Univariate			Univariate			Univariate		
		p-value	HR	(95% CI)	p-value	HR	(95% CI)	p-value	HR	(95% CI)	p-value	HR	(95% CI)
Group 1		0.1			*0.07*			**0.05**			0.1		
Group 2		0.2	0.71	(0.44-1.15)	0.3	0.73	(0.37-1.41)	0.2	0.66	(0.36-1.19)	0.6	0.85	(0.46-1.58)
Group 3		0.2	0.67	(0.39-1.16)	0.1	0.55	(0.25-1.19)	**0.04**	0.48	(0.24-0.98)	0.2	0.63	(0.30-1.31)
Group 4		*0.08*	0.65	(0.40-1.06)	**0.04**	0.48	(0.24-0.95)	**0.04**	0.54	(0.29-0.98)	0.2	0.67	(0.36-1.26)
Group 5		**0.01**	0.44	(0.23-0.84)	**0.01**	0.25	(0.08-0.75)	**0.007**	0.29	(0.12-0.71)	**0.01**	0.29	(0.11-0.77)
		Multivariate			Multivariate			Multivariate			Multivariate		
		p-value	HR	(95% CI)	p-value	HR	(95% CI)	p-value	HR	(95% CI)	p-value	HR	(95% CI)
Group 1		**0.005**			**0.02**			**0.001**			**0.02**		
Group 2		**0.003**	0.45	(0.27-0.76)	*0.06*	0.52	(0.26-1.04)	**0.006**	0.39	(0.21-0.77)	*0.07*	0.54	(0.28-1.05)
Group 3		**0.03**	0.54	(0.31-0.94)	**0.04**	0.43	(0.19-0.97)	**0.009**	0.37	(0.18-0.78)	*0.09*	0.53	(0.25-1.13)
Group 4		**0.003**	0.47	(0.29-0.78)	**0.01**	0.38	(0.19-0.80)	**0.005**	0.41	(0.22-0.77)	0.1	0.59	(0.31-1.13)
Group 5		**0.001**	0.33	(0.17-0.65)	**0.004**	0.19	(0.06-0.58)	**<0.001**	0.16	(0.06-0.41)	**0.001**	0.16	(0.06-0.45)
Age at operation		**0**	1.89	(1.57-2.27)	**0.01**	1.36	(1.08-1.72)	**0.05**	1.22	(0.99-1.49)	**0.03**	1.28	(1.03-1.58)
Gender		0.7	1.06	(0.75-1.51)	0.9	1.01	(0.60-1.71)	0.8	0.94	(0.59-1.49)	0.8	0.94	(0.59-1.52)
TNM stage	*1*	**0.001**			**<0.001**			**<0.001**			**<0.001**		
	2	*0.1*	1.57	(0.93-2.67)	*0.06*	2.58	(0.95-7.01)	0.2	1.68	(0.80-3.53)	0.2	1.78	(0.78-4.06)
	3	**0.001**	2.75	(1.55-4.89)	**<0.001**	8.65	(3.13-23.92)	**<0.001**	4.42	(2.02-9.70)	**0.001**	4.47	(1.92-10.42)
Tumor location		0.2	1.28	(0.86-1.89)	0.1	1.62	(0.91-2.89)	**0.04**	1.67	(1.02-2.73)	0.3	1.34	(0.79-2.25)
Tumor size		**0.01**	1.09	(1.02-1.18)	**0.04**	1.12	(1.00-1.24)	**0.005**	1.14	(1.04-1.24)	**0.003**	1.15	(1.05-1.27)
MSS status	*MSS*	0.5			0.2			0.2			0.5		
	MSI	0.2	0.73	(0.43-1.24)	0.1	0.5	(0.21-1.22)	0.2	0.58	(0.28-1.23)	0.2	0.64	(0.30-1.34)
	Unknown	0.9	0.98	(0.59-1.59)	0.6	1.2	(0.59-2.45)	0.3	1.35	(0.73-2.48)	0.9	1.06	(0.55-2.03)
Tumor in follow-up *		**0.001**	2.26	(1.37-3.74)	0.2	1.78	(0.71-4.44)	**<0.001**	3.78	(2.03-7.06)	**<0.001**	4.68	(2.46-8.91)
Adjuvant therapy		0.9	0.97	(0.59-1.61)	*0.07*	0.53	(0.26-1.06)	**0.04**	0.5	(0.26-0.97)	0.1	0.57	(0.29-1.11)

SIRT1, HDAC1, HDAC2 and **H4K16Ac**		OS			DSS			LRRFS			DRFS		
		Univariate			Univariate			Univariate			Univariate		
		p-value	HR	(95% CI)	p-value	HR	(95% CI)	p-value	HR	(95% CI)	p-value	HR	(95% CI)
Group 1		0.3			**0.04**			**0.05**			**0.03**		
Group 2		0.1	0.68	(0.43-1.09)	0.4	0.76	(0.40-1.45)	0.3	0.71	(0.39-1.27)	0.5	0.83	(0.45-1.52)
Group 3		0.3	0.74	(0.42-1.28)	0.5	0.79	(0.37-1.70)	0.4	0.77	(0.39-1.51)	0.7	1.12	(0.58-2.17)
Group 4		*0.07*	0.64	(0.40-1.04)	**0.01**	0.37	(0.17-0.81)	**0.009**	0.42	(0.22-0.80)	**0.01**	0.41	(0.20-0.84)
Group 5		**0.05**	0.58	(0.34-0.99)	**0.01**	0.34	(0.14-0.80)	**0.02**	0.42	(0.21-0.86)	*0.06*	0.49	(0.24-1.03)
		Multivariate			Multivariate			Multivariate			Multivariate		
		p-value	HR	(95% CI)	p-value	HR	(95% CI)	p-value	HR	(95% CI)	p-value	HR	(95% CI)
Group 1		**0.04**			**0.008**			**0.02**			**0.009**		
Group 2		**0.03**	0.56	(0.34-0.94)	**0.03**	0.47	(0.23-0.94)	0.2	0.64	(0.35-1.18)	0.3	0.73	(0.39-1.39)
Group 3		*0.06*	0.58	(0.33-1.03)	0.2	0.61	(0.28-1.35)	0.6	0.81	(0.40-1.63)	0.4	1.32	(0.66-2.64)
Group 4		*0.09*	0.65	(0.39-1.06)	**0.03**	0.39	(0.18-0.88)	**0.03**	0.46	(0.23-0.92)	**0.04**	0.46	(0.22-0.98)
Group 5		**0.003**	0.43	(0.25-0.75)	**0.001**	0.21	(0.09-0.51)	**0.002**	0.3	(0.14-0.63)	**0.006**	0.34	(0.16-0.74)
Age at operation		**<0.001**	1.89	(1.58-2.27)	**0.005**	1.39	(1.10-1.77)	**0.04**	1.24	(1.01-1.52)	**0.02**	1.29	(1.05-1.60)
Gender		0.9	1.02	(0.72-1.44)	0.6	0.87	(0.51-1.47)	0.3	0.79	(0.50-1.27)	0.3	0.79	(0.49-1.28)
TNM stage	*1*	**0.001**			**<0.001**			**0.001**			**0.001**		
	2	*0.07*	1.63	(0.96-2.78)	*0.07*	2.56	(0.93-7.01)	0.3	1.51	(0.71-3.19)	0.4	1.48	(0.65-3.41)
	3	**0.001**	2.87	(1.59-5.19)	**<0.001**	8.9	(3.11-25.51)	**0.002**	3.55	(1.61-7.84)	**0.003**	3.62	(1.53-8.53)
Tumor location		0.21	1.29	(0.86-1.92)	*0.07*	1.73	(0.96-3.09)	**0.05**	1.63	(0.99-2.68)	0.3	1.3	(0.77-2.20)
Tumor size		**0.005**	1.11	1.03-1.19)	**0.007**	1.15	(1.04-1.27)	**0.001**	1.17	(1.07-1.28)	**<0.001**	1.19	(1.08-1.31)
MSS status	*MSS*	0.7			0.4			0.5			0.8		
	MSI	0.4	0.82	(0.49-1.38)	0.2	0.55	(0.23-1.35)	0.4	0.75	(0.36-1.56)	0.5	0.77	(0.37-1.60)
	Unknown	0.8	0.95	(0.58-1.55)	0.7	1.14	(0.56-2.31)	0.4	1.29	(0.71-2.37)	0.9	1.03	(0.54-1.97)
Tumor in follow-up *		**0.01**	1.92	(1.17-3.14)	0.2	1.81	(0.73-4.47)	**<0.001**	3.33	(1.78-6.24)	**<0.001**	4.42	(2.31-8.45)
Adjuvant therapy		0.9	0.99	(0.59-1.64)	*0.08*	0.53	(0.26-1.07)	0.1	0.61	(0.32-1.17)	0.3	0.69	(0.35-1.34)

Chapter 5

Prognostic value of Polycomb proteins EZH2, BMI1 and SUZ12 and histone modification H3K27me3 in colorectal cancer

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Abstract

Numerous changes in epigenetic mechanisms have been described in various types of tumors. In search for new biomarkers, we investigated the expression of Polycomb-group (PcG) proteins EZH2, BMI1 and SUZ12 and associated histone modification H3K27me3 in colorectal cancer. PcG proteins and histone modification H3K27me3 were immunohistochemically (IHC) stained on a tissue microarray (TMA), including 247 tumor tissues and 47 normal tissues, and nuclear expression was scored using the semi-automated Ariol system. Tumor tissues showed higher expression of EZH2 ($p=0.05$) and H3K27me3 ($p<0.001$) as compared to their normal counterparts. Combined marker trend analyses indicated that an increase in the number of markers showing high expression was associated with better prognosis. High expression of all four markers in the combined marker analyses was correlated with the best patient survival and the longest recurrence-free survival, with overall survival ($p=0.01$, HR 0.42 (0.21-0.84)), disease-free survival ($p=0.007$, HR 0.23 (0.08-0.67)) and local recurrence-free survival ($p=0.02$, HR 0.30 (0.11-0.84)). In conclusion, we found that expression of PcG proteins and H3K27me3 showed prognostic value in our study cohort. Better stratification of patients was obtained by combining the expression data of the investigated biomarkers as compared to the individual markers, underlining the importance of investigating multiple markers simultaneously.

Introduction

New prognostic biomarkers are warranted in colorectal cancer that could improve decisions for treatment of individual patients in addition to the current TNM (American Joint Committee on Cancer, AJCC (1)) staging system, as even patients with the same TNM classification present with large differences in patient survival and tumor recurrence (2,3). Epigenetic mechanisms have been identified as factors frequently deregulated in tumors and are attractive targets for biomarker research, because of their roles in regulating gene expression and their potentially reversible nature. Numerous changes in DNA methylation, histone modifications and their modifying enzymes have been described in various tumor types, including colorectal cancer (4-6). In this study, we focused on expression of histone-modifying enzymes of the Polycomb-group (PcG) and their associated histone modification, trimethylation of lysine 27 on histone H3 (H3K27me3), in colorectal cancer tissues.

The PcG proteins act in large multi-protein complexes, the so-called Polycomb repressive complexes (PRC) 1 and 2 (7). PcG proteins play an important role in embryonic development and cell proliferation (8,9), and are also involved in inducing epithelial-mesenchymal transition (EMT) (10). Aberrant expression of several PcG proteins and correlations with patient outcome have been reported in various cancers. For example, expression of BMI1 polycomb ring finger oncogene (BMI1), a component of PRC1 and an important factor in stem cells (11,12), was found to be correlated to patient outcome in several types of cancer (13-16). Enhancer of zeste homolog 2 (EZH2), a key protein in the PRC2 complex, was also found to have prognostic value in several types of cancer (17-20). SUZ12 polycomb repressive complex 2 subunit (SUZ12), another key component of the PRC2 complex, was found to have tumor-promoting functions in several cancers, including colon cancer (21-23). The associated histone modification H3K27me3 was found to be higher expressed in tumor tissues, and to be associated with better prognosis in non-small cell lung cancer (24) and breast cancer (19).

Using immunohistochemical staining (IHC) and semi-automated scoring, we studied the expression of PcG proteins EZH2, BMI1 and SUZ12 and their associated histone modification H3K27me3 in a cohort of 247 TNM stage I-III colorectal cancer patients, in correlation with clinical outcome. As the PcG proteins act together on the same histone modification, we hypothesized the combination of all four markers would be more informative with respect to clinical outcome as compared to each of the individual markers.

Materials and methods

Patient selection

Tumor tissues were collected from a consecutive series of 408 colorectal cancer patients who underwent surgical resection of their primary tumor at the Leiden University Medical Center (LUMC) between 1991 and 2001. Patients who underwent pre-operative treatment, who had bilateral tumors, or a history of cancer other than basal cell carcinoma or *in situ* tumors, were excluded from the study analyses. In addition, we included only patients with a histologically proven TNM stage I-III colorectal carcinoma, as determined by an experienced

pathologist. This resulted in a study cohort of 259 patients, with a mean follow-up of 8.6 years. Clinicopathological data were available for all patients in the study cohort. Data were right-censored when patients were alive or free of recurrence at their last follow-up date. Patient characteristics are displayed in Table 1. Patient records information was anonymized and de-identified prior to analysis according to national ethical guidelines ("Code for Proper Secondary Use of Human Tissue", Dutch Federation of Medical Scientific Societies), and approved by the Medical Ethical Committee of the Leiden University Medical Center (LUMC). This study was performed according to the REMARK guidelines (NCI-EORTC) (25).

Tissue microarray construction and immunohistochemistry

Formalin-fixed paraffin-embedded (FFPE) tumor tissues from each of the patients in the consecutive series of colorectal cancer patients (n=408) were collected from the LUMC pathology archives and used to construct a tissue microarray (TMA), as described previously (26). Sections of 4μm were cut from each TMA block and used for IHC staining. Histologically normal colorectal tissues, as determined by an experienced pathologist, from 47 patients with corresponding tumor tissues included in this study were also collected and prepared for IHC. The following antibodies were used for IHC: anti-EZH2 (612667, BD Biosciences, San Jose, CA, USA), anti-BMI1 (ab14389, Abcam, Cambridge, UK), anti-SUZ12 (ab12073, Abcam) and anti-H3K27me3 (ab6002, Abcam). All antibodies have been validated for use in immunohistochemistry by Western blot (27-30). All primary antibodies were used at predetermined optimal dilutions and IHC was performed using a standard IHC protocol (31). Briefly, endogenous peroxidase was blocked by incubating the sections in a 0.3% solution of hydrogen peroxide (in PBS) for 20 min. Antigen retrieval was performed by heating the sections for 10 min at 95ºC in a citrate buffer (pH 6; pH Low Target Retrieval Solution, Dako, Glostrup, Denmark) for EZH2, BMI-1 and H3K27me3 and by heating the sections for 10 min at 95ºC in a Tris-EDTA buffer (pH 9; pH High Target Retrieval Solution, Dako) for SUZ12. TMA sections were incubated with the respective primary antibodies overnight (16 hrs). Staining was visualized using the Dako REAL™ EnVision™ Detection System, Peroxidase/DAB+, Rabbit/Mouse (Dako). The stained TMA sections were scanned using a 20x magnification on the semi-automated Ariol system (Leica Microsystems, Wetzlar, Germany). Tumor cell areas (tumor tissues) and colon epithelium (in normal tissues) were marked on the computer screen upon visual inspection, followed by careful training of the Ariol system to correctly identify positively stained and negative nuclei within the marked tissue areas, for each of the markers separately. Nuclear expression, defined as the percentage of positively stained nuclei in the marked area of each tissue core, was then assessed by the Ariol software. Several random cores were evaluated for each TMA section by visual inspection after automated analysis in order to verify correct identification of positively stained nuclei.

Statistical analyses

Data were analyzed in consultation with a statistician (H.P.) using SPSS 20.0 for Windows (SPSS Inc, Chicago, USA). 12 patients were excluded from the statistical analyses, as not all data of all four markers was available for these patients, resulting in a final patient cohort consisted of 247 patients. As the individual marker data were not normally distributed (Shapiro-Wilk test),

TABLE 1. Patient characteristics of the study cohort

		All patients (n=408)		Study cohort (n=247)		
		n	(%)	n	(%)	p-values
Age at randomization						
	<50	45	11	32	13	
	50-75	267	65.4	155	62.8	
	>=75	96	23.6	60	24.3	0.69
Gender						
	Male	202	49.5	127	51.4	
	Female	206	50.5	120	48.6	0.66
TNM stage						
	I	78	19	52	21.1	
	II	149	36.7	110	44.5	
	III	114	27.9	85	34.4	0.21
	IV	67	16.4			
Tumor location						
	Colon	289	71	181	73.3	
	Rectum	119	29	66	26.7	0.6
Tumor size						
	Mean (cm)	4.68		4.71		
	Standard error	2.22		1.53		0.95
MSS status						
	MSS	275	67.2	169	68.4	
	MSI	46	11.2	34	13.8	
	Unknown	87	21.6	44	17.8	0.76
Tumor in follow up						
	No	347	85	209	84.6	
	Yes	61	15	38	15.4	0.91
Adjuvant therapy						
	No	323	79.2	199	80.6	
	Yes	85	20.8	48	19.4	0.97

Patient characteristics are shown for both the omplete colorectal cancer series (n=408) and the study cohort (n=247). Patient selection was based on availability of FFPE tissues and available data for all four studied markers. The study cohort selection was representative for the entire colorectal cancer series. P-values represent the results of Student's t-tests. For TNM stage, only tumor stage I-III of the complete patient cohort were compared to the patients in the study cohort.

non-parametric Wilcoxon signed-rank tests were performed to assess the differences in nuclear expression between tumor and paired normal tissues (n=47) for each of the markers. Spearman's signed rank correlation analyses were performed to investigate the correlation between nuclear expression of the individual PcG proteins and histone modification H3K27me3. Cox proportional hazard trend analyses were performed for univariate and multivariate survival analyses of individual markers. Covariates included in all multivariate analyses were age at operation, gender, TNM tumor stage (tumor stages I-III), tumor location, tumor size, microsatellite stability (MSS) status. Covariates "tumor in the follow up" and "adjuvant therapy" were entered as time-dependent covariates. Overall survival (OS) was defined as the time from surgery until death (by any cause). Disease-free survival (DFS) was defined as the time from surgery until the occurrence of a second primary colorectal tumor, locoregional recurrence or distant recurrence, or death by colorectal cancer. Locoregional recurrence-free survival (LRRFS) was defined as the time from surgery until the occurrence of a locoregional recurrence or death by cancer. Distant recurrence-free survival (DRFS) was defined as the time from surgery until the occurrence of a distant recurrence or death by cancer.

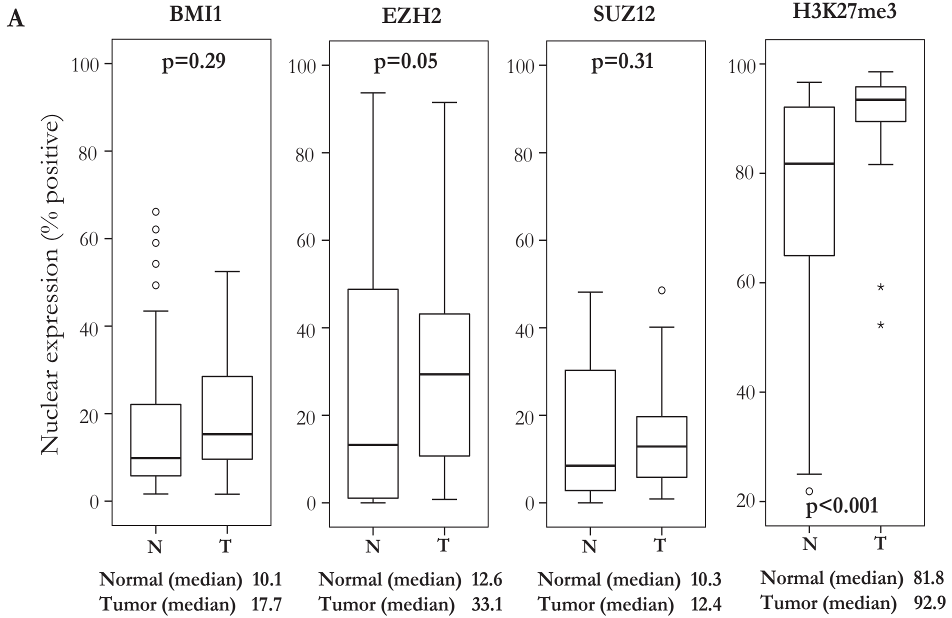
On the basis of the skewed distribution of expression data of each of the individual markers, the median expression was used to divide the patients into high expression (above-median) and low expression (below-median) groups. The four markers were then combined into a new variable, based on the number of markers showing high nuclear expression, resulting in the following grouping: all low (group 1), 1 high (group 2), 2 high (group 3), 3 high (group 4) and all high (group 5). Univariate and multivariate Cox proportional hazard analyses were performed using the group numbers as a categorical variable, using group 1 (all low) as the reference group. On the basis of these results we decided to combine patient groups 2, 3, and 4 into one patient group; all further statistical analyses were performed using three patient groups. In addition to the Cox proportional hazard analyses, trend analyses were performed using the group numbers as continuous variables to assess the influence of the combined markers on patient survival and tumor recurrence. Resulting hazard ratios (HR) represent the HR for each unit of increase (increase in group number). Cumulative incidence curves were made for DFS, LRRFS and DRFS, accounting for competing risks (32). Kaplan-Meier curves were used to visualize differences between the three patient groups for OS. For all statistical analyses, two-sided p-values ≤ 0.05 were considered statistically significant, and p-values $0.05 < p \leq 0.1$ were considered a trend.

Results

Expression in tumor versus paired normal colorectal tissues

Nuclear expression of all individual markers (EZH2, BMI1, SUZ12 and H3K27me3) in tumor tissues was compared to nuclear expression in paired normal colorectal tissues. When analyzing expression differences in the study cohort as a whole, only median H3K27me3 and EZH2 expression were significantly different between tumor and normal tissues ($p<0.001$ and $p=0.05$, respectively; Figure 1A). In individual tumors, however, all markers showed marked differences in expression compared to their normal counterparts (Figure 1B). Survival analyses based on below- or above-median expression in the normal tissues did not show differences in patient survival or tumor recurrence (data not shown).

Figure 1



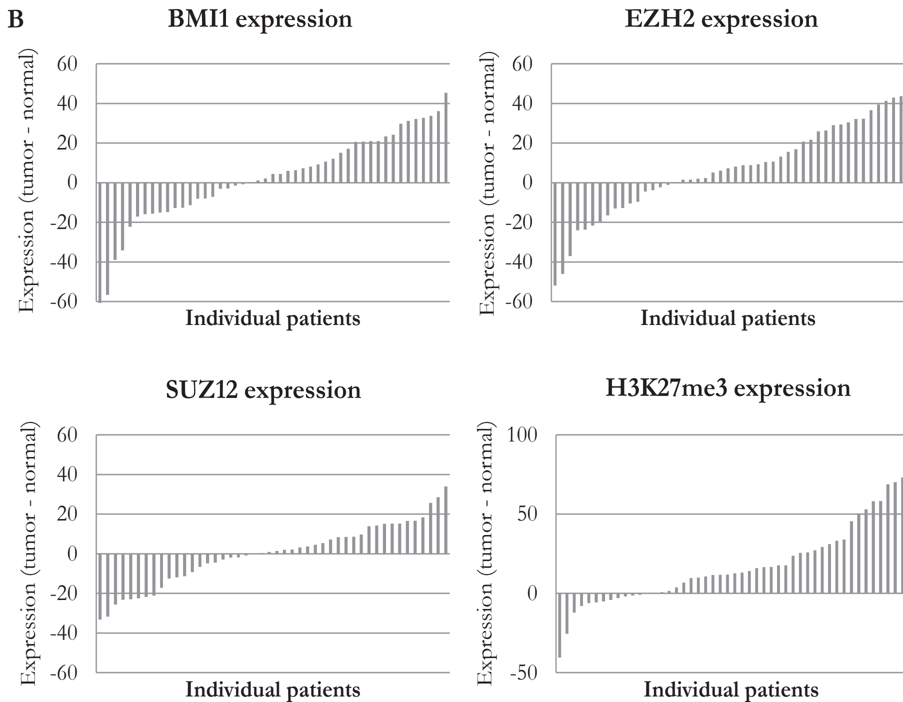


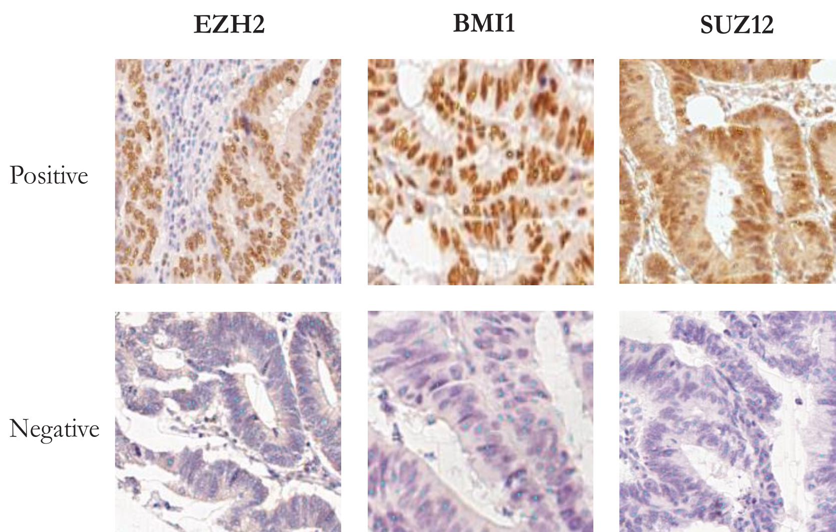
Figure legends: see next page

Figure 1 (see previous page) ***A***. *Differences in nuclear expression between normal and tumor tissues of individual markers.. Displayed are differences in nuclear expression, measured as the percentage of positively stained nuclei, between normal and tumor tissues (n=47). Boxplots show the median and range of expression of each of the individual markers in normal (N) and tumor (T) samples. The median percentages of positive nuclei are given for each of the markers. P-values represent statistical differences between normal and tumor samples, calculated using the Wilcoxon signed rank test..* ***B***. *Histograms show the difference in expression between tumor and paired normal tissues (y-axis) for each of the individual patients (x-axis). Differences in expression were calculated as follows: expression difference = expression in tumor tissue – expression in normal tissue. Negative values indicate higher expression in normal tissues, positive values indicate higher expression in tumor tissues.*

Individual marker analyses in tumor tissues

Examples of identification of positive and negative tumor cell nuclei for each of the individual markers by the Ariol system are shown in Figure 2. We first analyzed if the expression of histone modification H3K27me3 was correlated to the expression of the individual PcG proteins. The nuclear expression (percentage of positive nuclei) of H3K27me3 was indeed positively correlated with expression of EZH2 ($p<0.001$), BMI1 ($p<0.001$) and SUZ12 ($p=0.05$). No correlation was observed between the expression of the individual markers and TNM tumor stage. For survival analyses, patients were divided into low and high expression groups on the basis of the median expression of each of the individual markers, as given in Figure 1A. In survival analyses of individual markers, BMI1 showed strong correlations to patient survival (OS and DFS) and tumor recurrence (LRRFS and DRFS) in both univariate and multivariate analyses (Table 2). EZH2 and H3K27me3 showed significant correlations for DFS only.

Figure 2



Identification of positive and negative tumor cell nuclei by the Ariol system. *The Ariol system trainer overlay shows correct identification of positive (indicated by yellow dots) and negative (blue dots) nuclei in tumor cores. TMA slides were scanned using a 20x magnification. Shown for all markers are positively stained tumor cores (top row) and negative tumor cores (bottom row). The Ariol system was trained to identify positive and negative cells for each marker individually.*

TABLE 2. Univariate and multivariate survival analyses individual markers

			EZH2	BMI1	SUZ12	H3K27me3
OS	Univariate	p-value	*0.07*	**0.05**	0.9	0.5
		HR	0.74	0.73	1.03	0.89
		(95% CI)	(0.54-1.03)	(0.53-1.00)	(0.73-1.47)	(0.64-1.24)
	Multivariate	p-value	0.3	**0.009**	0.3	0.5
		HR	0.84	0.62	0.83	0.88
		(95% CI)	(0.60-1.18)	(0.44-0.89)	(0.57-1.20)	(0.62-1.24)
DFS	Univariate	p-value	**0.04**	*0.08*	0.8	*0.07*
		HR	0.64	0.68	0.95	0.66
		(95% CI)	(0.42-0.99)	(0.44-1.04)	(0.60-1.49)	(0.42-1.03)
	Multivariate	p-value	*0.08*	**0.03**	0.2	**0.05**
		HR	0.67	0.61	0.71	0.64
		(95% CI)	(0.43-1.05)	(0.39-0.96)	(0.44-1.17)	(0.41-0.99)
LRRFS	Univariate	p-value	*0.06*	**0.03**	0.8	0.2
		HR	0.67	0.63	1.06	0.76
		(95% CI)	(0.44-1.03)	(0.41-0.96)	(0.67-1.68)	(0.49-1.18)
	Multivariate	p-value	*0.1*	**0.005**	0.6	0.2
		HR	0.71	0.52	0.88	0.76
		(95% CI)	(0.46-1.11)	(0.33-0.82)	(0.55-1.42)	(0.49-1.19)
DRFS	Univariate	p-value	**0.04**	*0.06*	0.6	0.4
		HR	0.64	0.66	1.11	0.83
		(95% CI)	(0.42-0.99)	(0.48-1.02)	(0.69-1.77)	(0.53-1.29)
	Multivariate	p-value	*0.1*	**0.02**	0.7	0.6
		HR	0.73	0.58	0.91	0.89
		(95% CI)	(0.46-1.15)	(0.36-0.92)	(0.56-1.48)	(0.57-1.42)

Shown are the results of the univariate and multivariate analyses of all individual markers, with all p-values and hazard ratios (HR) and their 95% confidence intervals (95% CI). The "low expression" group was used as the reference group. OS = overall survival, DSS = disease-specific survival, LRRFS = locoregional recurrence-free survival, DRFS = distant recurrence-free survival. Significant p-values are indicated in ***bold****, p-values showing a trend (between 0.5 and 1.0) in Italic.*

For all three markers (BMI1, EZH2 and H3K27me3), high expression was associated with better patient survival as compared to the patients showing low expression, with p-values for DFS of p=0.07 (BMI1), p=0.04 (EZH2) and p=0.06 (H3K27me3). SUZ12 did not show any differences in patient survival or tumor recurrence based on low or high expression of the marker in tumor tissues.

Combined markers

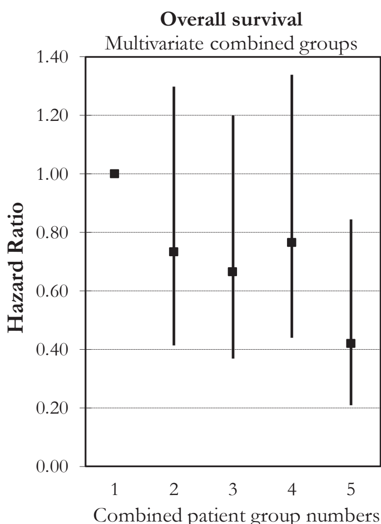
We hypothesized that combining multiple markers would result in better stratification of patients. Therefore, we performed statistical analyses on combinations of the histone-modifying enzymes. These analyses showed that combining multiple markers indeed resulted in statistically significant differences between the patient groups and more pronounced hazard ratios, indicating a more pronounced effect on patient survival. The combination of histone-modifying enzymes EZH2 and BMI1 showed significant differences for both patient survival and recurrence-free survival, with p=0.02 (HR=0.72; 95% CI 0.54-0.94) for DFS and p=0.012 (HR=0.71; 95% CI 0.54-0.92) for LRRFS in multivariate analyses. Combining EZH2 and SUZ12 showed a trend for DFS in multivariate analyses, with p=0.08 (HR=0.77; 95% CI 0.57-1.04). The combination of BMI1 and SUZ12 showed significant differences for patient survival, with p=0.02 (HR=0.76; 95% CI 0.61-0.96) for overall survival and p=0.05 (HR=0.73; 95% CI 0.54-1.00) for DFS.

Figure 3

A Trend analysis combined markers

EZH2, BMI1, SUZ12 and H3K27me3	OS			DSS			LRRFS			DRFS		
	p-value	HR	(95% CI)	p-value	HR	(95% CI)	p-value	HR	(95% CI)	p-value	HR	(95% CI)
Univariate	*0.09*	0.88	(0.77-1.02)	**0.04**	0.83	(0.69-0.99)	*0.08*	0.85	(0.71-1.02)	0.12	0.86	(0.72-1.04)
Multivariate	**0.05**	0.87	(0.76-1.00)	**0.01**	0.79	(0.65-0.95)	**0.04**	0.83	(0.69-0.99)	0.12	0.86	(0.71-1.04)

B



C **Overall survival combined markers**

Univariate	p-value	HR	(95% CI)
Group 1	*0.07*		
Group 2	**0.02**	0.53	(0.31-0.92)
Group 3	**0.02**	0.52	(0.29-0.90)
Group 4	*0.07*	0.62	(0.36-1.04)
Group 5	**0.01**	0.41	(0.21-0.80)

Multivariate	p-value	HR	(95% CI)
Group 1	0.2		
Group 2	0.3	0.73	(0.41-1.29)
Group 3	0.2	0.67	(0.37-1.20)
Group 4	0.3	0.77	(0.44-1.34)
Group 5	**0.02**	0.42	(0.21-0.84)

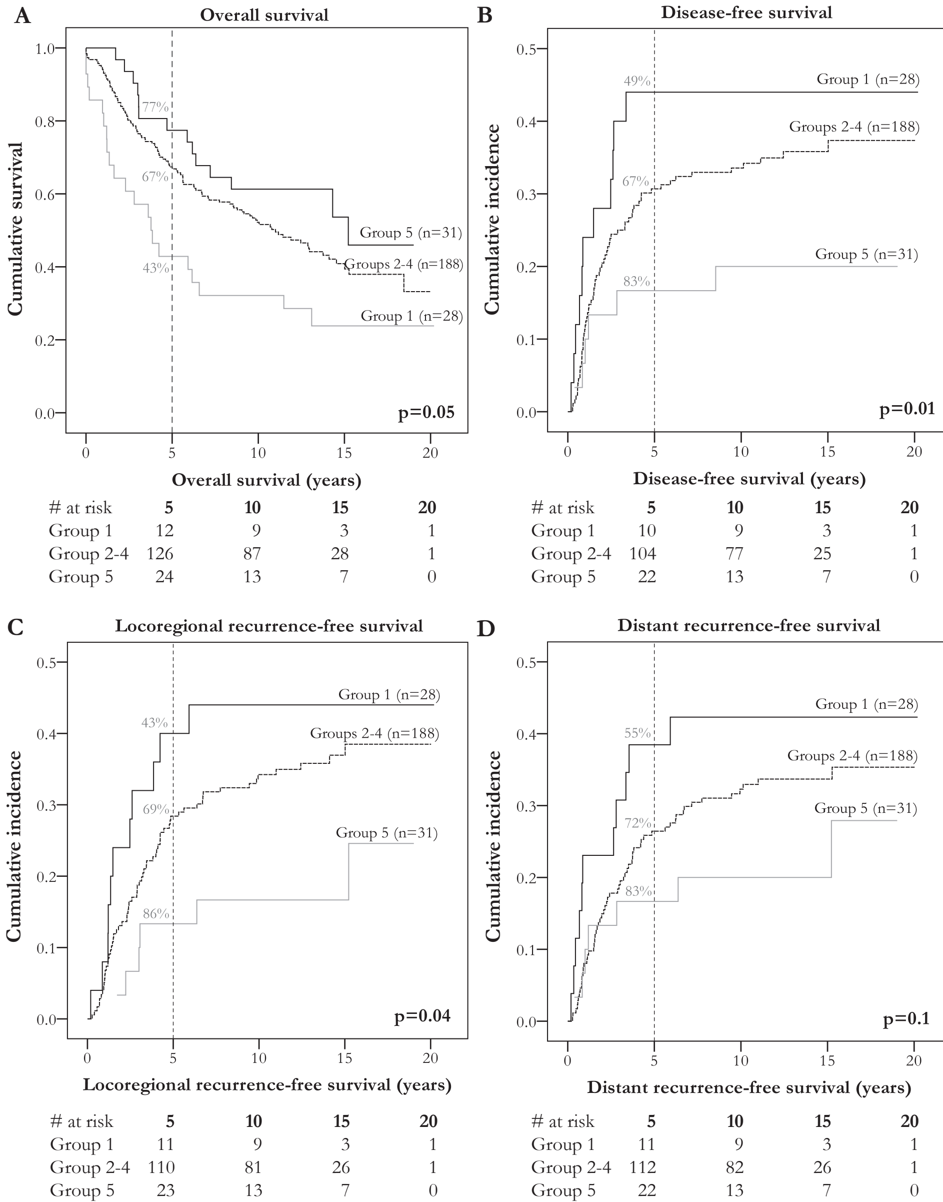
Trend analyses and hazard ratios of combined marker groups. *Patient groups were made on the basis of the number of markers showing high expression: all low (group 1), one high (group 2), two high (group 3), three high (group 4) and all high (group 5).* ***A****. Univariate and multivariate Cox regression trend analyses were performed using the combined marker groups as continuous variables. Hazard ratios per unit increase of each of the patient groups were plotted (***B***) and listed (***C***) for both the univariate and multivariate Cox regression trend analyses using the combined marker groups as categorical variables. The numbers of patients in the individual patient groups were: group 1 (n=28), group 2 (n=59), group 3 (n=55), group 4 (n=74) and group 5 (n=31).*

Because the three PcG proteins act together in multi-protein complexes to regulate H3K27me3 expression, we hypothesized that combining all markers (BMI1, EZH2, SUZ12 and H3K27me3) into one variable would result in even better stratification of patients. Patients were divided into five groups on the basis of the number of markers showing high nuclear expression. This resulted in the following patient groups: all low (group 1), one high (group 2), two high (group 3), three high (group 4) and all high (group 5). Patient characteristics of the 5 patient groups were comparable to the study cohort (Table S1). Multivariate trend analyses of the combined markers, using the patient group numbers as continuous variables, showed overall hazard ratios of 0.79-0.88 for each additional marker showing high nuclear expression in both univariate and multivariate analyses, indicating better patient survival and lower chances of tumor recurrence for each additional marker showing high expression (Figure 3A). When the patient group numbers were entered as categorical variables, a similar trend was observed (Figures 3B and 3C). Generally, hazard ratios for OS decreased with increasing group number, indicating a better patient survival when more markers were highly expressed compared to the "all low" expression group (group 1). Patients showing high expression of all markers, the "all high" group (group 5), showed the best overall survival (p=0.01, HR=0.42, 95% CI 0.21-0.84) as compared to reference group 1, which showed the shortest survival. Groups 2, 3 and 4 showed similar hazard ratios (Figures 3B and 3C), which was also reflected in the Kaplan-Meier survival curves that run close together as compared to the survival curves of groups 1 and 5. Therefore, we decided to combine the three patient groups 2,3 and 4 into one group, resulting in three patient groups (group 1, groups 2-4 and group 5). Kaplan-Meier curves and cumulative incidence plots showed significant differences between the three resulting patient groups for OS, DFS, LRRFS and a trend for DRFS, which were also reflected in the 5-year survival rates (Figure 4). The best patient survival and longest recurrence-free periods were observed for patients showing high expression of all four markers ("all high", group 5) in the tumor samples, with 5-year survival rates of 77% for OS, 83% for DFS and DRFS, and 86% for LRFS. Patients in combined groups 2-4 showed shorter OS, DFS and LRFS, with 5-year survival rates of 67% for OS and DFS, 69% for LRFS and 72% for DRFS. Patients in the "all low" group (group 1) showed significantly shorter OS, DFS and LRFS compared to either of the other patient groups, with 5-year survival rates of 43% for OS and LRFS, 49% for DFS and 55% for DRFS. Taken together, 5-year survival rates were lower when more markers showed low expression. The hazard ratios in both univariate and multivariate also reflected these findings (Table 3): group 5 shows the lowest hazard ratio as compared to reference group 1 (for example, multivariate HR=0.23 (0.08-0.67) for DFS). This indicates a lower risk of an event (patient death or locoregional tumor recurrence) for patients in the "all high" group for OS, DFS and LRFS. For DRFS, statistically significant results were only observed in univariate analyses.

Discussion

In addition to gene mutations, aberrant expression patterns of epigenetic regulators have been recognized as crucial events in the tumorigenic process, resulting in marked changes in gene expression. Changes in the expression of these epigenetic regulators include DNA methyltransferases and consequent changes in DNA methylation profiles, and histone-modifying enzymes and resulting changes in their corresponding histone modifications. In this study, we

Figure 4



Survival curves for the combined marker groups. *Combined marker (EZH2, BMI1, SUZ12 and H3K27me3) expression groups were divided into three patient groups: group 1, groups 2-4 and group 5. The numbers of patients in the individual patient groups were: group 1 (n=28), group 2 (n=59), group 3 (n=55), group 4 (n=74) and group 5 (n=31). Kaplan-Meier curves were made for overall survival (**A**) and cumulative incidence curves are shown for disease-free survival (**B**), locoregional recurrence-free survival (**C**) and distant recurrence-free survival (**D**). 5-year survival rates are given for each patient group. Tables below the curves indicate the numbers at risk (#) per group for the different time points.*

TABLE 3. Univariate and multivariate analyses combined markers

		Univariate			Multivariate		
		p-value	HR	(95% CI)	p-value	HR	(95% CI)
OS	Group 1	**0.018**			**0.05**		
	Groups 2-4	**0.014**	0.56	(0.35-0.89)	0.21	0.72	(0.44-1.19)
	Group 5	**0.009**	0.41	(0.21-0.80)	**0.01**	0.42	(0.21-0.84)
DFS	Group 1	*0.07*			**0.03**		
	Groups 2-4	0.1	0.64	(0.33-1.21)	0.2	0.61	(0.31-1.22)
	Group 5	**0.02**	0.32	(0.12-0.86)	**0.007**	0.23	(0.08-0.67)
LRRFS	Group 1	*0.06*			**0.03**		
	Groups 2-4	0.2	0.64	(0.34-1.22)	0.96	1.02	(0.49-2.09)
	Group 5	**0.02**	0.31	(0.11-0.83)	**0.02**	0.30	(0.11-0.84)
DRFS	Group 1	0.12			0.24		
	Groups 2-4	0.11	0.59	(0.31-1.13)	0.66	0.85	(0.41-1.75)
	Group 5	**0.05**	0.38	(0.15-0.98)	0.11	0.44	(0.16-1.20)

Results of Cox proportional hazard univariate and multivariate analyses are shown for combined markers EZH2, BMI1, SUZ12 and H3K27me3, with p-values and hazard ratios with their 95% confidence intervals. Patient groups were made based on the number of markers showing high expression: all low (group 1), one, two or three high (groups 2-4) and all high (group 5). Group 1 was used as the reference group. Covariates included in all multivariate analyses were age at operation, gender, TNM tumor stage, tumor location, tumor size, microsatellite stability (MSS) status, tumor in the follow up and adjuvant therapy. Significant values are shown in ***bold****, p-values showing a trend (between 0.5 and 1.0) in Italic.*

investigated the expression of three PcG proteins (EZH2, BMI1 and SUZ12) and associated histone modification H3K27me3 in colorectal cancer tissues. Aberrant expression of each of these histone-modifying enzymes, and of histone modification H3K27me3, has been indicated to contribute to tumorigenesis in several types of cancer and has been correlated to patient outcome (11-24). Studies in literature show conflicting results regarding the prognostic value of the Polycomb-group proteins in colorectal cancer. For example, high EZH2 expression has been associated with poor prognosis in a series of colorectal cancer patients by Wang *et al.* (33), whereas high EZH2 expression was found to be associated with better relapse-free survival in colon cancer patients (but not in rectal cancer patients) by Fluge *et al.* (34). In addition, high expression of BMI1 was found to correlate with good prognosis in breast cancer in a study by Pietersen *et al.* (35), whereas high BMI1 was associated with poor prognosis in colon cancer in a study by Du *et al.* (36). In our study cohort, survival data for the individual markers showed that high expression of all markers was correlated with better patient survival and longer recurrence-free periods as compared to patients showing low expression. The results found in this study correspond to our previous findings that high expression of H3K27me3 was associated with better patient survival in rectal tumors (4). In this study, we showed that high expression of H3K27me3 was indeed associated with better patient survival and longer recurrence-free

periods. As we showed that the expression of the PcG proteins was directly related to the expression of H3K27me3, we expected a similar correlation of expression of the PcG proteins with clinical outcome, which was indeed confirmed by the results presented in this manuscript. High levels of H3K27me3, because of aberrant expression of PcG proteins, might prevent aberrant expression of oncogenes, activation of retrotransposon sequences (such as LINE-1; 4), and result in other (epi)genomic events that promote tumor aggressiveness.

In addition to the individual markers, combinations of PcG proteins in correlation with patient outcome have been studied by several research groups. For example, co-expression of EZH2 and BMI1 was reported to be associated with poor prognosis in various cancers (37-39). In contrast, overexpression of EZH2 and BMI1 were reported to have different influences on patient prognosis in breast cancer (35), and was found to have no prognostic value in urothelial carcinoma of the bladder (40). In our colorectal cancer study cohort, all combinations of histone-modifying enzymes showed prognostic value. In order to obtain more information about epigenetic pathways with potential prognostic value in colorectal cancer, we performed multivariate survival analyses using combined expression data of multiple PcG proteins (EZH2, BMI1 and SUZ12) and their associated histone modification H3K27me3. Combining the three PcG proteins and their associated histone modification resulted in significantly better stratification of patient groups as compared to the individual markers. In combined marker analyses, the best patient survival and longest recurrence-free periods were observed for patients showing high expression of all four markers ("all high") in the tumor samples. Patients in the "all low" group showed significantly shorter OS, DFS and LRFS compared to either of the other patient groups. The results of the combined marker analyses underline the co-operation of these three enzymes in PcG complexes, and thus provide a better risk stratification of patients.

In addition to the roles of EZH2, BMI1 and SUZ12 in epigenetic regulation of chromatin structure and gene expression, direct regulation of protein function has been described for EZH2 and BMI1, including protein phosphorylation and ubiquitination. A cytosolic EZH2 and SUZ12-containing methyltransferase complex has been linked to actin polymerization, an important process in cell proliferation (41). Shuttling of the EZH2 and SUZ12 containing complex between different cellular compartments may explain the weak cytosolic staining observed for EZH2 and SUZ12 in addition to the strong nuclear staining for these markers, as compared to the strict nuclear staining observed for BMI1. Another non-histone protein methylated by EZH2 is cardiac transcription factor GATA4. Methylation reduces its transcriptional activity, resulting in inhibition of proper cardiac development (42). These examples indicate that aberrant expression of these PcG proteins influences key processes such as gene transcription and cell proliferation, promoting the transformation of normal cells into tumor cells.

In conclusion, we showed that combined expression of PcG proteins EZH2, BMI1 and SUZ12 and their associated histone modification H3K27me3 has prognostic value in our colorectal cancer study cohort. Combined marker expression resulted in better stratification of patients as compared to the individual markers and hence provides more insight into the roles of these epigenetic proteins and –modifications in colorectal cancer. Other combinations of epigenetic mechanisms should be investigated in colorectal cancer to further unravel the underlying biology in individual tumors. This will advance the search for new biomarkers to be used in a clinical setting in order to better classify patients for treatment.

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Supplementary TABLE 1 Patient characteristics of all patient groups used in combined-marker analyses

	Group 1 *(n=28)*		Group 2 *(n=59)*		Group 3 *(n=55)*		Group 4 *(n=74)*		Group 5 *(n=31)*		
	n	**(%)**	**n**	**(%)**	**n**	**(%)**	**n**	**(%)**	**n**	**(%)**	**p-values**
Age at randomization											
<50	1	3.6	9	15.3	9	16.4	10	13.5	3	9.7	
50-75	15	53.6	41	69.5	36	65.5	47	63.5	16	51.6	
>=75	12	42.9	9	15.3	10	18.2	17	23	12	38.7	0.81
Gender											
Male	14	50	28	47.5	27	49.1	36	48.6	15	48.4	
Female	14	50	31	52.5	28	50.9	38	51.4	16	51.6	0.99
TNM stage											
I	7	25	12	20.3	12	21.8	14	18.9	7	22.6	
II	9	32.1	27	45.8	25	45.5	31	41.9	18	58.1	
III	12	42.9	20	33.9	18	32.7	29	39.2	6	19.4	0.56
Tumor location											
Colon	18	64.3	44	74.6	40	72.7	61	82.4	18	58.1	
Rectum	10	35.7	15	25.4	15	27.3	13	17.6	13	41.9	0.85
Tumor size											
Mean (cm)	4.55		4.5		4.72		4.89		4.77		
Standard error	0.55		0.31		0.32		0.37		0.41		0.91
MSS status											
MSS	18	64.2	44	74.6	35	63.6	51	68.9	21	67.7	
MSI	5	17.9	7	11.9	10	18.2	8	10.8	4	12.9	
Unknown	5	17.9	8	13.6	10	18.2	15	20.3	6	19.4	0.73
Tumor in follow up*											
No	25	89.3	52	88.1	45	81.8	65	87.8	22	71	
Yes	3	10.7	7	11.9	10	18.2	9	12.2	9	29	0.52
Adjuvant therapy											
No	25	89.3	45	76.3	48	87.3	55	74.3	26	83.9	
Yes	3	10.7	14	23.7	7	12.7	19	25.7	5	16.1	0.13

*Patient characteristics are shown for all patient groups as used in the combined-marker analyses. The patient groups show comparable patient characteristics to the complete study cohort of 247 patients (Table 1). P-values represent the Jonckheere-Terpstra test used to test if samples came from the same distribution. For the variable "tumor size", a one-way ANOVA test was performed to test for statistical differences between the patient groups. * = second primary tumor during follow-up period. MSS = microsatellite stable, MSI = microsatellite instable.*

Chapter 6

Histone trimethylation at H3K4, H3K9 and H4K20 correlates with patient survival and tumor recurrence in early-stage colon cancer

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Abstract

Background: Post-translational modification of histone tails by methylation plays an important role in tumorigenesis. In this study, we investigated the nuclear expression of H3K4me3, H3K9me3 and H4K20me3 in early-stage colon cancer in relation to clinical outcome.

Methods: Tumor tissue cores of 254 TNM stage I-III colorectal cancer patients were immunohistochemically stained for H3K4me3, H3K9me3 and H4K20me3 and scored using the semi-automated Ariol system. Cox proportional hazard trend analyses were performed to assess the prognostic value of the combined markers with respect to patient survival and tumor recurrence.

Results: The histone methylation markers only showed prognostic value in early-stage (TNM stage I and II) colon cancer. Therefore, only this patient set (n=121) was used for further statistical analyses. Low nuclear expression of H3K4me3, and high expression of H3K9me3 and H4K20me3 were associated with good prognosis. In combined marker analyses, the patient group showing most favorable expression (low H3K4me3, high H3K9me3 and high H4K20me3) was associated with the best prognosis. Multivariate trend analyses showed significantly increased hazard ratios (HR) for each additional marker showing unfavorable expression, as compared to the "all favorable" reference group. The HR for disease-free survival was 3.81 (1.72-8.45; p=0.001), for locoregional recurrence-free survival 2.86 (1.59-5.13; p<0.001) and for distant recurrence-free survival 2.94 (1.66-5.22; p<0.001).

Conclusions: Combined nuclear expression of histone modifications H3K4me3, H3K9me3 and H4K20me3 is prognostic in early-stage colon cancer. The combination of expression of the three histone modifications provides better stratification of patient groups as compared to the individual markers and provides a good risk assessment for each patient group.

Introduction

In tumor cells, numerous changes in epigenetic regulation of gene expression have been reported (1). As epigenetic mechanisms are potentially reversible, they represent suitable targets for the development of new anti-cancer therapies. Both DNA methylation and histone modifications might therefore present as possible new biomarkers in cancer. In this study, we investigated the clinical prognostic value of several histone modifications in early-stage (TNM stage I and II) colon cancer.

Epigenetic regulation of gene expression through post-translational modification of histone proteins by methylation plays an important role in many biological processes, including cell-cycle regulation, DNA damage- and stress response, embryonic development and cellular differentiation (2). The most extensively studied histone methylation sites include histone H3 lysine 4 (H3K4), H3K9 and H4K20. Altered expression of these - and other - histone modifications has been reported in cancer (3). For example, expression of H3K4me3 was shown to have prognostic value in hepatocellular carcinoma (4) and renal cell carcinoma (5). Cancer-associated upregulation of H3K9me3 was prognostic in acute myeloid leukemia (6), salivary carcinoma (7) and bladder cancer(8). Expression of H4K20me3 was shown to be correlated to tumor progression and prognosis in non-small cell lung cancer (9). Marión *et al*. showed that loss of H4K20me3 contributed to telomere reprogramming and hence a higher tumorigenic potential (10). As these three histone methylation markers have been found to contribute to the tumorigenic process in various cancers, we hypothesized that these histone modifications would correlate to clinical outcome in colon cancer.

In addition to the individual functions of the histone modifications, they work together regulating gene expression and chromatin structure in different regions of the genome. H3K4me3 and H3K9me3 both regulate gene promoter activity and are mutually exclusive at promoter regions (11). H4K20me3 and H3K9me3 are both present on pericentric regions (12,13) and are critical for condensation of chromatin at these regions. Both H3K9me3 and H4K20me3 have also been found to be enriched on imprinted genes (14). The study by McEwen *et al.* also showed that all three histone methylation marks H3K4me3, H3K9me3 and H4K20me3 form a tri-mark signature on imprinting control regions (14). On the basis of the overlapping functions of the three histone methylation marks, we hypothesized that combining these three modifications in survival analyses would be more informative than the individual markers with respect to patient survival and tumor recurrence. A combination of high expression of activating histone modification H3K4me3 and low expression of silencing modifications H3K9me3 and H4K20me3 was expected to correlate with poor clinical outcome in colon cancer. Using immunohistochemistry and semi-automated scoring, nuclear expression of H3K4me3, H3K9me3 and H4K20me3 was determined on a tissue microarray of colorectal cancer patients, and subsequently correlated to clinical outcome.

Materials and methods

Patient selection

Tumor tissues were collected from a consecutive series of 409 colorectal cancer patients who underwent surgical resection of a primary colorectal tumor at the Leiden University Medical

Center (LUMC) between 1991 and 2001. Patients were excluded from the study analyses when patients had a history of cancer other than basal cell carcinoma or *in situ* tumors, had multifocal tumors or received preoperative treatment. Data were right-censored when patients were alive or free of recurrence at their last follow-up date. Patient records information was anonymized and de-identified prior to analysis according to national ethical guidelines ("Code for Proper Secondary Use of Human Tissue", Dutch Federation of Medical Scientific Societies), and approved by the Medical Ethical Committee of the LUMC. In the study cohort, we only included patients with TNM stage I-III tumors (n=259). Of 254 patients, complete data on all the studied markers were available. This study was performed according to the REMARK guidelines (NCI-EORTC) (15).

Tissue microarray construction and immunohistochemistry

Formalin-fixed paraffin-embedded (FFPE) tumor tissues from 409 colorectal cancer patients were collected from the LUMC pathology archives and used to construct a tissue microarray (TMA), as described previously (16). Three tumor tissue cores, and if available one normal tissue core, were included in the TMA for each patient. Sections of 4μm were cut from each TMA block and used for immunohistochemical (IHC) staining. Histologically normal colon tissues, as determined by an experienced pathologist, from 29 patients were also included and IHC stained. The following antibodies were used for IHC: anti-H3K4me3 (ab8580, ABcam, Cambridge, UK), anti-H3K9me3 (07-442, Millipore, Billerica, MA, USA) and anti-H4K20me3 (ab9053, Abcam). All primary antibodies were used at predetermined optimal dilutions and IHC was performed using a standard IHC protocol (17). Briefly, endogenous peroxidase was blocked by incubating the sections in a 0.3% solution of hydrogen peroxide (in PBS) for 20 min. Antigen retrieval was performed by heating the sections for 10 min at 95ºC in a citrate buffer (pH 6; pH Low Target Retrieval Solution, Dako, Glostrup, Denmark), followed by overnight (16 hrs) incubation of the respective primary antibodies. Staining was visualized using the Dako REAL™ EnVision™ Detection System, Peroxidase/DAB+, Rabbit (Dako). The stained TMA slides were scanned using a 20x magnification on the Ariol system (Leica Microsystems, Wetzlar, Germany), followed by marking the tumor cell areas or normal colon epithelium for each tissue punch upon visual inspection on the computer screen. The Ariol system is specifically designed to recognize cells, nuclei, cell membranes and pixel intensity. For each type of staining (membranous, cytoplasmatic or nuclear), different software packages are available. In the nuclear staining package, the system can be trained to recognize nuclei with a minimum pixel intensity that corresponds to positive staining. By carefully fine-tuning of the shape and intensity settings for each individual immunohistochemical staining, we verified that the system only counted positively stained nuclei. For each TMA section, several random cores were evaluated by visual inspection after automatic analysis in order to verify that the system correctly identified positively stained nuclei. Automatic analysis of the percentage of positively stained nuclei (nuclear expression) was performed by the Ariol system for each individual tissue core.

Statistical analyses

Data were analyzed in consultation with a statistician (H.P.) using SPSS 20.0 for Windows (SPSS Inc, Chicago, USA). The mean percentage of positive nuclei of the three tumor cores was calculated for each individual patient and this percentage was used for all statistical analyses.

TABLE 1. Patient characteristics of the study cohort

		Study cohort *(n=254)*		**Colon stage I+II** *(n=121)*	
		n	**(%)**	**n**	**(%)**
Age at randomization					
	<50	32	12.6	11	9.1
	50-75	161	63.4	80	66.1
	>75	61	24	30	24.8
Gender					
	Male	128	50.4	61	50.4
	Female	126	49.6	60	49.6
TNM stage					
	I	53	20.9	30	24.8
	II	113	44.5	91	75.2
	III	88	34.6		
Tumor location					
	Colon	187	73.6	121	100
	Rectum	67	26.4		
Tumor size					
	Mean	4.69		4.57	
	Standard error	2.32		0.21	
MSS status					
	MSS	175	68.9	20	16.5
	MSI	34	13.4	75	62
	Unknown	45	17.7	26	21.5
Tumor in follow up*					
	No	215	84.6	105	86.8
	Yes	39	15.4	16	13.2
Adjuvant therapy					
	No	206	81.1	117	96.7
	Yes	48	18.9	4	3.3

*Patient characteristics are shown for both the study cohort (n=254) and the patients with TNM stage I and II colon cancer as used for the statistical analyses (n=121). Patient selection was based on availability of FFPE tissues. available data for all three markers. and information about the listed covariables. The study cohort selection was representative for the entire colorectal cancer series. * = second primary tumor during follow-up period. MSS = microsatellite stable, MSI = microsatellite instable.*

Normality of the data was tested using the Shapiro-Wilk test. Non-parametric Wilcoxon signed-rank tests were performed to assess the differences in mean nuclear expression between the paired tumor and normal tissues (n=29) for each of the individual markers. The Cox proportional hazard model was used for univariate and multivariate survival analyses of individual and combined markers. Covariates included in all multivariate analyses were age at operation, gender, TNM tumor stage (tumor stages I-III), tumor location, tumor size, microsatellite stability (MSS) status. Additionally, covariates tumor in the follow up and adjuvant therapy were entered as time-dependent covariates. Patients in the study cohort (TNM stage I and II colon patients only) were divided into high and low expression groups based on the median expression of each of the markers separately. On the basis of the cellular function of each of the histone modifications, we expected low H3K4me3, high H3K9me3 and high H4K20me3 to be associated with a better prognosis ("all favorable"). For combinatorial analyses, patients were divided into groups on the basis of the number of favorable markers (all favorable, 1 favorable, 2 favorable and all unfavorable). Univariate and multivariate trend analyses were performed using the group numbers as continuous variables to assess the influence of the combined markers on patient survival and tumor recurrence. Resulting hazard ratios (HR) represent the HR for each unit of increase (increase in group number, and hence an increase in the number of markers showing unfavorable expression). Overall survival (OS) was defined as the time from surgery until death (by any cause). Disease-specific survival (DSS) was defined as the time from surgery until death by colorectal cancer. Loco-regional recurrence-free survival (LRRFS) was defined as the time from surgery until the occurrence of a (loco)regional recurrence or death by cancer. Distant recurrence-free survival (DRFS) was defined as the time from surgery until the occurrence of a distant recurrence or death by cancer. Cumulative incidence curves were made for DSS, LRRFS and DRFS, accounting for competing risks (18). Kaplan-Meier curves (for OS) or cumulative incidence curves (for DSS, LRRFS and DRFS) were used to visualize differences between the three patient groups for OS. For all statistical analyses, two-sided p-values ≤ 0.05 were considered as statistically significant, and p-values $0.05 < p \leq 0.1$ were considered a trend.

Results

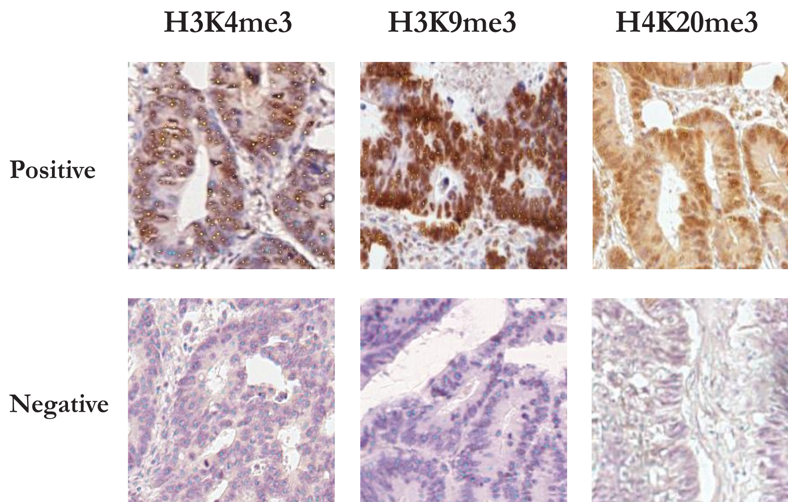
Patient selection for statistical analyses

In this study, we analyzed 254 patients with TNM stage I-III colorectal cancer, with no prior history of cancer or preoperative treatment and of whom complete clinicopathological data were available (Table 1). Combined marker analyses, on the basis of the number of favorable markers (as will be discussed below), showed statistically significant discrimination between patient groups in early-stage (TNM stage I and II) colon cancer (n=121). Multivariate trend analyses showed significant differences between the patient groups for patients with TNM stage I or II colon cancer (p=0.005), but no significant differences for patients with TNM stage I or II rectal cancer (p=0.256). For patients with TNM stage III, no significant differences were observed for patients with either colon (p=0.7) or rectal cancer (p=0.6). Together, these results indicate prognostic value of H3K4me3, H3K9me3 or H4K20me3 expression in early-stage colon cancer patients. Therefore, patients with TNM stage III colorectal cancer or TNM stage I and II rectum cancer were excluded from further analyses. The resulting patient cohort consisted of 121 patients with TNM stage I or II colon cancer, with a mean follow-up of 9.4 years.

Nuclear expression in tumor versus normal tissues

Comparison of expression between paired tumor and normal tissues was preceded by testing normality of expression distribution data in the stage I and II colon cancer tissues per histone modification using the Shapiro-Wilk test. As the data of the individual markers were not normally distributed, we used the Wilcoxon signed-rank test to compare the expression in the paired tumor and normal tissues (n=29). Marker expression was defined as the percentage of positively stained nuclei per tissue core. Representative staining of tumor tissue cores is shown in Figure 1. Statistically significant differences between tumor and normal samples were observed for H3K9me3 (p=0.001) and H4K20me3 (p=0.01), but not for H3K4me3 (p=0.9) (Figure 2A).

Figure 1

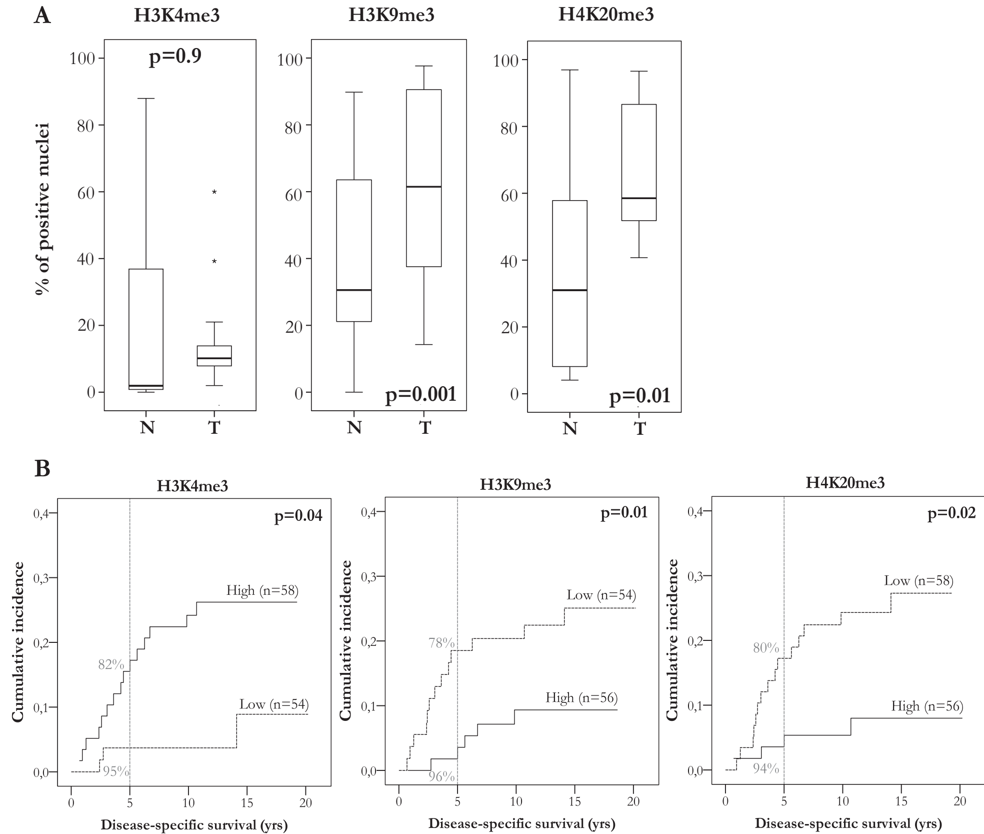


Correct identification of positively stained and negative nuclei for each individual marker. *The Ariol system trainer overlay shows correct identification of positive (indicated by yellow dots) and negative (blue dots) nuclei in tumor tissues. TMA slides were scanned using a 20x magnification. Shown for all individual markers are positively stained nuclei (top row) and negative tumor cores (bottom row).*

Survival analyses of individual markers

Median expression of each individual marker was used to divide the patients into high and low expression groups. The median expression for each of the individual markers in tumor tissues was 12.1% for H3K4me3, 65.5% for H3K9me3 and 65.4% for H4K20me3. Low expression of H3K4me3 was associated with better patient survival and lower chances of tumor recurrence (Figure 2B). In contrast, high expression of both H3K9me3 and H4K20me3 was associated with better patient survival and lower chances of tumor recurrence in our study cohort (Figure 2B). These findings are also reflected in the 5-year survival rates (Table 2). Both univariate and multivariate Cox regression analyses show significant differences between the low and high expression patient groups with respect to DSS, LRRFS and DRFS (Table 2).

Figure 2



Nuclear expression and survival analyses of individual markers. ***A****. Displayed are differences in nuclear expression, measured as the percentage of positively stained nuclei (y-axis), between normal and tumor tissues (n=29). Boxplots show the median and range of expression of each of the individual markers in normal (N) and tumor (T) samples (x-axis). P-values represent statistical differences between normal and tumor samples, calculated using the Wilcoxon signed rank test.* ***B****. Cumulative incidence curves, accounting for competing risks, showing the difference in survival between high and low expression groups of each of the individual markers. 5-year survival rates are included as percentages (in gray); p-values represent the statistical differences between the two patient groups in multivariate analyses. Numbers of patients in each group are indicated in each figure (n).*

Survival analyses of combinations of two markers

We analyzed the prognostic value of combinations of two of the histone methylation markers. As both H3K4me3 and H3K9me3 are mostly found on gene promoter regions, and H4K20me3 and H3K9me3 in constitutive chromatin at pericentric regions, we hypothesized that these combinations of two histone modifications would result in better stratification of patients as compared to the individual markers. Multivariate analyses showed that combining the histone modifications indeed resulted in better separation of the patient groups with respect to patient survival and tumor recurrence. For the combination of gene promoter-associated modifications H3K4me3 and H3K9me3, we observed that the patient group with the most unfavorable expression pattern (high H3K4me3 and low H3K9me3) showed the shortest disease-free

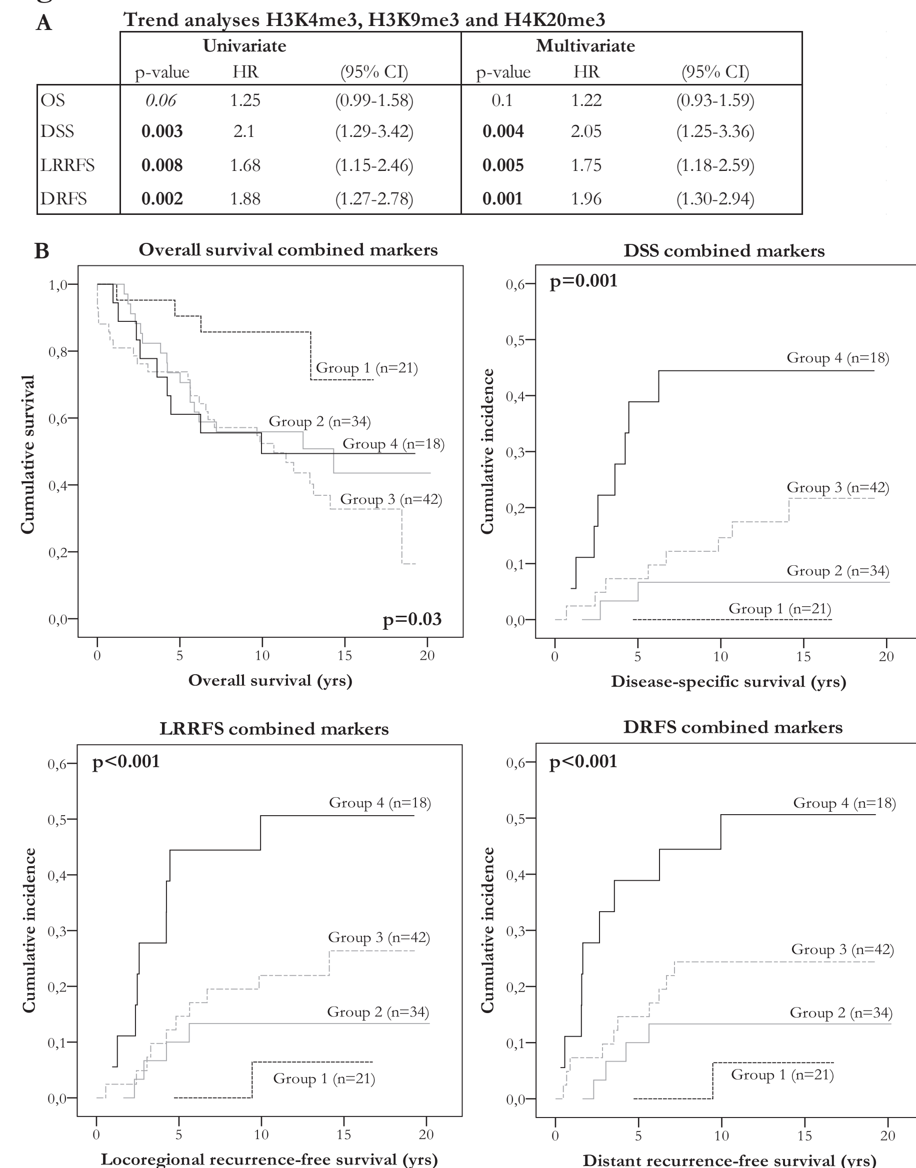
TABLE 2. Survival analyses single markers in TNM stage I and II colon cancer patients

		OS	DSS	LRRFS	DRFS
H3K4me3					
Univariate	p-value	0.4	**0.02**	**0.01**	**0.01**
	HR	1.26	4.45	3.54	3.63
	(95% CI)	(0.75-2.11)	(1.29-15.38)	(1.32-9.49)	(1.36-9.73)
Multivariate	p-value	0.3	**0.04**	**0.01**	**0.01**
	HR	1.36	3.79	3.86	3.57
	(95% CI)	(0.79-2.33)	(1.06-13.56)	(1.38-10.77)	(1.29-9.81)
5-year survival rates	Low expression	73%	95%	93%	94%
	High expression	76%	82%	74%	77%
H3K9me3					
Univariate	p-value	0.07	**0.02**	0.07	**0.02**
	HR	0.61	0.30	0.47	0.36
	(95% CI)	(0.36-1.04)	(0.12-0.86)	(0.21-1.08)	(0.16-0.85)
Multivariate	p-value	0.2	**0.01**	**0.05**	**0.01**
	HR	0.69	0.26	0.42	0.29
	(95% CI)	(0.39-1.24)	(0.09-0.77)	(0.17-1.01)	(0.12-0.75)
5-year survival rates	Low expression	64%	78%	71%	74%
	High expression	86%	96%	92%	92%
H4K20me3					
Univariate	p-value	0.1	**0.01**	**0.02**	**0.04**
	HR	0.67	0.24	0.34	0.41
	(95% CI)	(0.40-1.12)	(0.08-0.72)	(0.14-0.81)	(0.18-0.95)
Multivariate	p-value	**0.02**	**0.02**	**0.008**	**0.01**
	HR	0.51	0.21	0.29	0.31
	(95% CI)	(0.29-0.89)	(0.06-0.67)	(0.12-0.72)	(0.13-0.77)
5-year survival rates	Low expression	67%	80%	74%	77%
	High expression	80%	94%	90%	91%

Shown are the results of the univariate and multivariate analyses of all individual markers in TNM stage I and II colon cancer patients, with all p-values and hazard ratios (HR) with their 95% confidence intervals (95% CI). OS = overall survival, DSS = disease-specific survival, LRRFS = locoregional recurrence-free survival, DRFS = distant recurrence-free survival. The low expression group (below-median expression) was used as reference group. 5-year survival rates are given for both low and high expression groups. Statistically significant differences (defined as p<0.05) are shown in ***bold****, trends (p<0.1) in Italic.*

survival (trend analysis HR 2.05; p=0.004) and distant recurrence-free survival (trend analysis HR 1.96; p=0.001) as compared to the other patient groups. For the combination of pericentric region-associated modifications H4K20me3 and H3K9me3, the group with the most favorable expression (high expression of both markers) showed significantly better disease-free survival (HR 2.01; p=0.005) and distant recurrence-free survival (HR 1.77; p=0.004) as compared to the other patient groups.

Figure 3



A

Trend analyses H3K4me3, H3K9me3 and H4K20me3

	Univariate			Multivariate		
	p-value	HR	(95% CI)	p-value	HR	(95% CI)
OS	*0.06*	1.25	(0.99-1.58)	0.1	1.22	(0.93-1.59)
DSS	**0.003**	2.1	(1.29-3.42)	**0.004**	2.05	(1.25-3.36)
LRRFS	**0.008**	1.68	(1.15-2.46)	**0.005**	1.75	(1.18-2.59)
DRFS	**0.002**	1.88	(1.27-2.78)	**0.001**	1.96	(1.30-2.94)

***Trend and survival analyses of all markers combined**. **A**. Results of the univariate and multivariate trend analyses of combined markers H3K4me3, H3K9me3, and H4K20me3. HR represents the hazard ratio for each unit of increase, thus each additional marker showing unfavorable expression. 95% CI: 95% confidence interval for each HR. OS: overall survival; DSS: disease-specific survival; LRRFS: locoregional recurrence-free survival; DRFS: distant recurrence-free survival. **B**. Kaplan-Meier curves are shown for OS, including the number of patients in each patient group (n), based on the number of markers showing unfavorable expression. Patients were divided into the following groups: all favorable (group 1; H3K4me3 low and both H3K9me3 and H4K20me3 high), one out of three unfavorable (group 2), two out of three unfavorable (group 3), and all unfavorable (group 4; H3K4me3 high and both H3K9me3 and H4K20me3 low). Cumulative incidence curves, accounting for competing risks, are shown for DSS, LRRFS and DRFS. Multivariate p-values have been included in each of the combined marker graphs.*

Survival analyses of H3K4me3, H3K9me3 and H4K20me3 combined

To further improve the stratification of patients, we performed Cox regression survival analyses using the combined expression patterns of all three markers H3K4me3, H3K9me3 and H4K20me3. Low expression of activating modification H3K4me3 and high expression of silencing modifications H3K9me3 and H4K20me3 was expected to be associated with good prognosis, and was therefore used as the "all favorable" reference group. Patients were divided into 4 groups, on the basis of the number of markers showing clinically favorable or unfavorable expression. This resulted in the following grouping: all favorable (group 1; H3K4me3 low and both H3K9me3 and H4K20me3 high), one out of three unfavorable (group 2), two out of three unfavorable (group 3), and all unfavorable (group 4; H3K4me3 high and both H3K9me3 and H4K20me3 low). Both univariate and multivariate trend analyses showed that the more markers showed unfavorable expression, the shorter the patient survival (DSS) and recurrence-free survival times (both LRRFS and DRFS) (Figure 3A). The survival plots of OS, DSS, LRRFS and DRFS are shown in Figure 3B. Hazard ratios for the individual patient groups could not be calculated accurately, as not enough events (either death or recurrence of the tumor) occurred in the reference group (group 1; Figure 3B). Therefore, using multivariate trend analyses, we calculated hazard ratios for each additional marker showing unfavorable expression, as compared to the "all favorable" reference group. The calculated HRs were 1.46 (1.04-2.05; $p=0.03$) for OS, for DSS 3.81 (1.72-8.45; $p=0.001$) for DSS, 2.86 (1.59-5.13; $p<0.001$) for LRRFS and 2.94 (1.66-5.22; $p<0.001$) for DRFS. Combining all three markers resulted in better stratification and separation of the patient groups as compared to the single markers or the combinations of only two of the studied markers.

Discussion

Aberrant gene expression is a common feature of cancer cells, which is caused by a combination of gene mutations and aberrant regulation of gene expression by epigenetic mechanisms, including DNA methylation, microRNAs and histone modifications. Histone modifications play a crucial role in many cellular processes during embryonic development, cell proliferation and cellular differentiation (2). In cancer, aberrant expression of histone modifications has been described frequently (1). Therefore, we investigated the nuclear expression of three well-studied histone modifications in colon cancer.

In this study, we observed that nuclear expression of histone trimethylation on H3K4, H3K9 and H4K20 has prognostic value in early-stage colon cancer. Changes in expression of key histone modifications are found in early-stage tumors, which would be expected because tumor cells require instant changes in gene expression and chromatin structure in order to promote cell proliferation and tumor cell survival. Several epigenetic factors have been shown to be altered in early-stage cancer, including histone-modifying enzymes and histone modifications (19,20), DNA methylation (21,22) and microRNAs (23). We only observed differences between the patient groups in colon tumors, whereas in rectum tumors no difference was observed. The observed differences between the colon and rectum tumors with respect to the studied histone modifications may be due to differences in biology of the tissues of origin. Several other studies have suggested that rectum and colon tumors show differential gene expression signatures (24,25). This could be due to changes in epigenetic regulatory mechanisms. Detection

of aberrant expression of prognostic histone modifications, such as described in this study in early-stage colon cancer, could facilitate the risk assessment and subsequent decisions for treatment for specific patient groups at early stages of the disease.

The results of the survival analyses of the individual markers reflect our expected results based on the cellular functions of the respective histone modifications. Trimethylation of H3K4 is a modification found on gene promoter regions and is associated with activation of gene transcription (26), and higher expression of H3K4me3 in tumors could lead to aberrant gene transcription, including genes required for cell survival, proliferation and migration. In literature, poor prognosis was indeed reported for patients with tumors showing high expression of H3K4me3 (4). Histone modification H4K20me3 is a known repressive mark (13), and key modification regulating compaction of the chromatin in pericentric regions, which makes it crucial for proper chromosome segregation during cell division and for maintenance of genome integrity (12). Consequently, loss of H4K20me3 was expected to be associated with a worse prognosis for the patient, which has indeed been shown in literature (9). Finally, for H3K9me3, literature shows conflicting results with respect to patient survival and prognosis (6,7,27,28), depending on the type of cancer. Histone modification H3K9me3 is associated with silencing of gene transcription (26), and can hence be involved in aberrant silencing of tumor suppressor genes (i.e. DCC; 29). On the other hand, H3K9me3 prevents aberrant expression of (onco) genes and represses the abundant repetitive sequences in the genome (30-32). On the basis of the function of H3K9me3 as a silencing modification, we expected H3K9me3 expression to be comparable to H4K20me3 expression with respect to clinical outcome. Our results confirmed the hypotheses based on these individual functions, as high expression of H3K4me3 and low expression of H3K9me3 and H4K20me3 are correlated with shorter patient survival and higher chances of tumor recurrence.

Combined marker analyses showed that favorable expression of all markers (low H3K4me3, high H3K9me3 and high H4K20me3, as based on the individual marker analyses) was associated with the best prognosis with respect to patient survival and tumor recurrence. With each additional marker showing more unfavorable expression, the HR increased significantly about 3-fold for DSS, LRRFS and DRFS, indicating that combining all three methylation marks resulted in better separation of the patient groups as compared to individual markers. Combining multiple markers in survival analyses can thus be beneficial in identifying high-risk patient groups and to determine treatment strategies accordingly. To our knowledge, this is the first study to combine these three markers in survival analyses. In literature, multiple histone modifications have been studied in cancer tissues but have never been combined in survival analyses (27,33-36). In addition, expression of histone modifications was not always correlated to clinical outcome (37), or were found to have no prognostic value in cancer (38).

Conclusions

In conclusion, in this study we have shown that combined nuclear expression of histone trimethylation on H3K4, H3K9 and H4K20 is prognostic in early-stage colon cancer and that combined expression of the three histone modifications provides better stratification of patient groups and therefore provides a better risk assessment as compared to the individual markers. The clinically prognostic value of the histone modifications presented in this study underlines the consequences of epigenetic dysregulation in tumorigenesis.

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Chapter 7

Chromatin status of apoptosis genes correlates with sensitivity to chemo-, immune- and radiation therapy in colorectal cancer cell lines

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Abstract

The apoptosis pathway of programmed cell death is frequently deregulated in cancer. An intact apoptosis pathway is required for proper response to anti-cancer treatment. We investigated the chromatin status of key apoptosis genes in the apoptosis pathway in colorectal cancer cell lines in relation to apoptosis induced by chemo-, immune- or radiation therapy. Using chromatin immunoprecipitation (ChIP), we measured the presence of transcription-activating histone modifications H3Ac and H3K4me3 and silencing modifications H3K9me3 and H3K27me3 at the gene promoter regions of key apoptosis genes Bax, Bcl2, Caspase-9, Fas (CD95) and p53. Cell lines DLD1, SW620, Colo320, Caco2, Lovo and HT29 were treated with cisplatin, anti-Fas or radiation. The apoptotic response was measured by flow cytometry using propidium iodide and annexin V-FITC. The chromatin status of the apoptosis genes reflected the activation status of the intrinsic (Bax, Bcl2, Caspase-9 and p53) and extrinsic (Fas) pathways. An active intrinsic apoptotic pathway corresponded to sensitivity to cisplatin and radiation treatment in cell lines DLD1, SW620 and Colo320. An active Fas promoter corresponded to an active extrinsic apoptotic pathway in cell line DLD1. mRNA expression data correlated with the chromatin status of the apoptosis genes as measured by ChIP. In conclusion, the results presented in this study indicate that the balance between activating and silencing histone modifications, reflecting the chromatin status of apoptosis genes, can be used to predict the response of tumor cells to different anti-cancer therapies and could provide a novel target to sensitize tumors to obtain adequate treatment responses.

Introduction

Resistance to cell death is one of the capabilities acquired during tumor development and was therefore named as one of the hallmarks of cancer (1,2). The apoptosis pathway, responsible for programmed cell death, is indeed one of the pathways frequently deregulated in cancer (3). The level of apoptosis has been previously shown to have prognostic value in rectal cancer (4-6). As deregulation of the apoptotic pathway could lead to resistance to anti-cancer therapies, reactivation of the pathway is an attractive target to sensitize tumors for anti-cancer treatment (7-9). Both the extrinsic and intrinsic apoptotic pathways have been studied as possible targets for anti-cancer therapy (10), but more information about the complex regulation of the pathway is still warranted for the design of apoptosis-based anti-cancer therapies for solid tumors.

Anti-cancer treatments are directed towards inducing cell death in tumor cells by inducing DNA damage (activating the intrinsic apoptotic pathway) or by initiating an antitumor immune response (activating the extrinsic apoptotic pathway). An intact apoptotic response is required in order for these treatment regimens to have the intended effect of tumor cell death (11,12). Epigenetic mechanisms, including DNA methylation and histone modifications, are key regulators of gene expression and are frequently deregulated in cancer (13-15). Changes in epigenetic regulation of expression of apoptosis genes could influence the response of tumor cells to anti-cancer treatments. Therefore, in this study we investigated whether the chromatin status of key apoptosis genes in both the intrinsic and extrinsic apoptotic pathways could be used to predict the response of a tumor cell to anti-cancer treatment regimens.

We selected several apoptosis genes on the basis of their prognostic value in various cancers in literature (16,17), that are likely to play key roles in the apoptotic process. The selected genes were Fas (CD95) representing the extrinsic apoptotic pathway, and Bax, Bcl2, Caspase-9 (Casp9) and p53 representing the intrinsic pathway. We studied the cellular mRNA levels and the presence of both activating and silencing histone modifications at the promoter regions of each of these apoptosis genes using chromatin immunoprecipitation (ChIP) in six colorectal cancer cell lines. Subsequently, activation of the extrinsic apoptotic pathway was studied in the colorectal cancer cell lines using anti-Fas antibodies, and activation of the intrinsic pathway was studied using the chemotherapeutic agent cisplatin or by radiation treatment. The level of apoptosis induction upon treatment was then measured by flow cytometry and correlated to the chromatin status of the apoptosis genes as measured by ChIP. The chromatin status of each of the apoptosis genes was correlated to mRNA expression levels using gene expression assays.

Materials and methods

Cell lines and treatment

Colorectal cancer cell lines HT29, Lovo, Colo320, SW620, DLD1 and Caco2 were cultured under standard conditions, as described by the American Type Culture Collection (ATCC; Manassas, VA, USA), using T25 tissue culture flasks (Greiner-Bio One, Alphen aan den Rijn, The Netherlands). Dose-response curves were generated to determine an optimal dose and incubation time at which a maximum of 20% cell death (corresponding to 80% surviving cells) was measured. Cell lines were treated with different doses (based on findings in literature) and

incubation times tested were 8, 16, 24 and 48 hours. After incubation, cells were harvested and cell death was quantified using trypan blue staining. The percentage of surviving cells was determined as follows: 100% - (number of dead cells / total number of cells (dead+surviving)). Cells were treated with cisplatin (cis-Diammineplatinum (II) dichloride; Sigma-Aldrich, St. Louis, MO, USA) or anti-Fas (clone CH11; Millipore, Billerica, MA, USA) or were irradiated with X-rays at 6 Gy/min in tissue culture medium using an Andrex Smart 225 radiation instrument (200kV, 4mA; Andrex Radiation Products AS, Copenhagen). Following treatment with cisplatin, anti-Fas or radiation, the percentage of apoptotic cells was determined using flow cytometric analysis on a BD LSRII flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Apoptotic cells were defined as cells with a positive signal for annexin V-FITC (5µl in a total volume of 100µl; ImmunoTools 31490013, Friesoythe, Germany) and a low signal for propidium iodide (PI, 1µM; Calbiochem, San Diego, CA, USA). Cell lines were considered as sensitive to treatment when a ≥5% increase in the percentage of apoptotic cells between the untreated controls and the treated cell lines was detected.

Chromatin immunoprecipitation experiments

Chromatin immunoprecipitation (ChIP) experiments were performed for the following histone modifications: acetylated histone H3 (H3Ac, activating), trimethylation of lysine 4 at histone H3 (H3K4me3, activating), trimethylation of lysine 9 at histone H3 (H3K9me3, silencing), and trimethylation of lysine 27 at histone H3 (H3K27me3, silencing). ChIP experiments were performed according to a custom protocol, as described in (18), with some modifications. All ChIP experiments were performed in duplicate. Briefly, cell lines were trypsinized, centrifugated for 3 minutes at 1000 rpm and washed once with phosphate-buffered saline (PBS). Cells were then incubated in a 1% formaldehyde solution (in PBS) for 10 minutes at room temperature. The crosslinking reaction was stopped by adding 0.125M glycine solution and incubating for 5 minutes at room temperature. After centrifugation, cell pellets were washed twice with PBS supplemented with 20mM sodium butyrate. Cells were lysed by adding cell lysis buffer supplemented with 20mM sodium butyrate, 1:100 diluted proteinase inhibitor cocktail (PIC; Sigma-Aldrich) and 1:100 diluted phenylmethanesulfonyl fluoride (PMSF, 36978, Fisher Scientific, Landsmeer, The Netherlands) and incubating on ice for 5-10 minutes. After complete lysis of the cells, samples were sonicated using a Soniprep 150 ultrasonic disintegrator (MSE (UK) Ltd, London, UK), producing DNA fragments of 100-400 bp (checked by gel electrophoresis). The DNA concentration was measured using a nanodrop (Thermo Scientific; Waltham, MA, USA), and 10µg of chromatin was used as input for each of the ChIP reactions. Samples were incubated overnight (16 hrs), rotating at 4ºC, with one of the following antibodies: anti-H3K4me3 (ab8580, Abcam, Cambridge, UK), anti-H3K9me3 (ab8898, Abcam), anti-H3K27me3 (ab6002, Abcam), anti-H3Ac (06-599, Millipore) and anti-IgG (ab2410, Abcam). An "input" sample was used as the non-precipitated (no antibody) control. After overnight incubation, the chromatin-antibody complexes were incubated with protein G magnetic beads (Dynabeads, Thermo Scientific) for 4 hours, rotating at 4ºC. Magnetic beads with the bound chromatin-antibody complexes were washed with subsequently low salt buffer, high salt buffer, lithium-chloride buffer and twice with TE pH 8.0 buffer. Chromatin-antibody complexes were eluted using freshly prepared elution buffer. DNA-protein-antibody complexes were denatured by incubating the eluted samples with 0.2M NaCl for 15 min at 95ºC and subsequently with 0.5µg proteinase K (Sigma-Aldrich) for

15 min at 60ºC. DNA clean-up was performed using phenol-chloroform-isoamyl alcohol (Sigma-Aldrich), followed by chloroform-isoamyl alcohol (Sigma-Aldrich). DNA was precipitated overnight by adding 0.6M sodium acetate, 1 ml ethanol and 1 µl glycogen (20 mg/ml; 77534, Affymetrix, Cleveland, OH, USA) to a total volume of 1.4 ml. After centrifugation, cell pellets were resuspended in 30 µl TE and 3 µl of each sample was used for PCR.

PCR of apoptosis genes

PCR reactions of the duplicate experiments were performed using iQ™ SYBR ® Green supermix (2x) (Bio-Rad Laboratories, Hercules, CA, USA). Primers were designed in the promoter regions of each of the respective apoptosis genes and melting temperatures (Tm) were optimized for each of the individual primer sets. The following primer sequences were used: Bax-F 5'-TGCCCGAAACTTCTAAAA-3' and Bax –R 5'-CGTGACTGTCCAATGAGC-3' (Tm 58ºC); Bcl2-F 5'-GCAGAAGTCTGGGAATCG-3' and Bcl2-R 5'- GCATAAGGCAACGATCC-3' (Tm 58ºC); Casp9-F 5'-CGGAAGCGGACTGAG-3' and Casp9-R 5'-CAGAGCTGGTCCACCTG-3' (Tm 60ºC); Fas-F 5'-CCAACTTCCCAGGTTGAA-3' and Fas-R 5'-GCACAAATGGG CATTCC-3' (CD95; Tm 56ºC); p53-F 5'-CTTACTTGTCATGGCGACT-3' and p53-R 5'-CTGGACGGTGGCTCT-3' (Tm 60ºC). Housekeeping genes GAPDH (active) and MYT (silenced) were used as controls to verify accurate detection of the individual histone modifications compared to the input sample. Primer sequences were as follows (Tm for both PCR reactions was 60°C): GAPDH-F 5'-TACTAGCGGTTTTACGGGCG-3', GAPDH-R 5'-TCGAACAGGAGGAGCAGAGAGCGA-3' and MYT-F 5'-GCTGTGGGGAAAGGT AAGTC-3', MYT-R 5'-ATGTCTCCTCTGTCAGACGC-3'.

Thermal cycling conditions were as follows: hot start for 10 minutes at 95°C, followed by 40 cycles of denaturing for 10 sec at 95°C, annealing for 30 sec at the optimized Tm and extension for 30 sec at 72°C. Melting curves were generated after every PCR run to ensure a single PCR product was amplified. Relative enrichment (the percentage of product precipitated as compared to the non-precipitated "input" sample) of each the respective histone modifications was calculated using the following formula: 2^- (Ct value "input" sample – Ct value precipitated sample).

Real-time quantitative PCR for mRNA expression

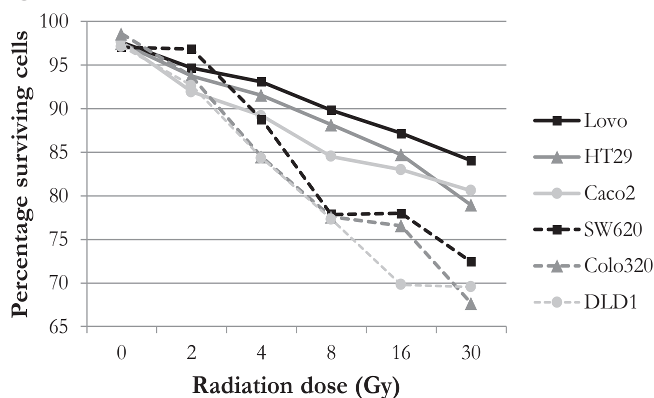
RNA was isolated from each of the colorectal cancer cell lines using TRI Reagent (Life Technologies, Bleiswijk, The Netherlands), according to the manufacturer's protocol. Reverse transcription of mRNA was performed using a High Capacity RNA-to-cDNA kit (Life Technologies). Quantitative duplex real-time PCR reactions were performed in duplicate using FAM-labeled commercially available Taqman Gene Expression assays (Life Technologies) for Casp9 (Hs00609647_m1), TP53 (Hs01034249_m1), Bcl2 (Hs00608023_m1), Bax (Hs000180269_m1), Fas (Hs00236330_m1), GAPDH (Hs99999905_m1) and MYT (Hs01027966_m1). VIC-labeled HPRT1 (Tamra quencher; Hs02800695_m1, Life Technologies) was used as an internal housekeeping gene control within each duplex PCR reaction. For each individual PCR reaction, Ct values were normalized against Ct values of HPRT1 in the same reaction (ΔCt). Real-time PCR reactions were performed on a 7900 HT Fast Real-time PCR system (Applied Biosystems, Foster City, CA, USA).

Results

Dose-response curves

For each of the six colorectal cell lines included in this study, dose-response curves were made to find the optimal dose and duration of treatment with cisplatin, anti-Fas and radiation. Dose-response curves following radiation treatment showed the most cell death in cell lines DLD1, SW620 and Colo320, and less response in cell lines HT29, Caco2 and Lovo (Figure 1). Cell lines DLD1, SW620 and Colo320 were sensitive to cisplatin treatment, showing higher percentages of dead cells as compared to cell lines Caco2, Lovo and HT29, that showed minimal cell death upon treatment with cisplatin (data not shown). Anti-Fas treatment induced marked cell death only in cell line DLD1, all other cell lines showed minimal cell death upon treatment with anti-Fas (data not shown). Treatment that resulted in a maximum of 20% cell death (corresponding to 80% of surviving cells at the time of harvest) in either of the cell lines after 24 hrs (for anti-Fas) or 48 hrs (for cisplatin and radiation treatment) was considered to be the optimal treatment procedure, indicating induction of apoptotic cell death but leaving a substantial surviving fraction to detect apoptotic cells that have initiated but have not finished the process at the time of harvest. On the basis of the survival curves obtained, we therefore chose for treatment of all cell lines with 40μM cisplatin for 48 hrs, with 250 ng/ml anti-Fas for 24 hrs and 6 Gy radiation followed by harvesting after 48 hrs.

Figure 1



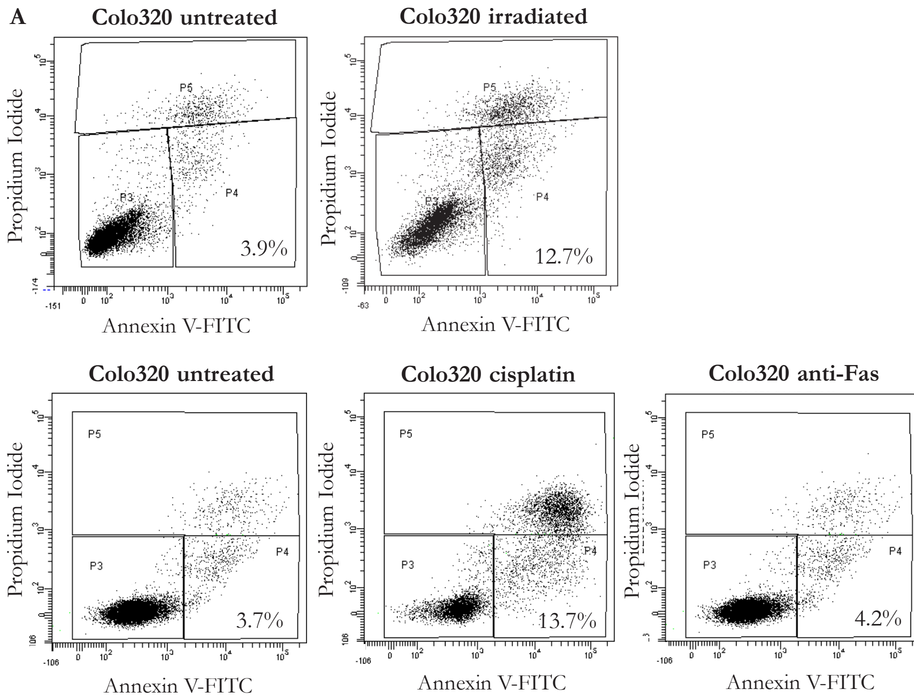
Dose-response curves and percentages of apoptotic cells of colorectal cancer cell lines following treatment. *The percentage of surviving cells (y-axis) was determined for cell lines Lovo, HT29. Caco2, SW620, Colo320 and DLD1, 48 hours after irradiation with different doses (x-axis). Cell lines were incubated with trypan blue and the surviving fraction was calculated as follows: 100% - (number of trypan blue positive (dead) cells / total number of cells (dead+surviving)).*

Flow cytometry detection of the apoptotic fraction after treatment

In order to quantify the percentage of apoptotic cells after treatment, flow cytometry analyses were performed to measure the apoptotic cell fraction of each of the cell lines upon treatment with cisplatin, anti-Fas or radiation. The apoptotic fraction was defined as the fraction of cells showing a positive signal for annexin V but a low signal for propidium iodide (19). The sensitivity of each of the cell lines to induction of apoptosis by each of the treatment regimens

was determined by comparing the apoptotic fractions before (untreated cell population) and after treatment. As for radiation and chemosensitivity, a large apoptotic fraction was observed after treatment as compared to the untreated population in cell lines DLD1, Colo320 (Figure 2A) and SW620. Only a limited response (small apoptotic fraction) was observed for cell lines HT29, Caco2 and Lovo.

Figure 2



B

	Cisplatin (40 µM) % apoptosis	**Radiation (6 Gy)** % apoptosis	**Anti-Fas (250 ng/ml)** % apoptosis
Caco2	0.1	3.1	2.5
Lovo	0.7	0.4	1.0
DLD1	**19.8**	**21.1**	**13.8**
HT29	3.1	0.4	0.6
SW620	**7.9**	**17.6**	0.1
Colo320	**9.8**	**8.8**	0.3

Flow cytometry results after treatment with cisplatin, radiation or anti-Fas. A. *Shown are the flow cytometry results of cell line Colo320 after treatment. Two experiments were performed, to measure the apoptotic cell fractions after treatment with radiation (upper panel) and after treatment with cisplatin or anti-Fas (lower panel). Untreated controls were included in both experiments. Cells in gate P4 were regarded as the apoptotic fraction (positive signal for annexin V and a low signal for propidium iodide). Cells in gate P3 are the living cell fraction, cells in gate P5 are dead cells. Cell debris has been excluded from the analyses (gates P1 and P2, not shown). The percentage of apoptotic cells is indicated in the right bottom corner of each figure.* ***B***. *Difference in percentages of apoptotic cells between treated cells and untreated controls are displayed for each of the cell lines after treatment with cisplatin (48 hrs), radiation (48 hrs) or Anti-Fas (24 hrs).*

Treatment with anti-Fas resulted in an apoptotic response only in cell line DLD1, the other cell lines did not respond to anti-Fas treatment. Each cell line showing a ≥ 5% increase in the percentage of apoptotic cells was considered "sensitive" to the respective treatment. Results are displayed in Figure 2B. The flow cytometry results confirmed the sensitivity of all cell lines for induction of apoptosis, as observed in the dose-response curves, to each of the treatment regimens.

Chromatin immunoprecipitation and mRNA expression levels of apoptotic genes

In order to investigate whether the chromatin status of genes involved in the apoptotic pathway could be used to predict the response to chemo-, immune- or radiation therapy, we performed ChIP experiments for five key apopotic genes (Bax, Bcl2, Casp9, Fas and p53). Immunoprecipitations were performed using antibodies against H3Ac, H3K4me3, H3K9me3 and H3K27me3, followed by real-time PCR for the genes of interest. All ChIP experiments were performed in duplicate, with a mean difference in Ct values between the two measurements of 1.93 (range 0.04-3.80), indicating sufficient reproducibility of the assay. In addition to the chromatin status of the genes, we measured mRNA expression levels of all genes (including control genes GAPDH and MYT) in each cell line and normalized the expression to the HPRT1 signal in each individual reaction (ΔCt). HPRT1 Ct values were very constant across the different genes and duplicate reactions, with average Ct values between 21.3 and 22.7 for the different cell lines and a standard deviation of 0.6 over all measurements.

ChIP results for control genes

Control genes GAPDH (active housekeeping gene) and MYT (myelin transcription factor, silenced in colorectal tissues) were included in each ChIP experiment for assay validation. As expected, at the GAPDH promoter higher relative enrichment (the percentage of precipitated DNA as compared to the non-precipitated "input" sample) of active modifications H3Ac and H3K4me3 was observed as compared to the silencing modifications H3K9me3 and H3K27me3 (Supplementary Figure 1A). At the MYT promoter, higher relative enrichment of silencing histone modifications was observed as compared to the activating histone modifications (Supplementary Figure 1B). mRNA expression data confirmed active expression of GAPDH, with higher relative expression (ΔCt values) compared to HPRT1, and silencing of MYT (Supplementary Figure 1C). As the control genes GAPDH and MYT showed valid results, we continued with the immunoprecipitation experiments on apoptosis gene promoter regions.

ChIP results for apoptosis genes

The activation status of the individual apoptosis genes, as determined by the ChIP experiments, was correlated with sensitivity of the cell lines to chemo-, immune- or radiation therapy (Figure 3). For example, cell line SW620 showed sensitivity to cisplatin and radiation treatment with differences in the percentage of apoptotic cells between controls and treated cells of 7.9% and 17.6%, respectively, but was not sensitive to anti-Fas treatment (difference 0.1% between controls and treated cells). Results from the ChIP experiments showed higher relative enrichment of silencing modification H3K9me3 on the Fas gene promoter as compared to the activating histone

modifications, and high relative enrichment of activating histone modifications for Casp9 and Bax in cell line SW620 (Figure 3A). These ChIP results corresponded to an active intrinsic pathway (the pathway is turned "on"), and hence an expected response to DNA damaging agents such as cisplatin and radiation, and a silenced extrinsic pathway (the pathway is turned "off"), which resulted in no response to anti-Fas treatment. For cell line DLD1, an active chromatin status of Casp9, Bax and Fas, as measured by higher relative enrichment of activating modifications H3Ac and H3K4me3 (Figure 3B), corresponded to sensitivity to all treatments. Cell line Caco2 did not show induction of apoptosis with any of the treatment regimens. This cell line indeed showed high relative enrichment of silencing modifications H3K9me3 and H3K27me3 as compared to the activating histone modifications for Casp9 and Fas, and bivalent histone modifications (both activating and silencing histone modifications present) on p53, Bcl2 and Bax (Figure 3C), corresponding to silenced intrinsic and extrinsic pathways. Cell lines Colo320, HT29 and Lovo, showed a similar correlation between the chromatin status of the apoptosis genes to the treatment response (Supplementary Figure 2). Table 1 provides an overview of the chromatin status of each of the genes coupled to the response of each of the cell lines to chemo-, immune- or radiation therapy. An active chromatin status was assigned when higher relative enrichment was observed for active histone modifications, a repressed chromatin status when higher relative enrichment for silencing histone modifications was observed. Many gene promoters, however, showed bivalent promoters where both activating and silencing histone modifications were present in similar amounts. For example, the p53 gene showed a bivalent chromatin status in all cell lines. Overall, the chromatin status of the predominantly active or silenced gene promoters did correspond well to the overall status of the pathway (on or off), which in turn corresponds to the sensitivity of the individual cell lines to treatment.

TABLE 1. Transcriptional status of apoptotis genes based on ChIP results in correlation with response to therapy

	Intrinsic pathway							Extrinsic pathway		
	Casp 9	**p53**	**Bcl2**	**Bax**	Pathway status	Cisplatin (% apoptotic cells)	Radiation (% apoptotic cells)	**Fas**	Pathway status	Anti-Fas (% apoptotic cells)
SW620	Active	Bivalent	Bivalent	Active	ON	**7.9%**	**17.6%**	Silenced	OFF	0.1%
DLD1	Active	Bivalent	Bivalent	Active	ON	**19.8%**	**21.1%**	Active	ON	**13.8%**
Caco2	Silenced	Bivalent	Bivalent	Bivalent	OFF	0.1%	3.1%	Silenced	OFF	2.5%
Colo320	Bivalent	Bivalent	Active	Active	ON	**9.8%**	**8.8%**	Silenced	OFF	0.3%
HT29	Bivalent	Bivalent	Active	Bivalent	Non-conclusive	3.1%	0.4%	Bivalent	Non-conclusive	0.6%
Lovo	Silenced	Bivalent	Bivalent	Silenced	OFF	0.7%	3.1%	Active	ON	2.5%

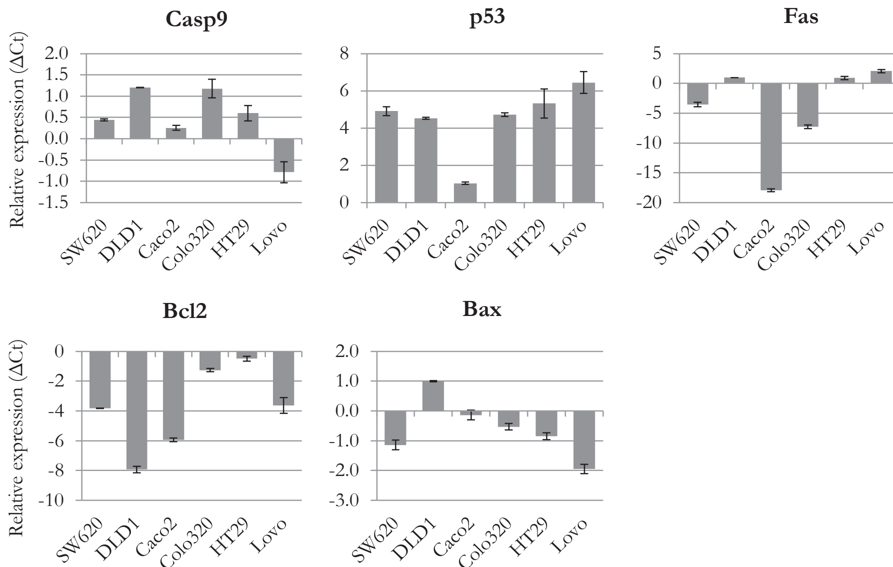
For all cell lines, the chromatin status for each of the apoptosis genes based on the ChIP results is shown, divided into the intrinsic and extrinsic apoptotic pathways. An "active" status was assigned to gene promoters showing higher relative enrichment for active modifications, a "silenced" status for gene promoters showing higher relative enrichment for silencing modifications. A "bivalent" status refers to gene promoters with both active and silencing modification present in equal amounts. The differences between the treated cell lines and untreated controls in percentages of apoptotic cells as measured by flow cytometry are indicated for each cell line. Increases of ≥5% apoptotic cells was defined as sensitivity to the respective treatments and are indicated in ***bold.***

In conclusion, these results indicate that the balance between activating and silencing histone modifications, reflecting the chromatin status of the apoptotic genes, can be used to predict the response of tumor cells to different anti-cancer therapies.

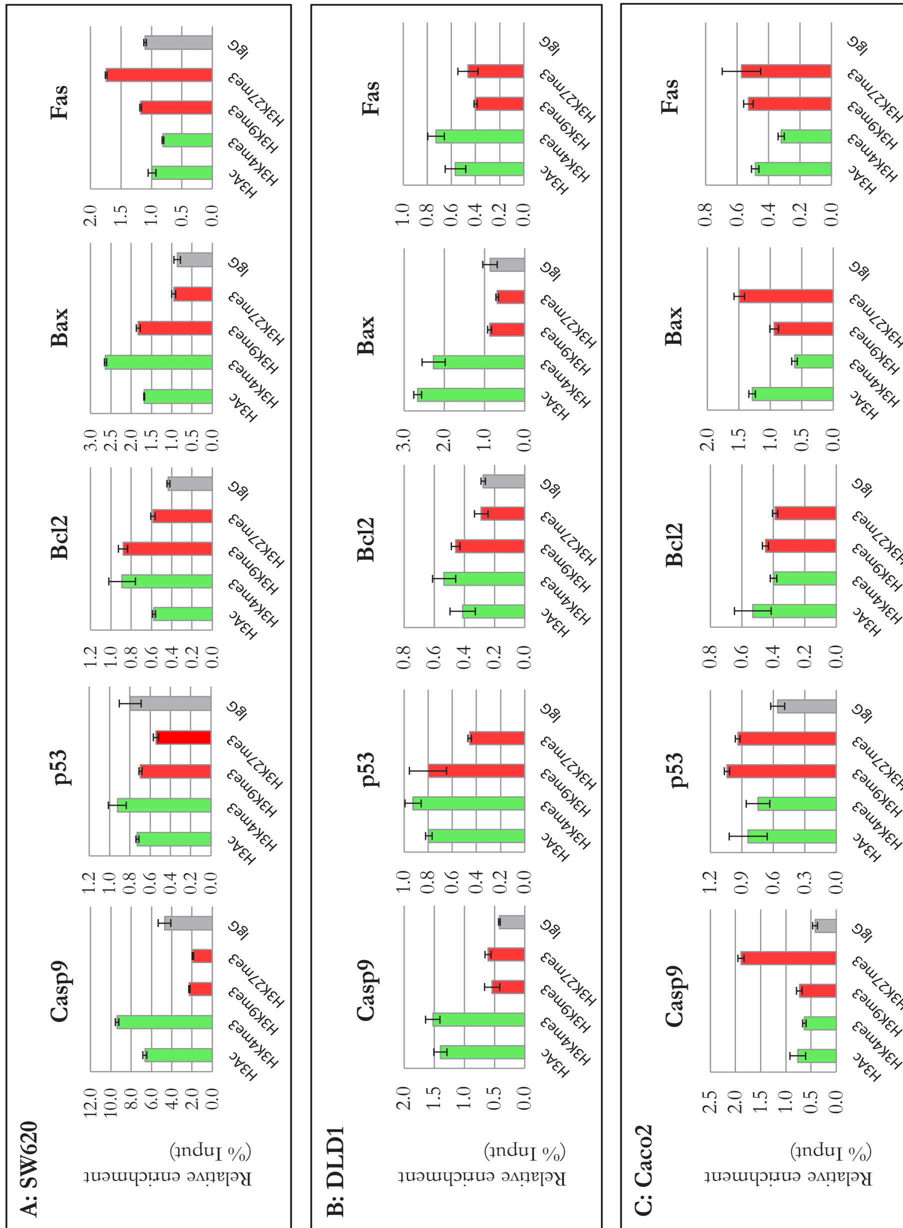
mRNA expression levels of apoptotic genes

To correlate the chromatin status of the genes as determined with the ChIP experiments with mRNA expression levels, we performed gene expression analyses for all apoptosis genes (Figure 4). Fas mRNA expression was observed in cell lines DLD1, HT29 and Lovo, which showed high relative enrichment of active histone modifications in the ChIP experiments. Low expression of Fas mRNA was observed in cell lines where high relative enrichment of silencing histone modifications was observed (SW620, Caco2 and Colo320). Caspase-9 showed high relative mRNA expression in all cell lines except Lovo, which indeed showed high relative enrichment of silencing histone modifications in the ChIP experiments. Bcl2 showed low relative mRNA expression in all cell lines, but cell lines Colo320 and HT29 that showed high relative enrichment of activating histone modifications showed only a slightly lower mRNA expression as compared to internal control HPRT1. Bax showed high relative mRNA expression in cell line DLD1 and only slightly lower relative expression as compared to HPRT1 in cell lines Caco2 and Colo320. Cell line Lovo showed a silenced Bax promoter in the ChIP experiments and indeed showed low relative mRNA expression. mRNA expression of p53 was high in all cell lines, except Caco2, despite the bivalent status of the p53 promoter in all cell lines. In conclusion, mRNA expression matches the chromatin status of the apoptosis gene promoters in most of the cell lines.

Figure 4



Messenger RNA expression of apoptosis gene promoters. *The average relative mRNA expression results (ΔCt) of the duplicate PCR reactions are shown for each of the individual apoptosis genes in all cell lines. All Ct values shown were normalized to the HPRT1 signal in each individual PCR reaction. Error bars indicate the maximum and minimum values of the duplicate measurements.*



Results of ChIP experiments for cell lines SW620, DLD1 and Caco2. *Results of the ChIP experiments are shown for cell lines SW620, DLD1 and Caco2. Immunoprecipitations have been performed using antibodies against active histone modifications H3Ac and H3K4me3 (indicated in green) and silencing histone modifications H3K9me3 and H3K27me3 (indicated in red). IgG was used as a control for non-selective binding (indicated in gray). PCR was performed for apoptosis genes Casp9, p53, Bcl2, Bax and Fas. The respective histone modifications are indicated on the x-axis, the relative enrichment (the percentage of precipitated DNA as compared to the non-precipitated input = % input) is indicated on the y-axis. Error bars represent the maximum and minimum values of the duplicate experiments. Corresponding percentages of apoptotic cells after each treatment are indicated in Figure 2B.*

Discussion

An intact and active apoptotic pathway is required for a proper response to anti-cancer therapies (11,12). We therefore investigated the chromatin status of several key apoptosis genes in colorectal cancer cell lines, in correlation with the apoptotic response upon treatment with cisplatin, radiation or anti-Fas.

Our results regarding sensitivity of the different cell lines to the treatment regimens were consistent with literature. Colorectal cancer cell lines DLD1, Colo320 and SW620 showed a strong apoptotic response upon treatment with cisplatin or radiation, whereas the other cell lines did not, which was also reported previously by other groups (20,21). As for sensitivity to anti-Fas treatment, only cell line DLD1 showed an apoptotic response, the other cell lines showed a resistance to this therapy (22). The sensitivity of each of the cell lines to chemo-, immune-, or radiation therapy was also reflected in the chromatin status of each of the apoptosis genes based on the presence of activating and/or silencing histone modifications at their promoter regions. The chromatin status of each of the apoptosis genes was also reflected in the mRNA expression profiles of the individual cell lines. As expected, an active chromatin status of the intrinsic pathway genes (Apaf1, Bax, Bcl2 and p53) was associated with sensitivity to treatment with cisplatin and radiation. Cell lines Lovo and DLD1 showed an active chromatin status of the Fas gene. However, only DLD1 showed a response to anti-Fas treatment. Differential expression of other extrinsic apoptotic pathway factors, that we have not included in our study, might affect the apoptotic response upon stimulation with anti-Fas and might hence explain the sensitivity of cell line DLD1 and the non-response of cell line Lovo upon treatment with anti-Fas.

Many genes showed a bivalent chromatin status, defined as the presence of both activating and silencing modifications at the same region. Bivalent chromatin marks key developmental genes and regulators, enabling the genes to be activated instantly when needed during development and differentiation (23,24). The bivalent promoter statuses as measured with our ChIP experiments are correlated with different effects on gene expression. For example, Bcl2 showed a bivalent chromatin status in four cell lines and showed repressed gene expression in these cell lines. On the contrary, the p53 gene showed a bivalent chromatin status in all cell lines but is associated with increased gene expression. As the bivalent chromatin status of Bcl2 and p53 does not specifically correlate to the differential responses to therapy, these genes might not play a crucial role determining sensitivity to therapy in the respective cell lines.

Epigenetic regulation of apoptosis genes has previously been studied (25,26) and expression of the studied genes has been correlated to prognosis in cancer (27-30). In addition, radiosensitization of colorectal cancer cell lines has been described upon treatment with HDAC inhibitors (31,32) or Aurora B kinase (33). HDAC inhibitors have also been shown to enhance the response to cisplatin treatment (34). These studies indicate that sensitization of cancer cells can be obtained by changing the epigenetic make-up in the cancer cell, by for instance changes in the epigenetic regulation of apoptosis genes involved in the process of cell death upon anti-cancer treatment. Knowledge of specific changes in epigenetic mechanisms in cancer cells might provide new possible targets for anti-cancer therapy.

In conclusion, histone modifications at apoptosis gene promoter regions, reflecting the transcriptional status of the gene, can be used to predict the sensitivity of colorectal cancer cell

lines to anti-cancer treatment. In current clinical practice, personalized treatment is warranted to prevent over- and undertreatment of colorectal cancer patients. Using information regarding the transcriptional activity of both the intrinsic and extrinsic apoptotic pathways may provide critical information that is required to make an informed treatment decision for each individual patient. Future research should focus on developing methods to detect these histone modifications on apoptosis gene promoters in patient tissues, such as snap-frozen tumor biopsies, in order to make this information available in the clinic. The chromatin status of the studied apoptosis genes might provide patient-specific information regarding the response to specific anti-cancer therapies. Because of the potentially reversible nature of epigenetic modifications, these modifications could provide novel targets to sensitize tumors to obtain adequate treatment responses.

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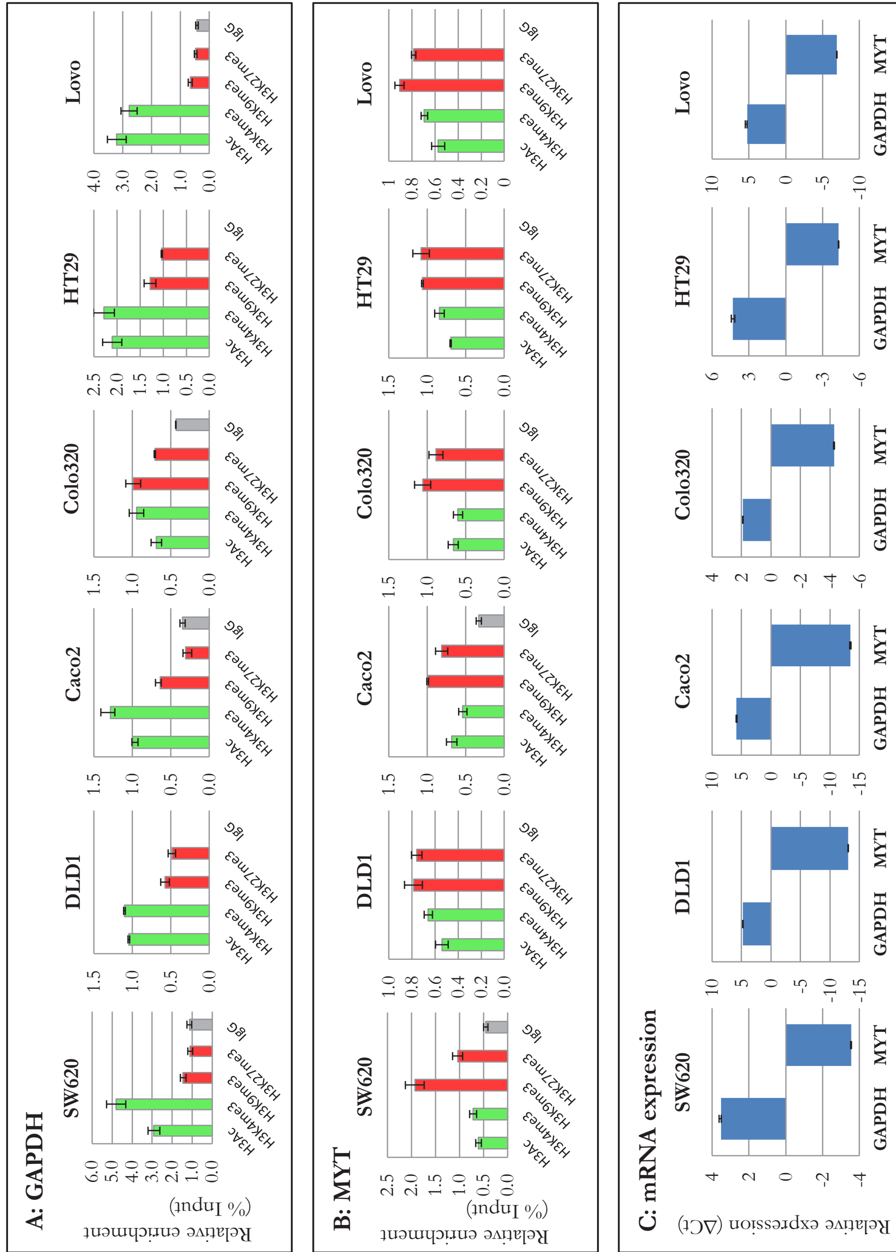
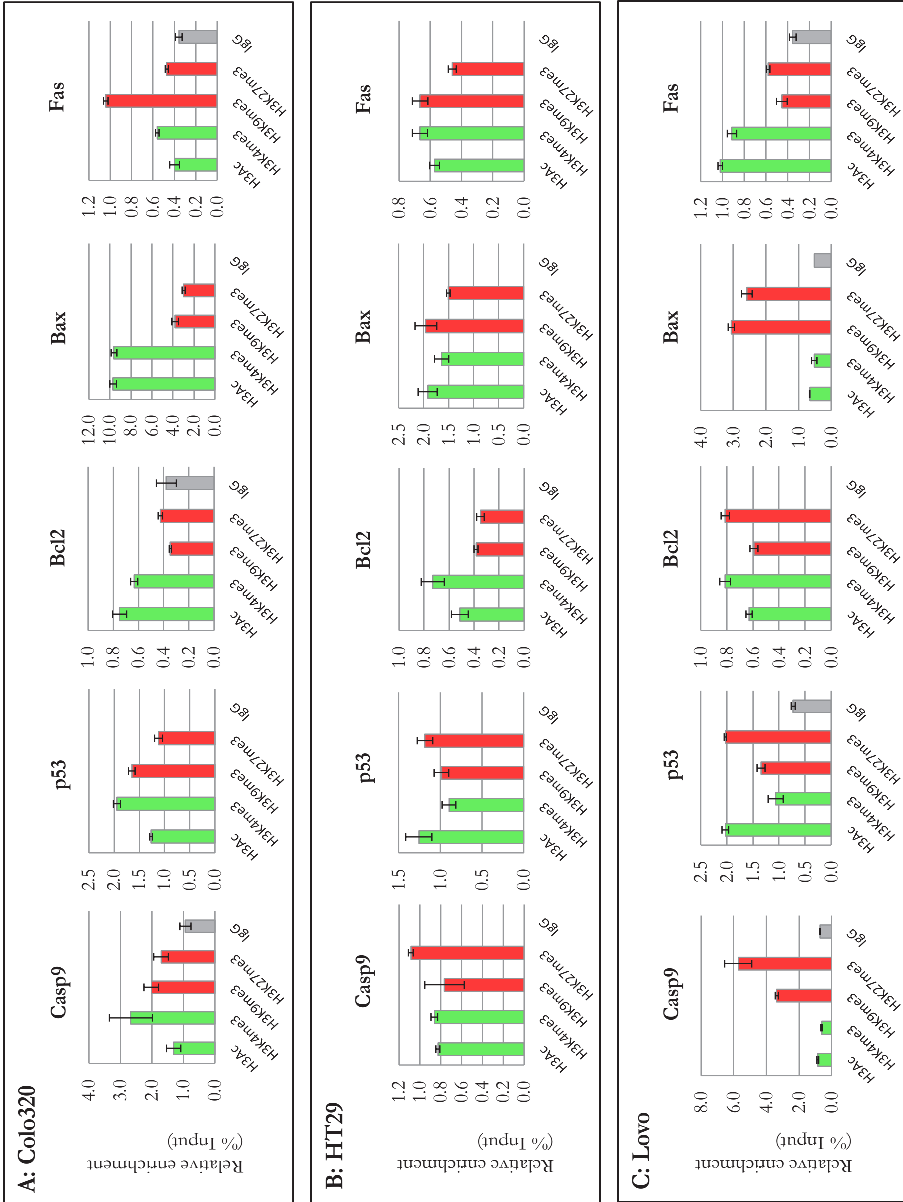
Supplementary files: see next two pages



ChIP results of active control gene GAPDH and silenced control gene MYT. *Results of ChIP experiments for active control gene GAPDH and silenced controlgene MYT are depicted for all cell lines (panels **A** and **B**). Immunoprecipitations have been performed using antibodies against active histone modifications H3Ac and H3K4me3 (indicated in green) andsilencing histone modifications H3K9me3 and H3K27me3 (indicated in red). IgG was used as a control for non-selective binding (indicated in gray). mRNA expression of both genes is given for all cell lines in panel (**C**).*



ChIP results for cell lines Colo320, HT29 and Lovo. *Results of the ChIP experiments are shown for cell lines Colo320, HT29 and Lovo. Immunoprecipitations have been performed using antibodies against active histone modifications H3Ac and H3K4me3 (indicated in green) and silencing histone modifications H3K9me3 and H3K27me3 (indicated in red). IgG was used as a control for non-selective binding (indicated in gray). PCR was performed for apoptosis genes Casp9, p53, Bcl2, Bax and Fas. The respective histone modifications are indicated on the x-axis, the relative enrichment (the percentage of precipitated DNA as compared to the non-precipitated input = % input) is indicated on the y-axis. Error bars represent the maximum and minimum values of the duplicate experiments. For each cell line, the percentages of apoptotic cells, as measured by flow cytometry, are given after each of the respective treatment regimens.*

Chapter 8

Summary

Summary

Colorectal cancer is one of the most common diagnosed cancers worldwide, and is the second most important cause of cancer mortality in Europe. Despite careful assessment of tumor stage to decide on treatment strategies using the tumor, nodes and metastasis (TNM) staging system, large differences in patient survival and tumor recurrence are observed among patients with tumors with the same TNM classification. The general aim of the work presented in this thesis was to identify new clinically prognostic biomarkers in colorectal cancer in order to better classify patients, which might aid in the decision-making process concerning anti-cancer therapies in the future. The work presented in this thesis is a starting point for finding new clinically prognostic biomarkers by studying epigenetic mechanisms regulating gene expression. Epigenetic mechanisms were studied at different levels: both DNA methylation and histone modifications were studied genome-wide and at specific gene promoter regions.

Chapters 2 and 3 report on DNA methylation studies that were performed in rectal cancer tissues from patients enrolled in the Dutch multicenter TME clinical trial. **Chapter 2** describes the prognostic value of DNA methylation of repetitive retrotransposon sequence long interspersed element 1 (LINE-1), but not of Alu repetitive sequences, in early-stage rectal cancer. Low methylation at LINE-1 sequences was shown to correlate with shorter patient survival and a higher probability of tumor recurrence, which can be attributed to activation of LINE-1 retrotransposons which can reintegrate at random sites into the genome and can thereby interrupt regular gene sequences or influence DNA methylation of neighboring genes. In addition, results suggested that high methylation at histone H3 lysine 27 (H3K27me3) when DNA methylation at LINE-1 was low could have a "protective" function in the cells by preventing deregulated gene expression when DNA methylation is absent. In contrast, high acetylation of H3K9 in combination with high levels of DNA methylation at LINE-1 sequences could be associated with a poor disease outcome through activation of aberrant gene expression. In **Chapter 3**, DNA methylation was studied at promoter regions of apoptosis genes functioning in both the intrinsic and extrinsic apoptosis pathway routes. Combined survival analyses of intrinsic apoptosis pathway genes Apaf1, Bcl2 and p53 showed shorter survival and recurrence-free periods when an increasing number of markers showed high methylation (all low, 1 high, 2 high or all high). The shortest survival, however, was observed for patients showing low methylation of all markers, which – as was expected - correlated with high apoptosis (M30), but also with high proliferation (Ki-67).

In addition to DNA methylation, histone modifications were studied in colorectal cancer tissues, both globally and at gene-specific promoter regions. **Chapter 4** describes the prognostic value of global nuclear expression of histone deacetylases SIRT1, HDAC1 and HDAC2 combined with histone modifications H3K56Ac and H4K16Ac. High expression of histone deacetylases prevents aberrant gene expression, high levels of H4K16Ac are associated with silenced repetitive sequences and high levels of H3K56Ac are essential for proper non-homologous end-joining. Indeed, better patient survival and less tumor recurrence were observed when more markers showed high nuclear expression in combined marker survival trend analyses. **Chapter 5** shows

the prognostic value of Polycomb-group proteins EZH2, BMI1 and SUZ12 with associated histone modification H3K27me3 in colorectal cancer tissues. As would be expected from the transcriptional silencing function of H3K27me3, high expression of the histone modification and the three Polycomb-group proteins showed better patient survival and longer recurrence-free survival in combined marker survival trend analyses. Better stratification of patients was obtained by combining the expression data of the investigated biomarkers as compared to the individual markers, underlining the importance of investigating multiple markers simultaneously. In **Chapter 6**, the prognostic value of histone methylation at several histone tail residues in early-stage (TNM stage I and II) colon cancer was investigated. Low nuclear expression of H3K4me3, and high expression of H3K9me3 and H4K20me3 were associated with good prognosis, both in individual marker analyses as well as in combined marker analyses. **Chapter 7** reports on the specific chromatin environment at apoptosis gene promoter regions that can be used to predict sensitivity of colorectal cancer cell lines to cisplatin, anti-Fas or radiation therapy. The results presented in this study indicate that the apoptotic response of individual cell lines is indeed correlated with the transcriptional status of the apoptotic genes, as measured by the balance between activating and silencing histone modifications.

In conclusion, the study of epigenetic mechanisms as presented in this thesis indeed resulted in the identification of clinically relevant prognostic biomarkers. On a genome-wide level, deregulated DNA methylation on repetitive sequences (LINE-1) and deregulated expression of histone modifications and -modifying enzymes showed strong correlations to clinical outcome. At specific gene promoter regions, all related to the process of apoptosis, DNA methylation was correlated to clinical outcome and the transcriptional status based on histone modifications was predictive for the response to therapy. Taken together, these results emphasize the importance of deregulated epigenetic mechanisms in tumorigenesis. In the future, the described potentially reversible epigenetic mechanisms might provide a starting point for the identification of important epigenetic biomarkers and might eventually lead to the development of tumor-specific treatment and hence individualized medicine.

Chapter 9

Discussion and future perspectives

Discussion and future perspectives

In the past decades, the knowledge of the tumorigenic process at the level of genetics (including mutations and single nucleotide polymorphisms), gene transcription (regulated by epigenetic mechanisms), gene translation (including non-coding RNAs) and proteins (comprising biochemical pathways) has increased exponentially. There is a need for new prognostic (providing information on the likely clinical outcome on the basis of tumor characteristics) and predictive (predicting the response to therapy on the basis of tumor characteristics) biomarkers to advance the field of individualized medicine for colorectal cancer patients. To date, only a few biomarkers that show changes in colorectal tumor tissues as compared to normal tissues have been implemented in clinical practice, including measuring CEA levels for postoperative surveillance (1) and assessment of KRAS mutations to predict the response to anti-EGFR therapy (2). No other biomarkers have been recommended for use in clinical practice by the European Group on Tumor Markers (EGTM) (3).

In order to find clinically relevant biomarkers, understanding the underlying tumor biology is of uttermost importance. Until recently, studies on finding new biomarkers have been focused on genetic changes in tumor tissues. Now it is becoming increasingly clear that genetics does not solely determine the course of tumor development and progression. Epigenetic mechanisms, including DNA methylation and histone modifications, play a significant role in cancer development, progression, metastasis and drug resistance, and are therefore potential new biomarkers in colorectal cancer. In this thesis, epigenetic mechanisms were identified as prognostic biomarkers. In addition, the studied changes in epigenetic regulation provide information about the underlying tumor biology.

The dynamic nature of epigenetic mechanisms – new options for therapy

Epigenetic mechanisms are dynamic modifications on DNA and histone proteins that are added or removed depending on the demands of the cell under specific conditions. For example, in order for the cells to differentiate during embryonic development, changes in DNA methylation and histone modifications allow for genes to be switched on or off at the correct stage of the developmental process (4-7). Following cellular differentiation, epigenetic modifications ensure cell- and tissue-specific gene expression patterns and regulate gene expression in response to environmental stimuli (8). In cancer cells, numerous changes in epigenetic modifications occur that promote tumor development, progression and metastasis. Because of their dynamic nature, epigenetic mechanisms are potentially reversible and therefore present as attractive targets for therapeutic intervention, especially since epigenetic alterations might also be the cause of drug resistance in human cancer. Several cellular processes that could contribute to drug resistance have been described to be affected by aberrant epigenetic modifications in cancer, including enzymatic drug-metabolism, drug efflux, DNA repair and apoptosis (9-13). Reversion of these aberrant epigenetic patterns might sensitize the tumor to anti-cancer treatments.

Epigenetic therapies in a clinical setting

Several epigenetic drugs have been extensively studied and are now tested in clinical trials for treatment of hematological malignancies, including FDA-approved drugs 5-aza-2'-deoxycytidine

(decitabine; 14,15) and several histone deacetylase (HDAC) inhibitors (16,17). To date, epigenetic drugs are most effective in hematological diseases and have not shown any conclusive effectiveness of antitumor activity of any of these drugs in solid tumors, including colorectal cancer (reviewed in ref 9). This suggests that more knowledge is needed concerning the complex tumor biology and the epigenetic mechanisms involved in the tumorigenic process in solid tumors. In addition to the limited effect of epigenetic drugs in solid tumors, major concerns of using epigenetic drugs such as DNA methyltransferase (DNMT) or HDAC inhibitors include their non-specificity and therefore the possibility of unwanted side-effects of the drugs. The inhibitors might reactivate genes that are normally silent and that might promote tumor aggressiveness in addition to or instead of the expected reactivation of tumor suppressor genes (18). Despite these concerns, increasing evidence suggests that combination treatment with HDAC inhibitors and DNMT inhibitors results in a synergistic response, leading to re-expression of silenced genes, increased apoptosis and reduced tumorigenesis (19,20). In addition, combination of epigenetic drugs and current standard therapy regimens might provide a more effective way of treatment. More research is needed concerning the specific epigenetic changes in (colorectal) tumors, in order to provide targets for the development of specific epigenetic drugs. In this thesis new potential biomarkers, including DNA methylation on specific genes or repetitive sequences and histone modifications and -modifying enzymes, were identified that might provide new targets for future epigenetic therapies, as will be discussed below.

Genome-wide DNA methylation

In this thesis, genome-wide DNA methylation was studied using LINE-1 and Alu repetitive sequences. Low levels of LINE-1 methylation were found to be associated with poor prognosis, which can be explained by activation of its retrotransposon activity and hence potential random insertion of the sequences and/or changes in methylation of surrounding gene regions. In contrast, Alu methylation was not found to correlate with prognosis, indicating that our findings in rectal cancer were LINE-1 sequence-specific and not generally applicable to the whole genome. This might explain why epigenetic drugs do not yet function optimally in solid tumors, including colorectal cancer: not all regions of the genome are equally affected by changes in epigenetic modifications. Identification of the (gene) regions that are differentially regulated and have prognostic or predictive value in colorectal cancer is therefore of key importance in the search for new, clinically useful, biomarkers. The findings in this study also underline the importance of the development of more specific epigenetic drugs, targeting single epigenetic modifying proteins or maybe even sequence-specific epigenetic alterations. Furthermore, measuring LINE-1 methylation in biopsy tissues of cancer patients might provide a good risk assessment for the potential development of a distant recurrent tumor in individual patients.

Global histone modifications and –modifying enzymes

The studies concerning global histone modifications presented in this thesis have identified multiple prognostic biomarkers in colorectal cancer. Histone modifying enzymes including HDACs and EZH2 present as promising targets for epigenetic therapies, but most likely need to be combined with other – existing – therapies, as discussed above. The studies presented in this thesis showed that combining histone-modifying enzymes and histone modifications resulted

in better stratification of patients into different risk groups as compared to individual markers, underlining the importance of pathway-focused approaches. Considering this, future research should focus on combining multiple drugs targeting epigenetic enzymes. This might lead the way to success for epigenetic therapy in solid tumors. Risk assessment for individual patients based on the expression of histone modifications and –modifying enzymes might add to the current TNM staging system.

Gene-specific epigenetic modifications

The transcriptional status (including both DNA methylation and histone modifications) of specific genes, as shown for apoptosis genes in this thesis, has prognostic and predictive value in colorectal cancer. DNA methylation of combined intrinsic apoptosis pathway genes Apaf1, Bcl2 and p53 was found to be prognostic in our rectal cancer patient cohort. In literature, methylation of apoptosis genes has also been linked to drug resistance. For example, methylation of apoptosis effectors BNIP3 and DAPK has been shown to predict lower response rates to fluoropyrimidine-based treatment in gastric cancer (21). In addition to DNA methylation, we also studied histone modifications on apoptosis gene promoters. The transcriptional status of the respective apoptosis genes, on the basis of the presence of activating and/or silencing histone modifications, were shown to correlate with the response to anti-cancer treatments, including chemotherapy, irradiation or immunotherapy. Determining the transcriptional status of key genes in for example the apoptosis pathway in patient tissues (biopsies) may lead to specific decisions for therapy on the basis of the biological make-up of individual tumors. Continuing on the discussion above, current epigenetic therapies lack specificity and may thereby fail to be effective in several types of solid tumors, including colorectal cancer. New therapies should be developed focused on reversing epigenetic alterations on specific genes or sequences in individual tumors that show prognostic and/or predictive value, thereby getting one step closer to individualized medicine. For this purpose, more research is needed to understand the mechanisms of gene- or pathway specific epigenetic regulation by epigenetic modifiers such as DNMTs and histone-modifying enzymes. This knowledge will aid in the identification of clinically relevant biomarkers that can be used to develop new - epigenetic - therapies for solid tumors.

Future perspectives

Several epigenetic clinically prognostic biomarkers were identified in colorectal cancer in this thesis, including both genome-wide and gene-specific patterns of DNA methylation and histone modifications. Knowledge of tumor biology is of key importance in the development of new therapies and the making of informed, patient-specific, treatment decisions. Pathway-focused approaches, as presented in this thesis, provide information regarding possible synergistic interactions of biomarkers. This information will lead to identification of new combinations of treatment regimens that enhance the anti-cancer effects in individual patients. Multidisciplinary medicine, combining the knowledge provided by biomarker research and results of clinical trials, is therefore important to advance the field of individualized medicine. In addition, newly validated biomarkers will add crucial information to tumor characterization instruments currently used in clinical practice, such as the TNM staging system. Adding information on epigenetic

modifications, will aid in identifying high-risk patients and predicting response to therapy based on tumor characteristics, and will thereby provide opportunities for individualized medicine.

In conclusion, the work presented in this thesis underlines the importance of epigenetics in tumor development. The various studies presented show that epigenetic alterations have a profound impact on patient survival and tumor recurrence. Even though most cancer research has focused on the effect of gene mutations on the tumorigenic process during the last decades, it is becoming increasingly clear that epigenetic mechanisms are important mediators in this process. A delicate interplay between genetics and epigenetics determines the fate of each individual cell in the human body. Gene mutations cause changes in gene expression or stability/interactions of the resulting mutated proteins, which all have downstream effects within pathways that are accompanied by changes in the epigenetic regulation of these downstream factors. In addition, gene mutations in epigenetic modifiers will have direct effects on the transcriptional regulation of many genes or gene regions. Therefore, epigenetic mechanisms are unquestionably tied to the tumorigenic process and should be considered as a grand new source of information not only for identification of prognostic and predictive biomarkers, but also for the development of new, possibly tumor- and therefore patient-specific, anti-cancer therapies.

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Appendices

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Dankwoord

Nederlandse samenvatting

Darmkanker is wereldwijd één van de meest voorkomende soorten van kanker, en neemt een tweede plaats in als het gaat om sterfte door kanker in Europa (1). Ongeveer twee derde van de colorectale tumoren (dikkedarmkanker) ontstaat in het colon, en een derde in het rectosigmoid of rectum. Colorectale tumoren worden geclassificeerd met behulp van het "tumor, nodes and metastasis" (TNM) stageringssysteem (2,3), en vervolgens wordt aan de hand van de geldende richtlijnen bepaald hoe de patiënt behandeld wordt. Colorectale tumoren worden primair verwijderd door middel van een operatie. Daarnaast wordt aan een deel van de patiënten aanvullende behandelingen gegeven in de vorm van chemotherapie en/of bestraling. Met de introductie van pre-operatieve bestraling en de "total mesorectal excision" (TME) operatietechniek voor rectale tumoren, is het percentage van patiënten bij wie de tumor lokaal terugkeert gereduceerd van 11% naar 5% (4). Dit houdt echter ook in dat een groot deel van de patiënten (minimaal 94%) onnodig behandeld wordt met pre-operatieve bestraling, met kans op bijwerkingen zoals seksuele dysfunctie of incontinentie (5,6). De huidige TNM classificatie van colorectale tumoren is vooralsnog ontoereikend, aangezien patiënten met tumoren in hetzelfde stadium grote verschillen laten zien in overleving en het terugkeren van een tumor (7). Er is dus een grote behoefte aan biomarkers die kunnen helpen bij het identificeren van hoog-risicopatiënten en bij het bepalen van de behandelingsstrategie. Biomarkers zijn biologische markers die gemeten kunnen worden in bijvoorbeeld bloed of tumorweefsel, en die gebruikt kunnen worden als indicatoren voor de aanwezigheid en het verloop van pathologische processen of om de respons op therapie te bepalen. Tot op heden is maar een klein aantal biomarkers goedgekeurd voor gebruik in de kliniek, waaronder "carcinoembryonic antigen" (CEA) in het bloed voor post-operatieve monitoring en KRAS mutaties voor het eventueel toedienen van anti-EGFR therapie (8,9). Vele andere biomarkers zijn beschreven, maar er is voor deze markers onvoldoende bewijs om deze in de kliniek te gebruiken. Voor het vinden van klinisch relevante biomarkers is het begrijpen van de onderliggende tumorbiologie van groot belang. Met deze informatie van individuele tumoren kan per patiënt gekeken worden wat het risicoprofiel is en kunnen nieuwe therapieën ontwikkeld worden die ingrijpen op de in de tumor verstoorde processen.

Doel van dit proefschrift

Het onderzoek beschreven in dit proefschrift richt zich op het vinden van nieuwe prognostische biomarkers in colorectale tumoren op het gebied van epigenetische mechanismen, die kunnen voorspellen wat het klinisch verloop zal zijn met betrekking tot lokaal terugkeren of metastasering (uitzaaien) van de tumor en overleving van de patiënt.

Genetische en epigenetische veranderingen in tumoren

Een kankercel ontstaat doordat een serie van opeenvolgende veranderingen plaatsvindt, die ervoor zorgen dat de cel zich ongeremd kan vermenigvuldigen, niet dood gaat, en waardoor de cel kan metastaseren (uitzaaien) naar andere organen. Deze eigenschappen zijn beschreven door Hanahan en Weinberg als de "hallmarks of cancer" (10,11). De veranderingen die leiden tot

tumorvorming kunnen hun oorsprong vinden op genetisch niveau, door bijvoorbeeld mutaties in specifieke genen, zoals beschreven in een model voor colorectale tumoren door Vogelstein (12). Deze veel voorkomende mutaties zijn in veel tumoren aanwezig, maar verklaren niet het ontstaan van alle tumoren.

Naast veranderingen in het DNA, is er in het wetenschappelijk onderzoek steeds meer aandacht voor de regulatie van genexpressie door epigenetische mechanismen. Epigenetica betreft de wetenschap die veranderingen in een organisme door modificatie van de genexpressie bestudeert, die niet worden veroorzaakt door veranderingen in de onderliggende DNA sequentie. De epigenetische mechanismen die in dit proefschrift aan bod komen beïnvloeden de genexpressie via regulatie van de lokale structuur van chromatine, het complex van histon eiwitten waar het DNA omheen gewikkeld is. In een "open" chromatine structuur kunnen transcriptiefactoren binden aan de specifieke genen in het DNA, wat vervolgens resulteert in expressie van het betreffende gen. Is de chromatinestructuur echter gesloten, zal er geen genexpressie plaatsvinden. De chromatinestructuur wordt gereguleerd door het toevoegen of verwijderen van chemische groepen aan het DNA (DNA-methylering) of aan histon-eiwitten (histonmodificaties). Deze modificaties worden hieronder verder besproken. Veranderingen in deze epigenetische mechanismen zijn in verschillende typen tumoren beschreven (13,14).

DNA-methylering

De modificatie van DNA door het toevoegen van methylgroepen op Cytosines (DNA-methylering) vertoont een specifiek patroon voor elk celtype (15,16) en is betrokken bij vele functies in de cel, zoals het uitschakelen van één van de twee X-chromosomen bij vrouwen, het onderdrukken van repetitieve sequenties en de regulatie van cellulaire differentiatie (17). DNA-methylering beschermt tegen het spontaan ontstaan van mutaties (18) en speelt ook een belangrijke rol tijdens de embryonale ontwikkeling, waar in de verschillende stadia op het juiste moment verschillende sets van genen tot expressie moeten komen (19,20).

In tumorcellen vinden een aantal veranderingen plaats in DNA-methylering ten opzichte van normale cellen. Op globaal niveau neemt de hoeveelheid DNA-methylering af (21), wat resulteert in activatie van sequenties die normaal gesproken onderdrukt worden, zoals repetitieve sequenties en oncogenen die celdeling bevorderen en celdood tegengaan (22-25). Daarentegen kunnen sequenties die normaal gesproken actief zijn (geen DNA-methylering aanwezig), waaronder tumorsuppressor genen, juist gemethyleerd worden waardoor deze niet meer tot expressie komen. De prognostische waarde van DNA-methylering van vele genen is inmiddels beschreven in verschillende tumoren (26-29).

In dit proefschrift worden zowel globale als gen-specifieke veranderingen in DNA-methylering besproken. In **hoofdstuk 2** wordt beschreven dat een lage mate van DNA-methylering in rectumtumoren op de repetitieve sequentie "long interspersed element 1" (LINE-1, dat 17% van het humane genoom beslaat) gecorreleerd is met kortere overleving en een grotere kans op het terugkeren van de tumor bij patiënten met rectumtumoren. LINE-1 sequenties, indien geactiveerd door verlies van DNA-methylering, kunnen zichzelf verplaatsen in het genoom en daarmee andere genen verstoren, wat kan leiden tot tumorvorming. Ook kan de DNA-methylering van omliggende genen worden veranderd door de aan- of afwezigheid van DNA-methylering op de LINE-1 sequenties, wat kan leiden tot bijvoorbeeld activatie van oncogenen.

DNA-methylering op een andere repetitieve sequentie, Alu (short interspersed elements), was niet voorspellend voor overleving en/of terugkeer van de tumor, wat aangeeft dat de gevonden correlatie voor LINE-1 methylering specifiek is voor deze sequentie. Op gen-specifiek niveau wordt in **hoofdstuk 3** beschreven dat methylering van drie genen betrokken bij apoptose (geprogrammeerde celdood), namelijk Apaf1, Bcl2 en p53, in rectumtumoren voorspellend is voor de overleving van de patiënt en terugkeer van de tumor. Een combinatie van veel celdood maar tegelijkertijd ook veel celdeling was gecorreleerd met de slechtste overleving en de hoogste kans op terugkeer van de tumor.

De studies in deze twee hoofdstukken laten zien dat DNA-methylering op zowel genoom-wijd als op gen-specifiek niveau prognostische waarde heeft in rectumtumoren. Een goede risico-inventarisatie voor individuele patiënten zal verkregen kunnen worden door het bepalen van DNA-methylering van de beschreven factoren in bijvoorbeeld biopsieweefsels.

Histonmodificaties

Acht histon-eiwitten vormen in vier paren een kern van eiwitten waar het DNA omheen gewikkeld is. Het uiteinde van deze histon-eiwitten kan gemodificeerd worden door de toevoeging of verwijdering van onder andere methyl- en acetylgroepen. Histon-acetylering is geassocieerd met een open chromatinestructuur en dus activatie van genexpressie. Wat betreft methylering van histon-eiwitten bepaalt de specifieke locatie van methylgroepen op de histon-eiwitten het effect van deze modificatie: 3 methylgroepen op het vierde aminozuur (lysine, K) op histon H3 (H3K4me3) zijn bijvoorbeeld activerend, terwijl diezelfde groepen op aminozuur 27 op histone H3 (H3K27me3) juist geassocieerd zijn met een gesloten chromatinestructuur en dus genexpressie tegengaan. Daarbij is het aantal methylgroepen (een, twee of drie) dat wordt toegevoegd ook van belang voor hun specifieke functie in de cel (30). De specifieke histonmodificaties worden toegevoegd of verwijderd door zogenaamde histon-modificerende eiwitten, waaronder DNA methyltransferases (DNMTs) die methylgroepen toevoegen aan het DNA en histon deacetylases (HDACs) die acetylgroepen verwijderen van histon-eiwitten. Veranderingen in expressie van deze modificerende eiwitten, met als gevolg veranderingen in de aanwezigheid van bepaalde histonmodificaties, zijn veelvuldig gerapporteerd in verschillende tumoren (31,32), en hebben ook prognostische waarde (33-36).

In dit proefschrift zijn een aantal studies beschreven waarin is gekeken naar globale expressie van histonmodificaties en de bijbehorende histon-modificerende eiwitten. **Hoofdstuk 4** beschrijft de prognostische waarde van histon deacetylases SIRT1, HDAC1 en HDAC2 samen met histonmodificaties H3K56Ac en H4K16Ac in colorectale tumoren, allen betrokken bij reparatie van het DNA en het onderdrukken van onder ander repetitieve sequenties. Hoge expressie van de combinatie van de drie histon deacteylases met een van beide histonmodificaties was geassocieerd met betere overleving en een kleinere kans op terugkeer van de tumor. In **hoofdstuk 5** wordt de prognostische waarde van Polycomb eiwitten EZH2, BMI1 and SUZ12 samen met de geassocieerde histonmodificatie H3K27me3 in colorectale tumoren beschreven. Hoge expressie van deze Polycomb eiwitten en H3K27me3 was gecorreleerd met betere overleving en een kleinere kans op terugkeer van de tumor. Expressie van histonmodificaties H3K4me3, H3K9me3 en H4K20me3, zoals beschreven in **hoofdstuk 6**, had alleen prognostische waarde in stadium I en II colon tumoren. Lage expressie van H3K4me3 en hoge expressie van H3K9me3

en H4K20me3 waren geassocieerd met een goede prognose, zowel in individuele marker analyses als in gecombineerde analyses.

Histonmodificaties hebben ook op gen-specifiek niveau predictieve waarde. In **hoofdstuk 7** wordt beschreven dat de aanwezigheid van activerende of onderdrukkende histonmodificaties invloed heeft op de genexpressie van apoptose genen en daarmee op de gevoeligheid van colorectale cellijnen voor chemotherapie, immuuntherapie of bestraling. Expressie van apoptose genen, door de aanwezigheid van activerende histonmodificaties, was gecorreleerd met een goede respons (celdood) op het toedienen van de verschillende therapieën.

In de beschreven studies naar histonmodificaties zijn verschillende prognostische factoren geïdentificeerd in colorectale tumoren. Deze studies onderstrepen het combineren van meerdere markers en dus op signaleringsroute-gerichte benaderingen (pathways) om de onderliggende tumorbiologie te kunnen ontrafelen. Vervolgstudies zullen zich dus ook moeten richten op het combineren van meerdere (epigenetische) therapieën om een beter behandelingseffect te verkrijgen. De bepaling van de aanwezigheid van histonmodificaties in biopsieweefsel kan belangrijke informatie opleveren wat betreft de keuze voor therapie voor individuele patiënten. Kennis van de regulatie van specifieke genen, zoals beschreven in het laatste hoofdstuk, zal in de toekomst hopelijk leiden tot het ontwikkelen van meer specifieke epigenetische therapieën, die gericht zijn op het behandelen van individuele tumoren.

Toekomstperspectieven

Vanwege de dynamische eigenschappen van epigenetische modificaties, die op elk moment toegevoegd of verwijderd kunnen worden afhankelijk van de behoeften van de cel, zijn epigenetische mechanismen aantrekkelijke kandidaten voor het ontwikkelen van nieuwe therapieën. Epigenetische therapieën gericht op DNA-methylering (37,38) of histon deacetylases (39,40) worden momenteel getest in klinische trials. Tot op heden zijn deze therapieën echter voornamelijk effectief in hematologische ziekten (leukemieën), maar beduidend minder in solide (waaronder colorectale) tumoren. Voor het ontwikkelen van effectievere therapieën voor individuele patiënten met colorectale tumoren, is kennis van de onderliggende tumorbiologie essentieel. Ook zullen signaleringsroute-gerichte benaderingen belangrijk zijn in het identificeren van prognostische en predictieve biomarkers in colorectale tumoren. Multidisciplinair onderzoek, waarbij clinici en onderzoekers nauw samenwerken, is hierbij van groot belang, om kennis uit het wetenschappelijk onderzoek te kunnen toepassen en potentieel nieuwe behandelingen te testen in klinische studies. Klinisch relevante biomarkers zullen moeten worden toegevoegd aan de classificatiesystemen die momenteel in de kliniek gebruikt worden, zoals het TNM classificatiesysteem. Identificatie en toepassing van nieuwe biomarkers in de kliniek zal bijdragen aan betere classificatie van patiënten en daarmee een kans op gerichte behandeling van individuele patiënten.

Samenvattend onderstrepen de verschillende studies gepresenteerd in dit proefschrift dat epigenetische mechanismen een belangrijke rol spelen in het proces van tumorvorming. Er is de afgelopen decennia veel onderzoek gedaan naar genetische mutaties in tumoren, maar het wordt steeds duidelijker dat andere factoren zoals epigenetische mechanismen hierbij ook een grote rol spelen. Genmutaties kunnen bijvoorbeeld leiden tot verhoogde of juist verlaagde expressie van het betreffende gen, waar dus ook veranderingen in de epigenetische regulatie van deze genen bij

betrokken zijn. Aangezien de expressie van een gen nooit op zichzelf staat en altijd is verbonden met andere genen in dezelfde signaleringsroute, zal ook de epigenetische regulatie van deze andere genen worden beïnvloed door mutaties of veranderingen in de epigenetische status van een individueel gen. Mutaties in epigenetische factoren zelf zullen uiteraard ook grote gevolgen hebben voor de epigenetische regulatie van individuele genen en daarmee signaleringsroutes, en beïnvloeden daarmee het proces van tumorvorming. Epigenetische mechanismen zijn dus onbetwistbaar betrokken bij het proces van tumorvorming en moeten worden beschouwd als een grote bron van informatie, niet alleen voor het identificeren van prognostische en predictieve biomarkers, maar ook voor de ontwikkeling van nieuwe, mogelijk tumor- en daardoor patiënt-specifieke, tumortherapieën.

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Curriculum Vitae

Anne Benard werd geboren op 30 oktober 1984 te ‘s-Gravenhage. In 2003 behaalde zij haar gymnasiumdiploma aan het Christelijk College de Populier te ’s-Gravenhage, waarna zij de studie Biomedische Wetenschappen begon aan de Universiteit Leiden. Zij behaalde haar Bachelor of Science diploma in 2006 en haar Master of Science diploma in 2008. Tijdens deze studie liep zij stage bij de afdelingen Toxicogenetica en Immunohematologie en Bloedtransfusie in het Leids Universitair Medisch Centrum (LUMC). Haar afstudeerstage deed zij op de afdeling Heelkunde in het LUMC, met als onderwerp apoptose in colorectale tumoren. Na het behalen van haar master-diploma, begon zij in November 2008 haar promotie-onderzoek naar epigenetische prognostische biomarkers in colorectale tumoren op de afdeling Heelkunde in het LUMC, onder leiding van prof. dr. C.J.H. van de Velde en dr. P.J.K. Kuppen. De eerste 3 jaar van haar promotie-onderzoek heeft zij in het kader van een samenwerkingsproject gewerkt op de afdeling Molecular Oncology van het John Wayne Cancer Institute (JWCI) in Santa Monica, California, United States of America, onder leiding van dr. D.S.B. Hoon. De onderzoeken die op het JWCI werden geïnitieerd en uitgevoerd, zijn vervolgens na terugkeer op de afdeling Heelkunde in het LUMC afgerond en verder uitgewerkt. De gecombineerde resultaten van de onderzoeken uitgevoerd in het JWCI en het LUMC zijn te lezen in dit proefschrift. Anne werkt momenteel als product developer op de afdeling Tumor Diagnostiek bij MRC Holland.

Curriculum Vitae

Anne Benard was born on October 30th 1984 in The Hague, The Netherlands. In 2003 she graduated high school and started her university studies in Biomedical Sciences at Leiden University (The Netherlands). She obtained her Bachelor of Science degree in 2006 and her Master of Science degree in 2008. During her studies, she did internships at the departments of Toxicology and Immunohematology and Blood Transfusion at the Leiden University Medical Center (LUMC, Leiden, The Netherlands). She did her final master internship at the department of Surgery at the LUMC, studying apoptosis in colorectal cancer. After obtaining her Master of Science degree, she started her PhD studies at the department of Surgery at the LUMC (supervised by prof. dr. C.J.H. van de Velde and dr. P.J.K. Kuppen), studying epigenetic prognostic biomarkers in colorectal cancer. The first three years of her PhD studies, she worked at the department of Molecular Oncology of the John Wayne Cancer Institute (JWCI) in Santa Monica, CA, USA (supervised by dr. D.S.B. Hoon), in close collaboration with the department of Surgery of the LUMC. The studies initiated and performed at the JWCI were finished and further developed at the LUMC from 2012 onwards. The combined results of the research projects performed at both the JWCI and the LUMC are presented in this thesis. Anne currently works as a product developer at the department of Tumor Diagnostics at MRC Holland.

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